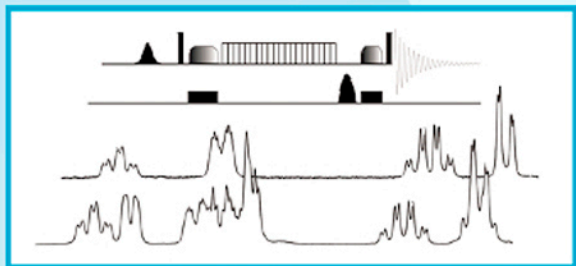




Tetrahedron Organic Chemistry • Volume 27

High-Resolution NMR Techniques in Organic Chemistry

Second Edition



TIMOTHY D W CLARIDGE

TETRAHEDRON ORGANIC CHEMISTRY SERIES
Series Editors: J.-E. Bäckvall, J.E. Baldwin and R.M. Williams

VOLUME 27

High-Resolution NMR Techniques in Organic Chemistry

Second Edition

Related Titles of Interest

JOURNALS

Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry Letters
Tetrahedron
Tetrahedron Letters
Tetrahedron: Asymmetry

BOOKS

BRUCKNER: Advanced Organic Chemistry
KATRITZKY: Handbook of Heterocyclic Chemistry, 2nd edition
KURTI & CZAKO: Strategic Applications of Named Reactions in Organic Synthesis
SILVERMAN: Organic Chemistry of Drug Design

BOOK SERIES

Annual Reports in Medicinal Chemistry

Best Synthetic Methods

Rodd's Chemistry of Carbon Compounds

Strategies and Tactics in Organic Synthesis

Studies in Natural Products Chemistry

Tetrahedron Organic Chemistry Series:

BOGDAL: Microwave-assisted Organic Synthesis: One Hundred Reaction Procedures

CARRUTHERS: Cycloaddition Reactions in Organic Synthesis

CLAYDEN: Organolithiums: Selectivity for Synthesis

GAWLEY & AUBÉ: Principles of Asymmetric Synthesis

HASSNER & STUMER: Organic Syntheses Based on Name Reactions and Unnamed Reactions, 2nd Edition

LI & GRIBBLE: Palladium in Heterocyclic Chemistry, 2nd Edition

McKILLOP: Advanced Problems in Organic Reaction Mechanisms

OBRECHT & VILLALGORDO: Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries

PIETRA: Biodiversity and Natural Product Diversity

PIRRUNG: Molecular Diversity and Combinatorial Chemistry

SESSLER & WEGHORN: Expanded, Contracted & Isomeric Porphyrins

TANG & LEVY: Chemistry of C-Glycosides

WONG & WHITESIDES: Enzymes in Synthetic Organic Chemistry

MAJOR REFERENCE WORKS

Comprehensive Organic Functional Group Transformations II
Comprehensive Heterocyclic Chemistry III
Comprehensive Medicinal Chemistry II

High-Resolution NMR Techniques in Organic Chemistry

Second Edition

TIMOTHY D W CLARIDGE

*Chemistry Research Laboratory, Department of Chemistry,
University of Oxford*



ELSEVIER

Amsterdam • Boston • Heidelberg • London • New York • Oxford
Paris • San Diego • San Francisco • Singapore • Sydney • Tokyo

Elsevier
Linacre House, Jordan Hill, Oxford OX2 8DP, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands

First edition 1999
Reprinted 2004, 2005, 2006
Second edition 2009

Copyright © 2009 Elsevier Ltd. All rights reserved

No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means electronic, mechanical, photocopying, recording or otherwise without the prior written permission of the publisher

Permissions may be sought directly from Elsevier's Science & Technology Rights Department in Oxford, UK: phone (+44) (0) 1865 843830; fax (+44) (0) 1865 853333; email: permissions@elsevier.com. Alternatively you can submit your request online by visiting the Elsevier web site at <http://elsevier.com/locate/permissions>, and selecting Obtaining permission to use Elsevier material

Notice

No responsibility is assumed by the publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein. Because of rapid advances in the medical sciences, in particular, independent verification of diagnoses and drug dosages should be made

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data

A catalog record for this book is available from the Library of Congress

ISBN-13: 978-0-08-054628-5 (hardbound)

ISBN-13: 978-0-08-054818-0 (paperback)

ISSN: 1460-1567 (series)

For information on all Elsevier publications
visit our website at books.elsevier.com

Printed and bound in Hungary

09 10 11 12 10 9 8 7 6 5 4 3 2

Working together to grow
libraries in developing countries

www.elsevier.com | www.bookaid.org | www.sabre.org

ELSEVIER

BOOK AID
International

Sabre Foundation

Contents

Preface to Second Edition	xi
Preface to First Edition	xiii

Chapter 1. Introduction	1

1.1. The development of high-resolution NMR	1
1.2. Modern high-resolution NMR and this book	3
1.2.1. What this book contains	4
1.2.2. Pulse sequence nomenclature	5
1.3. Applying modern NMR techniques	7
References	10

Chapter 2. Introducing high-resolution NMR	11

2.1. Nuclear spin and resonance	11
2.2. The vector model of NMR	13
2.2.1. The rotating frame of reference	13
2.2.2. Pulses	15
2.2.3. Chemical shifts and couplings	16
2.2.4. Spin-echoes	18
2.3. Time and frequency domains	19
2.4. Spin relaxation	20
2.4.1. Longitudinal relaxation: establishing equilibrium	21
2.4.2. Measuring T_1 with the inversion recovery sequence	22
2.4.3. Transverse Relaxation: loss of magnetisation in the x - y plane	24
2.4.4. Measuring T_2 with a spin-echo sequence	25
2.5. Mechanisms for relaxation	28
2.5.1. The path to relaxation	28
2.5.2. Dipole-dipole relaxation	30
2.5.3. Chemical shift anisotropy relaxation	31
2.5.4. Spin-rotation relaxation	32
2.5.5. Quadrupolar relaxation	32
References	34

Chapter 3. Practical aspects of high-resolution NMR	35

3.1. An overview of the NMR spectrometer	35
3.2. Data acquisition and processing	37
3.2.1. Pulse excitation	38
3.2.2. Signal detection	40
3.2.3. Sampling the FID	40
3.2.4. Quadrature detection	46
3.2.5. Phase cycling	50
3.2.6. Dynamic range and signal averaging	51
3.2.7. Window functions	55
3.2.8. Phase correction	58
3.3. Preparing the sample	59
3.3.1. Selecting the solvent	59
3.3.2. Reference compounds	61
3.3.3. Tubes and sample volumes	62
3.3.4. Filtering and degassing	64
3.4. Preparing the spectrometer	65
3.4.1. The probe	65
3.4.2. Probe design and sensitivity	67
3.4.3. Tuning the probe	73
3.4.4. The field-frequency lock	75
3.4.5. Optimising the field homogeneity: shimming	77
3.4.6. Reference deconvolution	82

3.5.	Spectrometer calibrations	83
3.5.1.	Radiofrequency pulses	83
3.5.2.	Pulsed field gradients	89
3.5.3.	Sample temperature	91
3.6.	Spectrometer performance tests	93
3.6.1.	Lineshape and resolution	94
3.6.2.	Sensitivity	94
3.6.3.	Solvent presaturation	96
	References	97
Chapter 4. One-dimensional techniques		99
4.1.	The single-pulse experiment	99
4.1.1.	Optimising sensitivity	99
4.1.2.	Quantitative measurements and integration	101
4.1.3.	Quantification with ERETIC	103
4.2.	Spin decoupling methods	105
4.2.1.	The basis of spin decoupling	105
4.2.2.	Homonuclear decoupling	105
4.2.3.	Heteronuclear decoupling	107
4.3.	Spectrum editing with spin-echoes	111
4.3.1.	The <i>J</i> -modulated spin-echo	111
4.3.2.	APT	113
4.4.	Sensitivity enhancement and spectrum editing	114
4.4.1.	Polarisation transfer	115
4.4.2.	INEPT	116
4.4.3.	DEPT	121
4.4.4.	DEPTQ	124
4.4.5.	PENDANT	125
4.5.	Observing quadrupolar nuclei	126
	References	127
Chapter 5. Correlations through the chemical bond I: Homonuclear shift correlation		129
5.1.	Introducing two-dimensional methods	130
5.1.1.	Generating a second dimension	130
5.2.	Correlation spectroscopy (COSY)	134
5.2.1.	Correlating coupled spins	134
5.2.2.	Interpreting COSY	135
5.2.3.	Peak fine structure	137
5.3.	Practical aspects of 2D NMR	138
5.3.1.	2D lineshapes and quadrature detection	138
5.3.2.	Axial peaks	142
5.3.3.	Instrumental artefacts	143
5.3.4.	2D data acquisition	145
5.3.5.	2D data processing	147
5.4.	Coherence and coherence transfer	148
5.4.1.	Coherence transfer pathways	150
5.5.	Gradient-selected spectroscopy	151
5.5.1.	Signal selection with PFGs	152
5.5.2.	Phase-sensitive experiments	155
5.5.3.	PFGs in high-resolution NMR	156
5.5.4.	Practical implementation of PFGs	157
5.6.	Alternative COSY sequences	158
5.6.1.	Which COSY approach?	158
5.6.2.	Double-quantum filtered COSY (DQF-COSY)	159
5.6.3.	COSY- β	165
5.6.4.	Delayed COSY: detecting small couplings	166
5.6.5.	Relayed COSY	167
5.7.	Total correlation spectroscopy (TOCSY)	168
5.7.1.	The TOCSY sequence	169
5.7.2.	Applying TOCSY	171
5.7.3.	Implementing TOCSY	174
5.7.4.	1D TOCSY	175

5.8. Correlating dilute spins with INADEQUATE	178
5.8.1. 2D INADEQUATE	178
5.8.2. 1D INADEQUATE	180
5.8.3. Implementing INADEQUATE	181
5.9. Correlating dilute spins with ADEQUATE	183
5.9.1. 2D ADEQUATE	183
5.9.2. Enhancements to ADEQUATE	185
References	187

Chapter 6. Correlations through the chemical bond II: Heteronuclear shift correlation 189

6.1. Introduction	189
6.2. Sensitivity	190
6.3. Heteronuclear single-bond correlation spectroscopy	191
6.3.1. Heteronuclear multiple-quantum correlation (HMQC)	192
6.3.2. Heteronuclear single-quantum correlation (HSQC)	195
6.3.3. Practical implementations	196
6.3.4. Hybrid experiments	203
6.4. Heteronuclear multiple-bond correlation spectroscopy	207
6.4.1. The HMBC sequence	208
6.4.2. Applying HMBC	210
6.4.3. HMBC extensions and variants	212
6.4.4. Measuring long-range $^nJ_{\text{CH}}$ coupling constants with HMBC	221
6.5. Heteronuclear X-detected correlation spectroscopy	223
6.5.1. Single-bond correlations	223
6.5.2. Multiple-bond correlations and small couplings	225
6.6. Heteronuclear X–Y correlations	226
6.6.1. Direct X–Y correlations	227
6.6.2. Indirect ^1H -detected X–Y correlations	228
References	230

Chapter 7. Separating shifts and couplings: J -resolved spectroscopy 233

7.1. Introduction	233
7.2. Heteronuclear J -resolved spectroscopy	233
7.2.1. Measuring long-range proton–carbon coupling constants	235
7.2.2. Practical considerations	238
7.3. Homonuclear J -resolved spectroscopy	238
7.3.1. Tilting, projections and symmetrisation	240
7.3.2. Applications	241
7.3.3. Broadband-decoupled ^1H spectroscopy	242
7.3.4. Practical considerations	244
7.4. ‘Indirect’ homonuclear J -resolved spectroscopy	245
References	246

Chapter 8. Correlations through space: The nuclear Overhauser effect 247

8.1. Introduction	247
8.2. Definition of the NOE	248
8.3. Steady-state NOEs	248
8.3.1. NOEs in a two-spin system	249
8.3.2. NOEs in a multispin system	255
8.3.3. Summary	260
8.3.4. Applications	262
8.4. Transient NOEs	266
8.4.1. NOE kinetics	267
8.4.2. Measuring internuclear separations	268
8.5. Rotating-frame NOEs	269
8.6. Measuring steady-state NOEs: NOE Difference	271
8.6.1. Optimising difference experiments	272
8.7. Measuring transient NOEs: NOESY	276
8.7.1. The 2D NOESY sequence	277
8.7.2. 1D NOESY sequences	282
8.7.3. Applications	286
8.7.4. Measuring chemical exchange: EXSY	290

8.8.	Measuring rotating-frame NOEs: ROESY	292
8.8.1.	The 2D ROESY sequence	292
8.8.2.	1D ROESY sequences	294
8.8.3.	Applications	295
8.9.	Measuring heteronuclear NOEs	297
8.9.1.	1D Heteronuclear NOEs	297
8.9.2.	2D Heteronuclear NOEs	298
8.9.3.	Applications	299
8.10.	Experimental considerations	300
	References	301

Chapter 9. Diffusion NMR spectroscopy.....303

9.1.	Introduction	303
9.1.1.	Diffusion and molecular size	304
9.2.	Measuring self-diffusion by NMR	304
9.2.1.	The PFG spin-echo	304
9.2.2.	The PFG stimulated-echo	306
9.2.3.	Enhancements to the stimulated-echo	306
9.2.4.	Data analysis: regression fitting	309
9.2.5.	Data analysis: pseudo-2D DOSY presentation	310
9.3.	Practical aspects of diffusion NMR spectroscopy	311
9.3.1.	The problem of convection	311
9.3.2.	Calibrating gradient amplitudes	316
9.3.3.	Optimising diffusion parameters	317
9.3.4.	Hydrodynamic radii and molecular weights	320
9.4.	Applications of diffusion NMR spectroscopy	321
9.4.1.	Signal suppression	321
9.4.2.	Hydrogen bonding	322
9.4.3.	Host-guest complexes	323
9.4.4.	Ion pairing	324
9.4.5.	Supramolecular assemblies	326
9.4.6.	Aggregation	327
9.4.7.	Mixture separation	328
9.4.8.	Macromolecular characterisation	329
9.5.	Hybrid diffusion sequences	330
9.5.1.	Sensitivity-enhanced heteronuclear methods	330
9.5.2.	Spectrum-edited methods	330
9.5.3.	Diffusion-encoded 2D methods (or 3D DOSY)	331
	References	333

Chapter 10. Experimental methods.....335

10.1.	Composite pulses	335
10.1.1.	A myriad of pulses	337
10.1.2.	Inversion versus refocusing	338
10.2.	Adiabatic and broadband pulses	338
10.2.1.	Common adiabatic pulses	340
10.2.2.	Broadband inversion pulses (BIPs)	342
10.3.	Broadband decoupling and spin locking	343
10.3.1.	Broadband adiabatic decoupling	344
10.3.2.	Spin-locking	345
10.4.	Selective excitation and soft pulses	345
10.4.1.	Shaped soft pulses	346
10.4.2.	Excitation sculpting	350
10.4.3.	DANTE sequences	351
10.4.4.	Practical considerations	352
10.5.	Solvent suppression	354
10.5.1.	Presaturation	355
10.5.2.	Zero excitation	356
10.5.3.	PFGs	357
10.6.	Suppression of zero-quantum coherences	359
10.6.1.	The variable-delay z-filter	359
10.6.2.	Zero-quantum dephasing	360
10.7.	Heterogeneous samples and MAS	362

10.8. Emerging methods	363
10.8.1. Fast data acquisition: single-scan 2D NMR	364
10.8.2. Hyperpolarisation: DNP	365
10.8.3. Residual dipolar couplings (RDCs)	368
10.8.4. Parallel acquisition NMR spectroscopy	370
References.	371
Appendix: Glossary of acronyms	375
Index	377

This page intentionally left blank

Preface to Second Edition

It is 9 years since the publication of the first edition of this book and in this period the discipline of NMR spectroscopy has continued to develop new methodology, improve instrumentation and expand in its applications. This second edition aims to reflect the key developments in the field that have relevance to the structure elucidation of small to mid-sized molecules. It encompasses new and enhanced pulse sequences, many of which build on the sequences presented in the first edition, offering the chemist improved performance, enhanced information content or higher-quality data. It also includes coverage of recent advances in NMR hardware that have led to improved instrument sensitivity and thus extended the boundaries of application. Many of the additions to the text reflect incremental developments in pulsed methods and are to be found spread across many chapters, whereas some of the more substantial additions are briefly highlighted below.

Chapters 1 and 2 provide the background to NMR spectroscopy and to the pulse methods presented in the following chapters and thus have been subject to only minor modification. As with the first edition, no attempt is made to introduce the basic parameters of NMR spectroscopy and how these may be correlated with chemical structure since these topics are adequately covered in many other texts. Chapter 3 again provides information on the practical aspects of NMR spectroscopy and on how to get the best out of your available instrumentation. It has been extended to reflect the most important hardware developments, notably the array of probe technologies now available, including cryogenic, microscale, and flow probes and, indeed, those incorporating combinations of these concepts. The latest methods for instrument calibrations are also described. Chapter 4, describing one-dimensional methods, has been extended a little to reflect the latest developments in spectrum editing methods and to approaches for the quantification of NMR spectra using an external reference. Chapter 5 again provides an introduction to 2D NMR and describes homonuclear correlation methods, and has been enhanced to include advances in methodology that lead to improved spectrum quality, such as the suppression of zero-quantum interferences. It also contains an extended section on methods for establishing carbon–carbon correlations, notably those benefiting from proton detection, which gain wider applicability as instrument sensitivity improves. Chapter 6, presenting heteronuclear correlation techniques, has been extended substantially to reflect developments in methods for establishing long-range proton–carbon correlations in molecules, one of the more versatile routes to identifying molecular connectivities. These include new approaches to improved filtering of one-bond responses, to enhanced sampling of long-range proton–carbon coupling constants, for overcoming spectral crowding, and to methods for differentiating two-bond from three-bond correlations. It also briefly considers the measurement of the magnitudes of heteronuclear coupling constants themselves. The section on triple-resonance methods for exploiting correlations between two heteronucleides (termed X–Y correlations, where neither is a proton) has also been extended to include the more recent methods utilising proton detection. Chapter 7 on *J*-resolved methods has only a single addition in the form of an absorption-mode variant of the homonuclear method that has potential application for the generation of ‘proton-decoupled proton spectra’ and in the separation of proton multiplets. Rather like Chapter 5, that covering the NOE (Chapter 8) reflects incremental advances in established methods, such as those for cleaner NOESY data. It also extends methods for the observation of heteronuclear NOEs, a topic of increasing interest. Chapter 9 presents a completely new chapter dedicated to diffusion NMR spectroscopy and 2D diffusion-ordered spectroscopy (DOSY), mentioned only briefly in the first edition. These methods have become routinely available on modern instruments equipped with pulsed field gradients and have found increasing application in many areas of chemistry. The principal techniques are described and their practical implementation discussed, including the detrimental influence of convection and how this may be recognised and dealt with. A range of applications are presented and in the final section methods for editing or extending the basic sequences are briefly introduced. Descriptions of the components that make up modern NMR experiments are to be found in Chapter 10. This also contains the more recent developments in experimental methodologies such as the application of adiabatic frequency sweeps for pulsing and decoupling and the suppression of unwelcome artefacts through zero-quantum dephasing. The chapter concludes by considering some ‘emerging methods’, technologies that are presently generating significant interest within the NMR community and may have significant impact in the future, at least in some branches of chemistry, but remain subject to future development. These include techniques for acquiring 2D spectra in only a single scan, potentially offering significant time savings; those for generating highly polarised NMR samples, thus greatly enhancing detection sensitivity; and those for exploiting residual dipolar

couplings between nuclei in weakly aligned samples as an alternative means of providing stereochemical information. The book again concludes with an extensive glossary of the many acronyms that permeate the language of NMR spectroscopy.

The preparation of the second edition has again benefited from the assistance and generosity of many people in Oxford and elsewhere. I thank my colleagues in the NMR facility of the Oxford Department of Chemistry for their support and assistance, namely Dr Barbara Odell, Tina Jackson, Dr Guo-Liang Ping and Dr Nick Rees, and in particular I acknowledge the input of Barbara and Nick in commenting on draft sections of the text. I also thank Sam Kay for assistance with the HMBC data fitting routines used in Chapter 6 and Dr James Keeler of the University of Cambridge for making these available to us. I am grateful to Bruker Biospin for providing information on magnet development and probe performance and to Oxford Instruments Molecular Biotoools for providing the dynamic nuclear polarisation (DNP) data for Chapter 10. I also thank Deirdre Clark, Suja Narayana and Adrian Shell of Elsevier Science for their input and assistance during the production of the book.

Finally, I again thank my wife Rachael and my daughter Emma for their patience and support during the revision of this book. I apologise for sacrificing the many evenings and weekends that the project demanded and look to avoid this in the future. Until, perhaps, the next time.

Tim Claridge
Oxford, September 2008
tim.claridge@chem.ox.ac.uk

Preface to First Edition

From the initial observation of proton magnetic resonance in water and in paraffin, the discipline of nuclear magnetic resonance (NMR) has seen unparalleled growth as an analytical method and now, in numerous different guises, finds application in chemistry, biology, medicine, materials science and geology. Despite its inception in the laboratories of physicists, it is in the chemical laboratory that NMR spectroscopy has found the greatest use and, it may be argued, has provided the foundations on which modern organic chemistry has developed. Modern NMR is now a highly developed, yet still evolving, subject that all organic chemists need to understand, and appreciate the potential of, if they are to be effective in and able to progress their current research. An ability to keep abreast of developments in NMR techniques is, however, a daunting task, made difficult not only by the sheer number of available techniques but also by the way in which these new methods first appear. These are spread across the chemical literature in both specialised magnetic resonance journals and those dedicated to specific areas of chemistry, as well as the more general entities. They are often referred to by esoteric acronyms and described in a seemingly complex mathematical language that does little to endear them to the research chemist. The myriad of sequences can be wholly bewildering for the uninitiated and can leave one wondering where to start and which technique to select for the problem at hand. In this book I have attempted to gather together the most valuable techniques for the research chemist and to describe the operation of these using pictorial models. Even this level of understanding is perhaps more than some chemists may consider necessary, but only from this can one fully appreciate the *capabilities* and (of equal if not greater importance) the *limitations* of these techniques. Throughout, the emphasis is on the more recently developed methods that have, or undoubtedly will, establish themselves as the principal techniques for the elucidation and investigation of chemical structures in solution.

NMR spectroscopy is, above all, a practical subject that is most rewarding when one has an interesting sample to investigate, a spectrometer at one's disposal and the knowledge to make the most of this (sometimes alarmingly!) expensive instrumentation. As such, this book contains a considerable amount of information and guidance on how one implements and executes the techniques that are described and thus should be equally at home in the NMR laboratory as at the chemist's or spectroscopist's desk.

This book is written from the perspective of an NMR facility manager in an academic research laboratory and as such the topics included are naturally influenced by the areas of chemistry I encounter. The methods are chosen, however, for their wide applicability and robustness, and because, in many cases, they have already become established techniques in NMR laboratories in both academic and industrial establishments. This is not intended as a review of all recent developments in NMR techniques. Not only would this be too immense to fit within a single volume, but the majority of the methods would have little significance for most research chemists. Instead, this is a distillation of the very many methods developed over the years, with only the most appropriate fractions retained. It should find use in academic and industrial research laboratories alike, and could provide the foundation for graduate level courses on NMR techniques in chemical research.

The preparation of this book has benefited from the cooperation, assistance, patience, understanding and knowledge of many people, for which I am deeply grateful. I must thank my colleagues, both past and present, in the NMR group of the Dyson Perrins Laboratory, in particular Elizabeth McGuinness and Tina Jackson for their first-class support and assistance, and Norman Gregory and Dr Guo-Liang Ping for the various repairs, modifications and improvements they have made to the instruments used to prepare many of the figures in this book. Most of these figures have been recorded specifically for the book and have been made possible by the generosity of various research groups and individuals who have made their data and samples available to me. For this, I would like to express my gratitude to Dr Harry Anderson, Prof. Jack Baldwin, Dr Paul Burn, Dr John Brown, Dr Duncan Carmichael, Dr Antony Fairbanks, Prof. George Fleet, Dr David Hodgson, Dr Mark Moloney, Dr Jo Peach and Prof. Chris Schofield, and to the members of their groups, too numerous to mention, who kindly prepared the samples; they will know who they are and I am indebted to each of them. I am similarly grateful to Prof. Jack Baldwin for allowing me to use the department's instrumentation for the collection of these illustrative spectra.

I would like to thank Drs Carolyn Carr and Nick Rees for their assistance in proofreading the manuscript and for being able to spot those annoying little mistakes that I overlooked time and time again but that still did not register. Naturally I accept responsibility for those that remain and would be grateful to hear of these, whether factual or typographical. I also thank Eileen

Morrell and Sharon Ward of Elsevier Science for their patience in waiting for this project to be completed and for their relaxed attitude as various deadlines failed to be met.

I imagine everyone venturing into a career in science has at some time been influenced or even inspired by one or a few individual(s) who may have acted as teacher, mentor or perhaps role model. Personally, I am indebted to Dr Jeremy Everett and to John Tyler, both formerly from (what was then) Beecham Pharmaceuticals, for accepting into their NMR laboratory for a year a “sandwich” student who was initially supposed to gain industrial experience elsewhere as a chromatographer analysing horse urine! My fortuitous escape from this and the subsequent time at Beecham Pharmaceuticals proved to be a seminal year for me and I thank Jeremy and John for their early encouragement that ignited my interest in NMR. My understanding of what this could really do came from graduate studies with the late Andy Derome, and I, like many others, remain eternally grateful for the insight and inspiration he provided.

Finally, I thank my wife Rachael for her undying patience, understanding and support throughout this long and sometimes tortuous project, one that I’m sure she thought, on occasions, she would never see the end of. I can only apologise for the neglect she has endured but not deserved.

Tim Claridge
Oxford, May 1999

Chapter 1

Introduction

From the initial observation of proton magnetic resonance in water [1] and in paraffin [2], the discipline of nuclear magnetic resonance (NMR) has seen unparalleled growth as an analytical method and now, in numerous different guises, finds application in chemistry, biology, medicine, materials science and geology. The founding pioneers of the subject, Felix Bloch and Edward Purcell, were recognised with a Nobel Prize in 1952 ‘for their development of new methods for nuclear magnetic precision measurements and discoveries in connection therewith’. The maturity of the discipline has since been recognised through the awarding of Nobel prizes to two of the pioneers of modern NMR methods and their application, Richard Ernst (1991, ‘for his contributions to the development of the methodology of high-resolution NMR spectroscopy’) and Kurt Wüthrich (2002, ‘for his development of NMR spectroscopy for determining the three-dimensional structure of biological macromolecules in solution’). Despite its inception in the laboratories of physicists, it is in the chemical and biochemical laboratories that NMR spectroscopy has found greatest use. To put into context the range of techniques now available in the modern organic laboratory, including those described in this book, we begin with a short overview of the evolution of high-resolution (solution-state) NMR spectroscopy and some of the landmark developments that have shaped the subject.

1.1. THE DEVELOPMENT OF HIGH-RESOLUTION NMR

It is now over 16 years since the first observations of NMR were made in both solid and liquid samples, from which the subject has evolved to become the principal structural technique of the research chemist, alongside mass spectrometry. During this time, there have been a number of key advances in high-resolution NMR that have guided the development of the subject [3–5] (Table 1.1) and consequently the work of organic chemists and their approaches to structure elucidation. The seminal step occurred during the early 1950s when it was realised that the resonant frequency of a nucleus is influenced by its chemical environment and that one nucleus could further influence the resonance of

Table 1.1. A summary of some key developments that have had a major influence on the practice and application of high-resolution NMR spectroscopy in chemical research

Decade	Notable advances
1940s	<i>First observation of NMR in solids and liquids (1945)</i>
1950s	<i>Development of chemical shifts and spin–spin coupling constants as structural tools</i>
1960s	<i>Use of signal averaging for improving sensitivity</i> <i>Application of the pulse-FT approach</i> <i>The NOE employed in structural investigations</i>
1970s	<i>Use of superconducting magnets and their combination with the FT approach</i> <i>Computer controlled instrumentation</i>
1980s	<i>Development of multipulse and two-dimensional NMR techniques</i> <i>Automated spectroscopy</i>
1990s	<i>Routine application of pulsed field gradients for signal selection</i> <i>Development of coupled analytical methods, e.g. LC-NMR</i>
2000–	<i>Use of high-sensitivity cryogenic probes</i> <i>Routine availability of actively shielded magnets for reduced stray fields</i> <i>Development of microscale tube and flow probes</i>
2010+	<i>Adoption of fast and parallel data acquisition methods...?</i>

FT, Fourier transformation; LC-NMR, liquid chromatography and nuclear magnetic resonance.

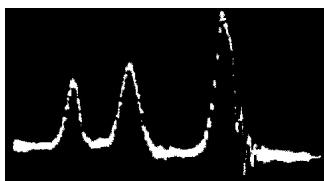


Figure 1.1. The first 'high-resolution' proton NMR spectrum, recorded at 30 MHz, displaying the proton chemical shifts in ethanol (reprinted with permission from [6], Copyright 1951, American Institute of Physics).

another through intervening chemical bonds. Although these observations were seen as unwelcome chemical complications by the investigating physicists, a few pioneering chemists immediately realised the significance of these chemical shifts and spin–spin couplings within the context of structural chemistry. The first high-resolution proton NMR spectrum (Fig. 1.1) clearly demonstrated how the features of an NMR spectrum, in this case chemical shifts, could be directly related to chemical structure, and it is from this that NMR has evolved to attain the significance it holds today.

The 1950s also saw a variety of instrumental developments that were to provide the chemist with even greater chemical insight. These included the use of sample spinning for averaging to zero field inhomogeneities, which provided a substantial increase in resolution, so revealing fine splittings from spin–spin coupling. Later, spin decoupling was able to provide more specific information by helping the chemists understand these interactions. With these improvements, sophisticated relationships could be developed between chemical structure and measurable parameters, leading to realisations such as the dependence of vicinal coupling constants on dihedral angles (the now well-known Karplus relationship). The inclusion of computers during the 1960s was also to play a major role in enhancing the influence of NMR on the chemical community. The practice of collecting the same continuous wave spectrum repeatedly and combining them with a CAT (computer of average transients) led to significant gains in sensitivity and made the observation of smaller sample quantities a practical realisation. When the idea of stimulating all spins simultaneously with a single pulse of radio frequency, collecting the time-domain response and converting this to the required frequency-domain spectrum by a process known as Fourier transformation (FT), was introduced, more rapid signal averaging became possible. This approach provided an enormous increase in signal-to-noise ratio and was to change completely the development of NMR spectroscopy. The mid-1960s also saw the application of the nuclear Overhauser effect (NOE) to conformational studies. Although described during the 1950s as a means of enhancing the sensitivity of nuclei through the simultaneous irradiation of electrons, the Overhauser effect has since found widest application in sensitivity enhancement between nuclei, or in the study of the spatial proximity of nuclei, and remains one of the most important tools of modern NMR. By the end of the 1960s, the first commercial FT spectrometer was available, operating at 90 MHz for protons. The next great advance in field strengths was provided by the introduction of superconducting magnets during the 1970s, which were able to provide significantly higher fields than the electromagnets previously employed. These, combined with the FT approach, made the observation of carbon-13 routine and provided the organic chemists with another probe of molecular structure. This also paved the way for the routine observation of a whole variety of previously inaccessible nuclei of low natural abundance and low magnetic moment. It was also in the early 1970s that the concept of spreading the information contained within the NMR spectrum into two separate frequency dimensions was proposed in a lecture. However, because of instrumental limitations, the quality of the first two-dimensional (2D) spectra was considered too poor to be published, and not until the mid-1970s, when instrument stability had improved and developments in computers made the necessary complex calculations feasible, did the development of 2D methods begin in earnest. These methods, together with the various multipulse one-dimensional (1D) methods that also became possible with the FT approach, did not have significant impact on the wider chemical community until the 1980s, from which point their development was nothing less than explosive. This period saw an enormous number of new pulse techniques presented that were capable of performing a variety of 'spin gymnastics', thus providing the chemist with ever more structural data, on smaller sample quantities and in less time. No longer was it necessary to rely on empirical correlations of chemical shifts and coupling constants with structural features to identify molecules, but instead a collection of spin interactions (through-bond, through-space and chemical exchange) could be mapped and used to determine structures more reliably and more rapidly. The evolution of new pulse methods continued throughout the 1990s, alongside which has emerged a fundamentally different way of extracting the desired information from molecular systems. Pulsed field gradient selected experiments have now become routine structural tools, providing better quality spectra, often in shorter times, than was previously possible. These came into widespread use not so much from a theoretical breakthrough (their use for signal selection was first demonstrated in 1980) but again as a result of progressive technological developments defeating practical difficulties. Similarly, the emergence of coupled analytical methods, such as liquid chromatography and NMR (LC-NMR), has come about after the experimental complexities of interfacing these very different techniques have been overcome, and these methods have established themselves for the analysis of complex mixtures. Developments in probe technologies over the last decade have led to the wider adoption of cryogenically cooled coils in probe heads that reduce significantly system noise and so

enhance signal-to-noise ratios. Probe coil miniaturisation has also provided a boost in signal-to-noise for mass-limited samples, and the marrying of this with cryogenic technology nowadays offers one of the most effective routes to higher detection sensitivity. Instrument miniaturisation has been a constant theme in recent years leading to smaller and more compact consoles driven by developments in solid-state electronics. Likewise, new generations of actively shielded superconducting magnets with significantly reduced stray fields are now commonplace, making the siting of instruments considerably easier and far less demanding on space. For example, the first generation unshielded magnets operating at 500 MHz possessed stray fields that would extend to over 3 m horizontally from the magnet centre when measured at the 0.5 mT (5 gauss) level, the point beyond which disturbances to the magnetic field are not considered problematic. Nowadays, the latest generation shielded magnets have this line sited at somewhat less than 1 m from the centre and only a little beyond the magnet cryostat itself. This is achieved through the use of compensating magnet coils that seek to counteract the stray field generated outside of the magnet assembly. Other developments are seeking to recycle the liquid cryogens needed to maintain the superconducting state of the magnet through the reliquification of helium and nitrogen. Recycling of helium in this manner is already established for imaging magnets but poses considerable challenges in the context of high-resolution NMR measurements.

Modern NMR spectroscopy is now a highly developed and technologically advanced subject. With so many advances in NMR methodology in recent years, it is understandably an overwhelming task for the research chemist, and even the dedicated spectroscopist, to appreciate what modern NMR has to offer. This text aims to assist in this task by presenting the principal modern NMR techniques and exemplifying their application.

1.2. MODERN HIGH-RESOLUTION NMR AND THIS BOOK

There can be little doubt that NMR spectroscopy now represents the most versatile and informative spectroscopic technique employed in the modern chemical research laboratory and that an NMR spectrometer represents one of the largest single investments in analytical instrumentation the laboratory is likely to make. For both these reasons, it is important that the research chemist is able to make the best use of the available spectrometer(s) and to harness modern developments in NMR spectroscopy in order to promote their chemical or biochemical investigations. Even the most basic modern spectrometer is equipped to perform a myriad of pulse techniques capable of providing the chemist with a variety of data on molecular structure and dynamics. Not always do these methods find their way into the hands of the practising chemist, remaining instead in the realms of the specialist, obscured behind esoteric acronyms or otherwise unfamiliar NMR jargon. Clearly, this should not be so, and the aim of this book is to gather up the most useful of these modern NMR methods and present them to the wider audience who should, after all, find greatest benefit from their applications.

The approach taken throughout is non-mathematical and is based firmly on using pictorial descriptions of NMR phenomena and methods wherever possible. In preparing and updating this work, I have attempted to keep in mind what I perceive to be the requirements of three major classes of potential readers:

1. those who use solution-state NMR as tool in their own research, but have little or no direct interaction with the spectrometer,
2. those who have undertaken training in directly using a spectrometer to acquire their own data, but otherwise have little to do with the upkeep and maintenance of the instrument, and
3. those who make use spectrometers and are responsible for the day-to-day upkeep of the instrument. This may include NMR laboratory managers, although in some cases the user may not consider themselves dedicated NMR spectroscopists.

The first of these could well be research chemists and students in an academic or industrial environment who need to know what modern techniques are available to assist them in their efforts, but otherwise feel they have little concern for the operation of a spectrometer. Their data is likely to be collected under fully automated conditions or provided by a central analytical facility. The second may be a chemist in an academic environment who has hands-on access to a spectrometer and has his or her own samples that demand specific studies that are perhaps not available from fully automated instrumentation. The third class of reader may work in a smaller chemical company or academic chemistry department who have invested in NMR instrumentation but may not employ a dedicated NMR spectroscopist for its upkeep, depending instead on, say, an analytical or synthetic chemist for this. This, it appears (in the UK at least), is often the case for new

start-up chemical companies. NMR laboratory managers may also find the text a useful reference source. With these in mind, the book contains a fair amount of practical guidance on both the execution of NMR experiments and the operation and upkeep of a modern spectrometer. Even if you see yourself in the first of the above categories, some rudimentary understanding of how a spectrometer collects the data of interest and how a sequence produces, say, the 2D correlation spectrum awaiting analysis on your desk can be enormously helpful in correctly extracting the information it contains or in identifying and eliminating artefacts that may arise from instrumental imperfections or the use of less than optimal conditions for your sample. Although not specifically aimed at dedicated spectroscopists, the book may still contain new information or may serve as a reminder of what was once understood but has somehow faded away. The text should be suitable for (UK) graduate level courses on NMR spectroscopy, and sections of the book may also be appropriate for use in advanced undergraduate courses. The book does not, however, contain descriptions of the basic NMR phenomena such as chemical shifts and coupling constants, and neither does it contain extensive discussions on how these may be correlated with chemical structures. These topics are already well documented in various introductory texts [7–12], and it is assumed that the reader is already familiar with such matters. Likewise, the book does not seek to provide a comprehensive physical description of the processes underlying the NMR techniques presented; the book by Keeler [13] presents this at an accessible yet rigorous level.

The emphasis is on techniques in solution-state (high-resolution) spectroscopy, principally those used throughout organic chemistry as appropriate for this series, although extensions of the discussions to inorganic systems should be straightforward, relying on the same principles and similar logic. Likewise, greater emphasis is placed on small to mid-sized molecules (with masses up to a few thousand say) since it is on such systems that the majority of organic chemistry research is performed. That is not to say that the methods described are not applicable to macromolecular systems, and appropriate considerations are given when these deserve special comment. Biological macromolecules are not covered however, but are addressed in a number of specialised texts [14–16].

1.2.1. What this book contains

The aim of this text is to present the most important NMR methods used for organic structure elucidation, to explain the information they provide, how they operate and to provide some guidance on their practical implementation. The choice of experiments is naturally a subjective one, partially based on personal experience, but also taking into account those methods most commonly encountered in the chemical literature and those recognised within the NMR community as being most informative and of widest applicability. The operation of many of these is described using pictorial models (equations appear infrequently and are only included when they serve a specific purpose) so that the chemist can gain some understanding of the methods they are using without recourse to uninviting mathematical descriptions. The sheer number of available NMR methods may make this seem an overwhelming task, but in reality, most experiments are composed of a smaller number of comprehensible building blocks pieced together, and once these have been mastered, an appreciation of more complex sequences becomes a far less daunting task. For those readers wishing to pursue a particular topic in greater detail, the original references are given but otherwise all descriptions are self-contained.

Following this introductory section, Chapter 2 introduces the basic model used throughout the book for the description of NMR methods and describes how this provides a simple picture of the behaviour of chemical shifts and spin–spin couplings during pulse experiments. This model is then used to visualise nuclear spin relaxation, a feature of central importance for the optimum execution of all NMR experiments (indeed, it seems early attempts to observe NMR failed most probably because of a lack of understanding at the time of the relaxation behaviour of the chosen samples). Methods for measuring relaxation rates also provide a simple introduction to multipulse NMR sequences. Chapter 3 describes the practical aspects of performing NMR spectroscopy. This is a chapter to dip into as and when necessary and is essentially broken down into self-contained sections relating to the operating principles of the spectrometer and the handling of NMR data, how to correctly prepare the sample and the spectrometer before attempting experiments, how to calibrate the instrument and how to monitor and measure its performance, should you have such responsibilities. It is clearly not possible to describe all aspects of experimental spectroscopy in a single chapter, but this (together with some of the descriptions in Chapter 10) should contain sufficient information to enable the execution of most modern experiments. These descriptions are kept general, and in these, I have deliberately attempted to avoid the use of a dialect specific to a particular instrument manufacturer. Chapter 4 contains the

most widely used 1D techniques, ranging from the optimisation of the single-pulse experiment to the multiplicity editing of heteronuclear spectra and the concept of polarisation transfer, another feature central to pulse NMR methods. This includes the universally employed methods for the editing of carbon spectra according to the number of attached protons. Specific requirements for the observation of certain quadrupolar nuclei that possess extremely broad resonances are also considered. The introduction of 2D methods is presented in Chapter 5, specifically in the context of determining homonuclear correlations. This is so that the 2D concept is introduced with a realistically useful experiment in mind, although it also becomes apparent that the general principles involved are common to any 2D experiment. Following this, a variety of correlation techniques are presented for identifying scalar (J) couplings between homonuclear spins, which for the most part means protons although methods for correlating spins of low abundance nuclides such as carbon are also discussed. Included in this chapter is an introduction to the operation and use of pulsed field gradients, again with a view to presenting them with a specific application in mind. Again, these discussions aim to make clear that the principles involved are quite general and can be applied to a variety of experiments. Those that benefit most from the gradient methodology are the heteronuclear correlation techniques given in Chapter 6. These correlations are used to map coupling interactions between, typically, protons and a heteroatom either through a single bond or across multiple bonds. In this chapter, most attention is given to the modern correlation methods based on proton excitation and detection, so-called ‘inverse’ spectroscopy. These provide significant gains in sensitivity over the traditional methods that use detection of the less sensitive heteroatom, which nevertheless warrant description because of specific advantages they provide for certain molecules. Chapter 7 considers methods for separating chemical shifts and coupling constants in spectra, which are again based on 2D methods. Chapter 8 moves away from through-bond couplings and onto through-space interactions in the form of the NOE. The principles behind the NOE are presented initially for a simple two-spin system, and then for more realistic multi-spin systems. The practical implementation of both 1D and 2D NOE experiments is described, including the widely used pulsed field gradient 1D NOE methods. Rotating-frame NOE (ROE) techniques are also described, which find greatest utility in the study of larger molecules for which the NOE can be poorly suited, having application in studies of host–guest complexes, oligomeric structures, supramolecular systems and so on. Chapter 9 extends into the study of self-diffusion, an area now routinely amenable to investigation on spectrometers equipped with standard pulsed field gradient hardware. This presents a new chapter for the second edition and reflects the wider use of diffusion NMR methods to studies of a variety of molecular interactions in solution, to the investigation of mixtures and to the classification of molecular size. The final chapter considers additional experimental methods that do not, on their own, constitute complete NMR experiments but are the tools with which modern methods are constructed. These are typically used as elements within the sequences described in the preceding chapters and include such topics as broadband decoupling, selective excitation of specific regions of a spectrum and solvent suppression. Finally, the chapter concludes with a brief overview of some emerging methods, including an approach to very fast 2D data acquisition, new methodology for boosting detection sensitivity and an alternative approach to defining stereochemistry in small organic molecules. At the end of the book is a glossary of some of the acronyms that permeate the language of modern NMR, and, it might be argued, have come to characterise the subject. Whether you love them or hate them they are clearly here to stay and although they provide a ready reference when speaking of pulse experiments to those in the know, they can also serve to confuse the uninitiated and leave them bewildered in the face of this NMR jargon. The glossary provides an immediate breakdown of the acronym together with a reference to its location in the book.

1.2.2. Pulse sequence nomenclature

Virtually all NMR experiments can be described in terms of a *pulse sequence*, which, as the name suggests, is a notation which describes the series of radiofrequency (rf) or field-gradient pulses used to manipulate nuclear spins and so tailor the experiment to provide the desired information. Over the years, a largely (although not completely) standard pictorial format has evolved for representing these sequences, not unlike the way a musical score is used to encode a symphony¹. As these crop up repeatedly throughout the text, the format and conventions used in this book deserve explanation. Only the definitions of the various

¹ Indeed, just as a skilled musician can read the score and ‘hear’ the symphony in his head, an experienced spectroscopist can often read the pulse sequence and picture the general form of the resulting spectrum.

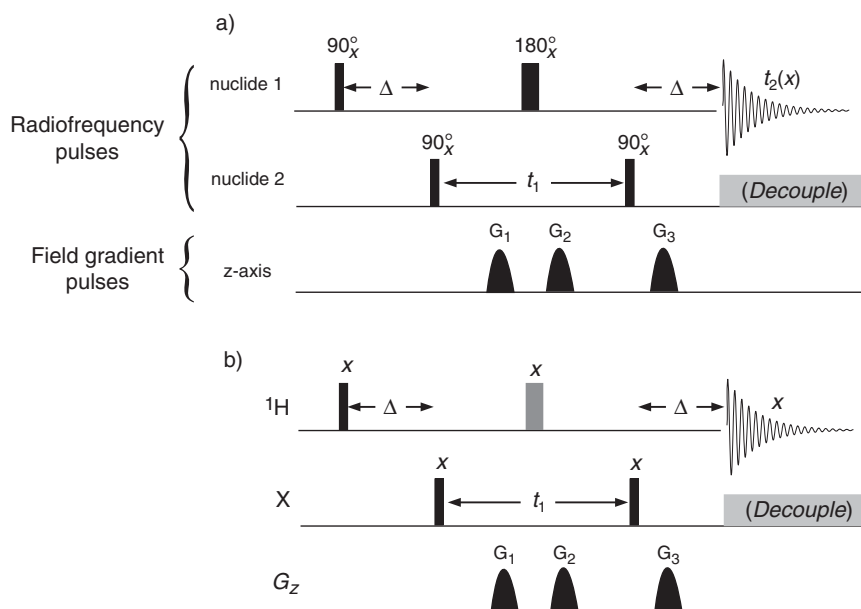


Figure 1.2. Pulse sequence nomenclature. (a) A complete pulse sequence and (b) the reduced representation used throughout the remainder of the book.

pictorial components of a sequence are given here, their physical significance in an NMR experiment will become apparent in later chapters.

An example of a reasonably complex sequence is shown in Fig. 1.2 (a heteronuclear correlation experiment from Chapter 6), and it illustrates most points of significance. Figure 1.2a represents a more detailed account of the sequence, whilst Fig. 1.2b is the reduced equivalent used throughout the book for reasons of clarity. The rf pulses applied to each nuclide involved in the experiment are presented on separate rows running left to right, in the order in which they are applied. Most experiments nowadays involve protons, and often one (and sometimes two) other nuclides as well. In organic chemistry, this is naturally most often carbon but can be any other, so is termed the X-nucleus. These rf pulses are most frequently applied as so-called 90° or 180° pulses (the significance of which is detailed in the following chapter), which are illustrated by a thin black bar and thick grey bars, respectively (Fig. 1.3). Pulses of other angles are marked with an appropriate Greek symbol that is described in the accompanying text. All rf pulses also have associated with them a particular phase, typically defined in units of 90° (0° , 90° , 180° , 270°), and indicated above each pulse by x , y , $-x$ or $-y$, respectively. If no phase is defined, the default will be x . Pulses that are effective over only a small frequency window and act only on a small number of resonances are differentiated as shaped rather than rectangular bars as this reflects the manner in which these are applied experimentally. These are the so-called *selective* or *shaped* pulses described in Chapter 10. Pulses that sweep over very wide bandwidths are indicated by horizontal shading to reflect the frequency sweep employed, as also explained in the final chapter. Segments that make use of a long series of very many closely spaced pulses, such as the decoupling shown in Fig. 1.2, are shown as a solid grey box, with the bracket indicating the use of the decoupling sequence is optional. Below the row(s) of rf pulses are shown field gradient pulses (G_z), whenever these are used, again drawn as shaped pulses where appropriate, and shown greyed when considered optional elements within a sequence.

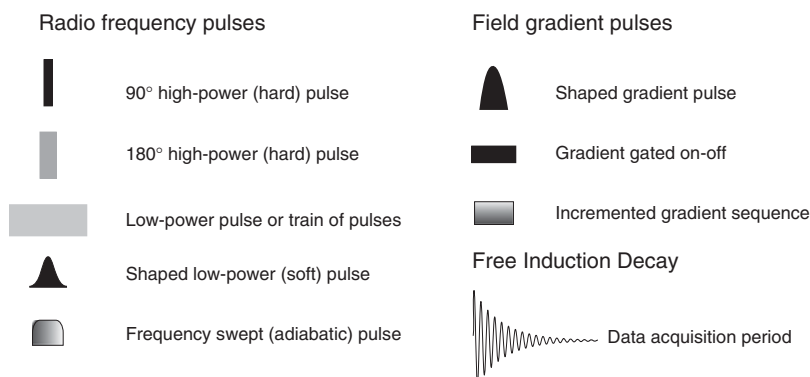


Figure 1.3. A summary of the pulse sequence elements used throughout the book.

The operation of very many NMR experiments is crucially dependent on the experiment being tuned to the value of specific coupling constants. This is achieved by defining certain delays within the sequence according to these values; these delays being indicated by the general symbol Δ . Other time periods within a sequence that are not tuned to J -values but are chosen according to other criteria, such as spin recovery (relaxation) rates, are given the symbol τ . The symbols t_1 and t_2 are reserved for the time periods, which ultimately correspond to the frequency axes f_1 and f_2 of 2D spectra, one of which (t_2) will always correspond to the data acquisition period when the NMR response is actually detected. The acquisition period is illustrated by a simple decaying sine wave in all experiments to represent the so-called free induction decay (FID). Again it should be stressed that although these sequences can have rather foreboding appearances, they are generally built up from much smaller and simpler segments that have well-defined and easily understood actions. A little perseverance can clarify what might at first seem a total enigma.

1.3. APPLYING MODERN NMR TECHNIQUES

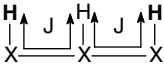
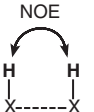
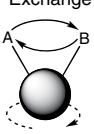
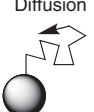
The tremendous growth in available NMR pulse methods over the last three decades can be bewildering and may leave one wondering just where to start or how best to make use of these new developments. The answer to this is not straightforward since it depends so much on the chemistry undertaken, on the nature of the molecule being handled and on the information required of it. It is also dependent on the amount of material and on the available instrumentation and its capabilities. The fact that NMR itself finds application in so many research areas means defined rules for experiment selection are largely intractable. A scheme that is suitable for tackling one type of problem may be wholly inappropriate for another. Nevertheless, it seems inappropriate that a book of this sort should contain no guidance on experiment selection other than the descriptions of the techniques in the following chapters. Here I attempt to broach this topic in rather general terms and present some loose guidelines to help weave a path through the maze of available techniques. Even so, only with a sound understanding of modern techniques can one truly be in a position to select the optimum experimental strategy for *your* molecule or system, and it is this understanding I hope to develop in the remaining chapters.

Most NMR investigations will begin with the analysis of the proton spectrum of the sample of interest, with the usual analysis of the chemical shifts, coupling constants and relative signal intensities, either manually or with the assistance of the various sophisticated computer-based structure spectrum databases now available. Beyond this, one encounters a plethora of available NMR methods to consider employing. The key to selecting appropriate experiments for the problem at hand is an appreciation of the type of information the principal NMR techniques can provide. Although there exist a huge number of pulse sequences, there are a relatively small number of what might be called core experiments, from which most others are derived by minor variation, of which only a rather small fraction ever find widespread use in the research laboratory. To begin, it is perhaps instructive to realise that the NMR methods presented in this book exploit four basic phenomena:

1. Through-bond interactions: scalar (J) spin coupling via bonding electrons.
2. Through-space interactions: the NOE mediated through dipole–dipole coupling and spin relaxation.
3. Chemical exchange: the physical exchange of one spin for another at a specific location.
4. Molecular self-diffusion: the translational movement of a molecule or complex.

When attempting to analyse the structure of a molecule and/or its behaviour in solution by NMR spectroscopy, one must therefore consider how to exploit these phenomena to gain the desired information, and from this select the appropriate technique(s). Thus, when building up the structure of a molecule, one typically first searches for evidence of scalar coupling between nuclei as this can be used to indicate the location of chemical bonds. When the location of all bonding relationships within the molecule has been established, the gross structure of the molecule is defined. Spatial proximities between nuclei, and between protons in particular, can be used to define stereochemical relationships within a molecule and thus address questions of configuration and conformation. The unique feature of NMR spectroscopy, and the principal reason for its superiority over any other solution-state technique for structure elucidation, is its ability to define relationships between specific nuclei within a molecule or even between molecules. Such exquisite detail is generally obtained by correlating one nucleus with another by exploiting the above phenomena. Despite the enormous power of NMR, there are, in fact, rather few types of correlation available to the chemist to employ for structural and conformational analysis. The principal

Table 1.2. The principal correlations or interactions established through NMR techniques. The correlated spins are shown in bold type for each correlation, with X indicating any spin- $\frac{1}{2}$ nucleus. The acronyms are explained in the glossary

Correlation	Principal technique(s)	Comments	Chapter
	^1H - ^1H COSY	Proton J -coupling typically over two or three bonds	5
	^1H - ^1H TOCSY	Relayed proton J -couplings within a coupled spin system. Remote protons may be correlated provided there is a continuous coupling network in between them	5
	^1H -X HMQC ^1H -X HSQC	One-bond heteronuclear couplings with proton observation	6
	^1H -X HMBC	Long-range heteronuclear couplings with proton observation. Typically over two or three bonds when $\text{X} = ^{13}\text{C}$	6
	X-X COSY X-X INADEQUATE H-X-X-H ADEQUATE	COSY only used when X spin natural abundance is greater than 20%. Sensitivity problems when X has low natural abundance; can be improved with proton detection methods	5
	^1H - ^1H NOE difference 1/2D NOESY 1/2D ROESY	Through-space correlations. NOE difference only applicable to 'small' molecules, ROESY applicable to 'mid-sized' molecules with masses of ca. 1–2 kDa	8
	^1H -X NOE difference 2D HOESY	Sensitivity limited by X spin observation. Care required to make NOEs specific in presence of proton decoupling	8
	Exchange 1D saturation or inversion transfer 2D EXSY	Interchange of spins at chemically distinct locations. Exchange must be slow on NMR timescale for separate resonances to be observed. Intermediate to fast exchange requires lineshape analysis	8
	Diffusion Spin-echo or stimulated-echo methods 2D DOSY	Measurement of molecular self-diffusion using pulsed field gradient technology. Used mainly in studies of molecular associations	9

1D, one-dimensional; 2D, two-dimensional; NOE, nuclear Overhauser effect.

spin interactions and the main techniques used to map these, which are frequently 2D methods, are summarised in Table 1.2 and further elaborated in the chapters that follow.

The homonuclear correlation experiment, correlation spectroscopy (COSY), identifies those nuclei that share a J -coupling, which, for protons, operate over two, three and, less frequently, four bonds. This information can therefore be used to indicate the presence of a bonding pathway. The correlation of protons that exist within the same coupled network or chain of spins, but do not themselves share a J -coupling, can be made with the total correlation spectroscopy (TOCSY) experiment. This can be used to identify groups of nuclei that sit within the same isolated spin system, such as the amino acid residue of a peptide or the sugar ring of an oligosaccharide. One-bond heteronuclear correlation methods (heteronuclear multiple-quantum correlation (HMQC) or heteronuclear single-quantum correlation (HSQC)) identify the heteroatoms to which the protons are directly attached and can, for example, provide carbon assignments from previously established proton assignments. Proton chemical shifts can also be dispersed according to the shift of the attached heteroatom, so aiding the assignment of the proton spectrum itself. Long-range heteronuclear correlations over typically two or three bonds (heteronuclear multiple-bond correlation (HMBC)) provide a wealth of information on the skeleton of the molecule and can be used to infer the location of carbon–carbon or carbon–heteroatom bonds. These correlations can be particularly valuable when proton–proton correlations are absent. The incredible natural abundance double quantum transfer experiment (INADEQUATE)-based experiments identify connectivity between like nuclei of low natural abundance, for which it is favoured over COSY. This can therefore correlate directly connected carbon centres, but as

this relies on the presence of neighbouring carbon-13 nuclei, it suffers from appallingly low sensitivity and thus finds little use. Modern variants that use proton detection, termed adequate sensitivity double-quantum spectroscopy (ADEQUATE), have greatly improved performance but are still less used than the heteronuclear correlation techniques. Measurements based on the NOE are most often applied after the gross structure is defined, and NMR assignments established, to define the three-dimensional (3D) stereochemistry of a molecule since these effects map through-space proximity between nuclei. It can also provide insights into the conformational folding of larger structures and the direct intermolecular interactions. The vast majority of these experiments investigate proton–proton NOEs, although in exceptional cases, heteronuclear NOEs involving a proton and a heteroatom have been applied successfully. Similar techniques to those used in the observation of NOEs can also be employed to correlate nuclei involved in chemical exchange processes that are slow on the NMR timescale and so give rise to distinct resonances for each exchanging species or site. Finally, methods for the quantification of molecular self-diffusion provide information on the nature and extent of molecular associations and provide a complimentary view of solution behaviour. They may also be used to separate the spectra of species with differing mobilities and have potential application in the characterisation of mixtures.

The greatest use of NMR in the chemical research laboratory is in the routine characterisation of synthetic starting materials, intermediates and final products. In these circumstances, it is often not so much full structure *elucidation* that is required, rather it is structure *confirmation* or *verification* since the synthetic reagents are known, which naturally limit what the products may be, and because the desired synthetic target is usually defined. Routine analysis of this sort typically follows a general procedure similar to that summarised in Table 1.3, which is supplemented with data from the other analytical techniques, most notably mass spectrometry and infrared spectroscopy. The execution of many of the experiments in Table 1.3 benefits nowadays from the incorporation of pulsed field gradients to speed data collection and to provide spectra of higher quality. Furthermore, experiments involving heteronuclear correlations typically employ proton observation to aid sensitivity rather the detection of the heteronuclide as was the case for earlier 2D methods. Nowadays, the collection of a 2D proton-detected ^1H – ^{13}C shift correlation experiment requires significantly less time than the 1D carbon spectrum of the same sample, providing both carbon shift data (of protonated centres) and correlation information. This can be a far more powerful tool for routine structure confirmation than the 1D carbon experiment alone. In addition, editing can be introduced to the 2D experiment to differentiate methine from methylene correlations for example, providing yet more data in a single experiment and in less time. Even greater gains can be made in the indirect observation of heteronuclides of still lower intrinsic sensitivity, for example, nitrogen-15, and when considering the observation of low abundance nuclides it is sensible to first consider adopting a proton-detected method for this. The structure confirmation process can also be enhanced through the measured use of spectrum prediction tools that are now widely available. The generation

Table 1.3. A typical protocol for the routine structure confirmation of synthetic organic materials. Not all these steps may be necessary, and the direct observation of a heteronuclide, such as carbon or nitrogen, can often be replaced through its indirect observation with the more sensitive proton-detected heteronuclear shift correlation techniques

Procedure	Technique	Information
1D ^1H spectrum ↓	1D	Information from chemical shifts, coupling constants, integrals
2D ^1H – ^1H correlation ↓	COSY	Identify <i>J</i> -coupling relationships between protons
1D ^{13}C (with spectrum editing) ↓	1D, (DEPT or APT)	Carbon count and multiplicity determination (C, CH, CH ₂ , CH ₃). Can often be avoided by using proton-detected heteronuclear 2D experiments
1D heteronuclide spectra e.g. ^{31}P , ^{19}F ↓	1D	Chemical shifts and homonuclear/heteronuclear coupling constants
2D ^1H – ^{13}C one-bond correlation (with spectrum editing) ↓	HMQC or HSQC (with editing)	Carbon assignments transposed from proton assignments. Proton spectrum dispersed by ^{13}C shifts. Carbon multiplicities from edited HSQC (faster than above 1D approach)
2D ^1H – ^{13}C long-range correlation ↓	HMBC	Correlations identified over two and three bonds. Correlations established across heteroatoms, e.g. N and O. Fragments of structure pieced together
Through-space NOE correlation	1D or 2D NOE	Stereochemical analysis: configuration and conformation

1D, one-dimensional; 2D, two-dimensional; NOE, nuclear Overhauser effect.

of a calculated spectrum from a proposed structure can provide useful guidance when considering the validity of the structure, and a number of computational packages are now available that predict at least ^{13}C and ^1H spectra, but also the more common nuclides including ^{19}F , ^{31}P and ^{15}N in some cases.

Even when dealing with unknown materials or with molecules of high structural complexity, the general scheme of Table 1.3 still represents an appropriate general protocol to follow. In such cases, the basic experiments of this table are still likely to be employed, but may require data to be collected under a variety of experimental conditions (solvent, temperature, pH, etc.) and/or may require additional support from other methods or extended versions of these techniques before a complete picture emerges. This book aims to explain the primary NMR techniques and some of their more useful variants and to describe their practical implementation, so that the research chemist may realise the full potential that modern NMR spectroscopy has to offer.

REFERENCES

- [1] F. Bloch, W. W. Hansen and M. E. Packard, *Phys. Rev.*, 1946, **69**, 127.
- [2] E. M. Purcell, H. C. Torrey and R. V. Pound, *Phys. Rev.*, 1946, **69**, 37.
- [3] J. W. Emsley and J. Feeney, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2007, **50**, 179–198.
- [4] J. W. Emsley and J. Feeney, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1995, **28**, 1–9.
- [5] J. N. Shoolery, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1995, **28**, 37–52.
- [6] J. T. Arnold, S. S. Dharmatti and M. E. Packard, *J. Chem. Phys.*, 1951, **19**, 507.
- [7] L. M. Harwood and T. D. W. Claridge, *Introduction to Organic Spectroscopy*, Oxford University Press, Oxford, 1997.
- [8] R. M. Silverstein, F. X. Webster and D. J. Kiemle, *Spectrometric Identification of Organic Compounds*, 7th ed., Wiley, New York, 2005.
- [9] H. Gunther, *NMR Spectroscopy: Basic Principles, Concepts and Applications in Chemistry*, 2nd ed., Wiley, Chichester, 1995.
- [10] J. W. Akitt and B. E. Mann, *NMR and Chemistry: An Introduction to Modern NMR Spectroscopy*, 4th ed. Stanley Thorne, Cheltenham, 2000.
- [11] M. Balci, *Basic ^1H – ^{13}C NMR Spectroscopy*, Elsevier, Amsterdam, 2005.
- [12] H. Friebolin, *Basic One- and Two-dimensional NMR Spectroscopy*, 4th ed., Wiley, Chichester, 2005.
- [13] J. Keeler, *Understanding NMR Spectroscopy*, Wiley, Chichester, 2005.
- [14] J. Cavanagh, W. J. Fairbrother, A. G. Palmer, N. J. Skelton and M. Rance, *Protein NMR Spectroscopy: Principles and Practice*, 2nd ed., Academic Press (Elsevier), San Diego, 2006.
- [15] J. N. S. Evans, *Biomolecular NMR Spectroscopy*, Oxford University Press, Oxford, 1995.
- [16] H. J. Dyson and P. E. Wright, Protein Structure Calculation Using NMR Restraints, in *Two-dimensional NMR Spectroscopy: Applications for Chemists and Biochemists*, 2nd ed., (Eds.: W.R. Croasmun and R.M.K. Carlson), VCH, New York, 1994.

Chapter 2

Introducing high-resolution NMR

For anyone wishing to gain a greater understanding of modern NMR techniques together with an appreciation of the information they can provide and hence their potential applications, it is necessary to develop an understanding of some elementary principles. These, if you like, provide the foundations from which the descriptions in subsequent chapters are developed, and many of the fundamental topics presented in this introductory chapter will be referred to throughout the remainder of the text. In keeping with the style of the book, all concepts are presented in a pictorial manner and avoid the more mathematical descriptions of NMR. Following a reminder of the phenomenon of nuclear spin, the Bloch vector model of NMR is introduced. This presents a convenient and comprehensible picture of how spins behave in an NMR experiment and provides the basic tool with which most experiments will be described.

2.1. NUCLEAR SPIN AND RESONANCE

The nuclei of all atoms may be characterised by a nuclear spin quantum number, I , which may have values greater than or equal to zero and which are multiples of $\frac{1}{2}$. Those with $I=0$ possess no nuclear spin and therefore cannot exhibit NMR, so are termed ‘NMR silent’. Unfortunately, from the organic chemists’ point of view, the nucleus likely to be of most interest, carbon-12, has zero spin, as do all nuclei with atomic mass and atomic number both even. However, the vast majority of chemical elements have at least one nuclide that does possess nuclear spin that is, in principle at least, observable by NMR (Table 2.1) and as a consolation the proton is a high-abundance NMR-active isotope. The property of nuclear spin is fundamental to the NMR phenomenon. The spinning nuclei possess angular momentum, P , and, of course, charge and the motion of this charge gives rise to an associated magnetic moment, μ , (Fig. 2.1) such that:

$$\mu = \gamma P \quad (2.1)$$

where the term γ is the *magnetogyric ratio*¹, which is constant for any given nuclide and may be viewed as a measure of how ‘magnetic’ a particular nuclide is. Both angular momentum and the magnetic moment are vector quantities, that is, they have both magnitude and direction. When placed in an external, static magnetic field (denoted B_0 , strictly the magnetic flux density), the microscopic magnetic moments align themselves relative to the field in a discrete number of orientations because the energy states involved are quantised. For a spin of magnetic quantum number I , there exist $2I + 1$ possible spin states, so for a spin- $\frac{1}{2}$ nucleus such as the proton, there are two possible states denoted $+\frac{1}{2}$ and $-\frac{1}{2}$, whilst for $I = 1$, for example, deuterium, the states are $+1$, 0 , -1 (Fig. 2.2) and so on. For the spin- $\frac{1}{2}$ nucleus, the two states correspond to the popular picture of a nucleus taking up two possible orientations with respect to the static field, either parallel (the α state) or antiparallel (the β state), the former being of lower energy. The effect of the static field on the magnetic moment can be described in terms of classical mechanics, with the field imposing a torque on the moment, which therefore traces a circular path about the applied field (Fig. 2.3). This motion is referred to as precession, or more specifically *Larmor precession* in this context. It is analogous to the familiar motion of a gyroscope in the Earth’s gravitational field, in which the gyroscope spins about its own axis, and this axis in turn precesses about the direction of the field. The rate of the precession as defined by the angular velocity (ω (rad s^{-1}) or ν (Hz)) is:

$$\omega = -\gamma B_0 (\text{rad s}^{-1}) \quad \text{or} \quad \nu = \frac{-\gamma B_0}{2\pi} \equiv -\gamma B_0 (\text{Hz}) \quad (2.2)$$

¹ The term gyromagnetic ratio is also in widespread use for γ although this does not conform to IUPAC recommendations [1–3].

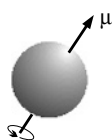


Figure 2.1. A nucleus carries charge and when spinning possesses a magnetic moment, μ .

Table 2.1. Properties of selected spin- $1/2$ nuclei. Frequencies are given for a 400 MHz spectrometer (9.4 T magnet), and sensitivities are given relative to proton observation and include terms for both intrinsic sensitivity of the nucleus and its natural abundance

Isotope	Natural abundance (%)	NMR frequency (MHz)	Relative sensitivity
^1H	99.98	400	1.0
^3H	0	426.7	1.2^*
^{13}C	1.11	100.6	1.76×10^{-4}
^{15}N	0.37	40.5	3.85×10^{-6}
^{19}F	100.00	376.3	0.83
^{29}Si	4.7	79.5	3.69×10^{-4}
^{31}P	100.00	161.9	6.63×10^{-2}
^{77}Se	7.58	76.3	5.25×10^{-4}
^{103}Rh	100.00	12.6	3.11×10^{-5}
^{113}Cd	12.16	88.7	1.33×10^{-3}
^{119}Sn	8.58	149.1	4.44×10^{-3}
^{183}W	14.40	16.6	1.03×10^{-5}
^{195}Pt	33.80	86.0	3.36×10^{-3}
^{207}Pb	22.60	83.7	2.07×10^{-3}

* Assuming 100% ^3H labelling. (Properties of quadrupolar nuclei are given in Table 2.3 below).

Figure 2.2. Nuclei with a magnetic quantum I may take up $2I + 1$ possible orientations relative to the applied static magnetic field B_0 . For spin- $1/2$ nuclei, this gives the familiar picture of the nucleus behaving as a microscopic bar magnet having two possible orientations, α and β .

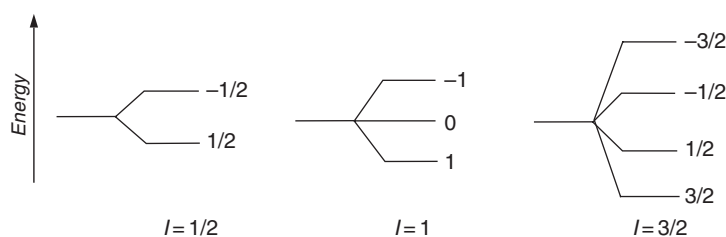
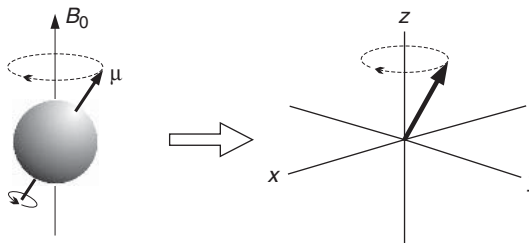


Figure 2.3. A static magnetic field applied to the nucleus causes it to precess at a rate dependent on the field strength and on the magnetogyric ratio of the spin. The field is conventionally applied along the z axis of a Cartesian co-ordinate frame and the motion of the nucleus represented as a vector moving on the surface of a cone.



This is known as the *Larmor frequency* of the nucleus. The direction of motion is determined by the sign of γ and may be clockwise or anticlockwise, but is always the same for any given nuclide. NMR occurs when the nucleus changes its spin state, driven by the absorption of a quantum of energy. This energy is applied as electromagnetic radiation, whose frequency must match that of the Larmor precession for the resonance condition to be satisfied with the energy involved being given by:

$$\Delta E = h\nu = \frac{h\gamma B_0}{2\pi} \quad (2.3)$$

where h is the Planck's constant. In other words, the resonant frequency of a spin is simply its Larmor frequency. Modern high-resolution NMR spectrometers currently (2008) employ field strengths up to 22.3 T, which, for protons, correspond to resonant frequencies up to 950 MHz that fall within the rf region of the electromagnetic spectrum. For other nuclei at similar field strengths, resonant frequencies will differ from those of protons (due to the dependence of ν on γ), but it is common practice to refer to a spectrometer's operating frequency in terms of the resonant frequencies of protons. Thus, one may refer to using a "400 MHz spectrometer", although this would equally operate at 100 MHz for carbon-13

² The symbol ν in place of $\gamma/2\pi$ will occur frequently throughout the text when representing frequencies in hertz.

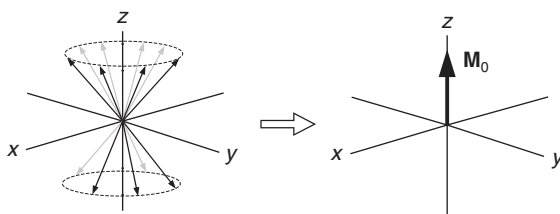


Figure 2.4. In the vector model of NMR, many like spins are represented by a bulk magnetisation vector. At equilibrium, the excess of spins in the α state places this parallel to the $+z$ axis.

since $\gamma_H/\gamma_C \approx 4$. It is also universal practice to define the direction of the static magnetic field as being along the z axis of a set of Cartesian co-ordinates, so that a *single* precessing spin- $1/2$ nucleus will have a component of its magnetic moment along the z axis (the longitudinal component) and an orthogonal component in the x - y plane (the transverse component) (Fig. 2.3).

Now consider a collection of similar spin- $1/2$ nuclei in the applied static field. As stated, the orientation parallel to the applied field, α , has slightly lower energy than the anti-parallel orientation, β , so at equilibrium there will be an excess of nuclei in the α state as defined by the Boltzmann distribution:

$$\frac{N_\alpha}{N_\beta} = e^{\Delta E/k_B T} \quad (2.4)$$

where $N_{\alpha,\beta}$ represents the number of nuclei in the spin orientation, k_B the Boltzmann constant and T the temperature. The differences between spin energy levels are rather small, so the corresponding population differences are similarly small and only about 1 part in 10^4 at the highest available field strengths. This is why NMR is so very insensitive relative to other techniques such as IR and UV, where the ground- and excited-state energy differences are substantially greater. The tiny population *excess* of nuclear spins can be represented as a collection of spins distributed randomly about the precessional cone and parallel to the z axis. These give rise to a resultant *bulk magnetisation vector* $\mathbf{M}_0$ along this axis (Fig. 2.4). It is important to realise that this z -magnetisation arises because of *population differences* between the possible spin states, a point we return to in Section 2.2. Since there is nothing to define a preferred orientation for the spins in the transverse direction, there exists a random distribution of individual magnetic moments about the cone and hence there is no *net magnetisation* in the transverse (x - y) plane. Thus, we can reduce our picture of many similar magnetic moments to one of a single bulk magnetisation vector $\mathbf{M}_0$ that behaves according to the rules of classical mechanics. This simplified picture is referred to as the Bloch vector model (after the pioneering spectroscopist Felix Bloch), or more generally as the *vector model* of NMR.

2.2. THE VECTOR MODEL OF NMR

Having developed the basic model for a collection of nuclear spins, we can now describe the behaviour of these spins in pulsed NMR experiments. There are essentially two parts to be considered, firstly the application of the rf pulse(s), and secondly the events that occur following this. The essential requirement to induce transitions between energy levels, that is, to cause NMR to occur, is the application of a time-dependent magnetic field oscillating at the Larmor frequency of the spin. This field is provided by the magnetic component of the applied rf, which is designated the B_1 field to distinguish it from the static B_0 field. This rf is transmitted via a coil surrounding the sample, the geometry of which is such that the B_1 field exists in the *transverse* plane, perpendicular to the static field. In trying to consider how this oscillating field operates on the bulk magnetisation vector, one is faced with a mind-boggling task involving simultaneous rotating fields and precessing vectors. To help visualise these events, it proves convenient to employ a simplified formalism, known as the *rotating frame* of reference, as opposed to the so-called *laboratory frame* of reference described thus far.

2.2.1. The rotating frame of reference

To aid the visualisation of processes occurring during an NMR experiment, a number of simple conceptual changes are employed. Firstly, the oscillating B_1 field is considered to be composed of two counter-rotating magnetic vectors in the x - y plane, the resultant

Figure 2.5. The rf pulse provides an oscillating magnetic field along one axis (here the x axis), which is equivalent to two counter-rotating vectors in the transverse plane.

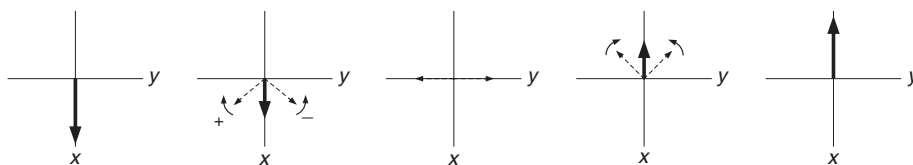
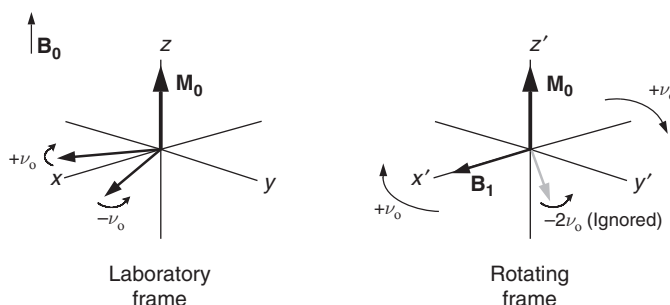


Figure 2.6. The laboratory and rotating frame representations. In the laboratory frame, the co-ordinate system is viewed as being static, whereas in the rotating frame it rotates at a rate equal to the applied rf frequency, ν_0 . In this representation, the motion of one component of the applied rf is frozen whereas the other is far from the resonance condition and may be ignored. This provides a simplified model for the description of pulsed NMR experiments.

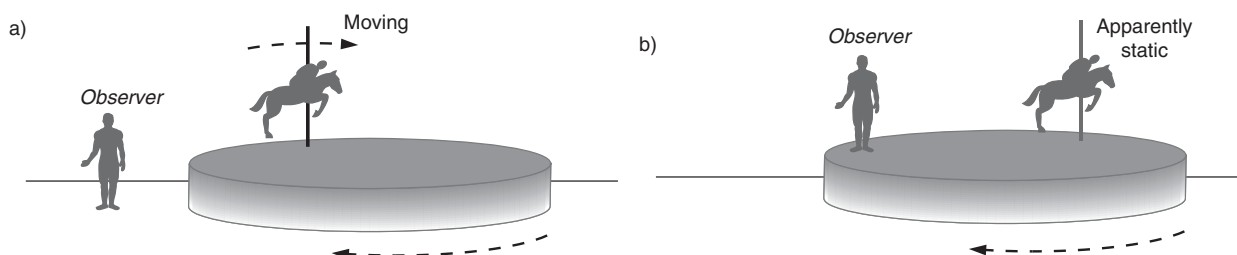


of which corresponds exactly to the applied oscillating field (Fig. 2.5). It is now possible to simplify things considerably by eliminating one of these and simultaneously freezing the motion of the other by picturing events in the *rotating frame* of reference (Fig. 2.6). In this, the set of x , y , z co-ordinates are viewed as rotating along with the nuclear precession, in the same sense and at the same rate. Since the frequency of oscillation of the rf field exactly matches that of nuclear precession (which it must for the magnetic resonance condition to be satisfied), the rotation of one of the rf vectors is now static in the rotating frame whereas the other is moving at *twice* the frequency in the *opposite* direction. The latter vector is far from resonance and is simply ignored. Similarly, the precessional motion of the spins has been frozen as these are moving with the same angular velocity as the rf vector and hence the co-ordinate frame. Since this precessional motion was induced by the static magnetic field B_0 , this is also no longer present in the rotating frame representation.

The concept of the rotating frame may be better pictured with the following analogy. Suppose you are at a fairground and are standing watching a child going round on the carousel. You see the child move towards you then away from you as the carousel turns and are thus aware of the circular path he follows. This corresponds to observing events from the so-called *laboratory frame* of reference (Fig. 2.7a). Now imagine what you see if you step onto the carousel as it turns. You are now travelling with the same angular velocity and in the same sense as the child, so their motion is no longer apparent. His precession has been frozen from your point of view and you are now observing events in the *rotating frame* of reference (Fig. 2.7b). Obviously, the child is still moving in the 'real' world but your perception of events has been greatly simplified. Likewise, this transposition simplifies our picture of events in an NMR experiment.

Strictly, one should use the different co-ordinate labelling scheme for the laboratory and the rotating frames, such as x , y , z and x' , y' , z' , respectively, as in Fig. 2.6. However, since we shall be dealing almost exclusively with a rotating frame description of events, the simpler x , y , z notations will be used throughout the remainder of the book, and explicit indication provided where the laboratory frame of reference is used.

Figure 2.7. A fairground carousel can be viewed from (a) the laboratory or (b) the rotating frame of reference.



2.2.2. Pulses

We are now in a position to visualise the effect of applying an rf pulse to the sample. The ‘pulse’ simply consists of turning on rf irradiation of a defined amplitude for a time period t_p , and then switching it off. As in the case of the static magnetic field, the rf electromagnetic field imposes a torque on the bulk magnetisation vector in a direction that is perpendicular to the direction of the B_1 field (the ‘motor rule’) that rotates the vector from the z axis toward the x - y plane (Fig. 2.8). Thus, applying the rf field along the x axis will drive the vector towards the y axis.³ The rate at which the vector moves is proportional to the strength of the rf field (γB_1) and so the angle θ through which the vector turns, colloquially known as the pulse flip or tip angle (but more formally as the nutation angle) will be dependent on the amplitude and duration of the pulse:

$$\theta = 360 \gamma B_1 t_p \text{ degrees} \quad (2.5)$$

If the rf was turned off just as the vector reached the y axis, this would represent a 90° pulse, if it reached the $-z$ axis, it would be a 180° pulse, and so on. Returning to consider the individual magnetic moments that make up the bulk magnetisation vector for a moment, we see that the 90° pulse corresponds to *equalising the populations* of the α and β states, as there is now no net z -magnetisation. However, there is a net magnetisation in the x - y plane, resulting from ‘bunching’ of the individual magnetisation vectors caused by the application of the rf pulse. The spins are said to possess *phase coherence* at this point, forced upon them by the rf pulse (Fig. 2.9). Note that this equalising of populations is not the same as the *saturation* of a resonance, a condition that will be encountered in various circumstances in this book. Saturation corresponds again to equal spin populations but with the phases of the individual spins distributed randomly about the transverse plane such that there exists no net transverse magnetisation and thus no observable signal. In other words, under conditions of saturation the spins lack phase coherence. The 180° pulse *inverts the populations* of the spin states, since there must now exist more spins in the β than in the α orientation to place the bulk vector anti-parallel to the static field. Only magnetisation in the x - y plane is ultimately able to induce a signal in the detection coil (see below) so that the 90° and 270° pulse will produce the maximum signal intensity, but the 180° and 360° pulse will produce none (this provides a useful means of ‘calibrating’ the pulses, as in Chapter 3). The vast majority of the multipulse experiments described in this book, and indeed throughout NMR, use only 90° and 180° pulses.

The example above made use of a 90_x pulse, that is a 90° pulse in which the B_1 field was applied along the x axis. It is, however, possible to apply the pulse with arbitrary phase, say along any of the axes x , y , $-x$, or $-y$ as required, which translates to a different starting phase of the excited magnetisation vector. The spectra provided by these pulses show resonances whose *phases* similarly differ by 90° . The detection system of the spectrometer designates one axis to represent the positive *absorption* signal (defined by a receiver

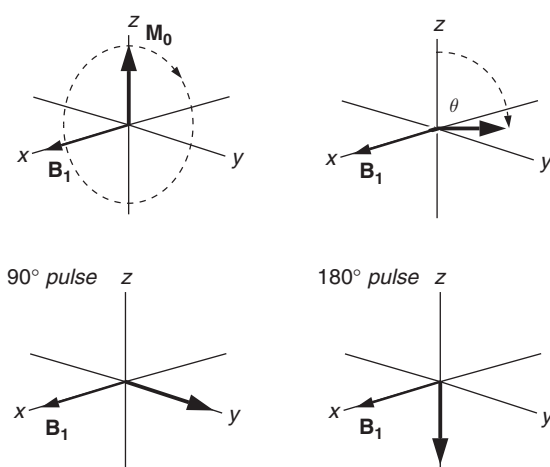


Figure 2.8. An rf pulse applies a torque to the bulk magnetisation vector and drives it towards the x - y plane from equilibrium. θ is the pulse tip or flip angle, which is most frequently 90 or 180° .

³ Strictly speaking, the sense of rotation is positive about the applied B_1 field so that the vector will be driven towards the $-y$ axis. For clarity of presentation, the vector will always been shown as coming out of the page towards the $+y$ axis for a pulse applied along the $+x$ axis.

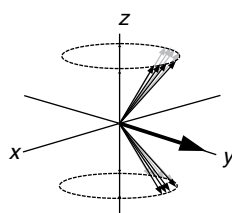


Figure 2.9. Following a 90° pulse, the individual spin vectors bunch along the y axis and are said to possess phase coherence.

reference phase, Section 3.2.2) meaning only magnetisation initially aligned with this axis will produce a pure absorption-mode resonance. Magnetisation that differs from this by $+90^\circ$ is said to represent the pure *dispersion* mode signal, that which differs by 180° is the negative absorption response and so on (Fig. 2.10). Magnetisation vectors initially between these positions result in resonances displaying a mixture of absorption and dispersion behaviour. For clarity and optimum resolution, all NMR resonances are displayed in the favoured absorption mode whenever possible (which is achieved through the process known as phase correction). Note that in all cases, the *detected* signals are those *emitted* from the nuclei as described below, and a negative phase signal does not imply a change from emission to absorption of radiation (the absorption corresponds to the initial excitation of the spins).

The idea of applying a sequence of pulses of different phase angles is of central importance to all NMR experiments. The process of repeating a multipulse experiment with different pulse phases and combining the collected data in an appropriate manner is termed *phase cycling* and is one of the most widely used procedures for selecting the signals of interest in an NMR experiment and rejecting those that are not required. We shall encounter this concept further in Chapter 3, and indeed throughout the remainder of the book.

Now consider what happens immediately after the application of, for example, a 90°_x pulse. We already know that in the rotating frame the precession of the spins is effectively frozen because the B_1 frequency ν_0 and hence the rotating frame frequency exactly match the spin Larmor frequency. Thus, the bulk magnetisation vector simply remains static along the $+y$ axis. However, if we step back from our convenient ‘fiction’ and return to consider events in the laboratory frame, we see that the vector starts to precess about the z axis at its Larmor frequency. This rotating magnetisation vector will produce a weak oscillating voltage in the coil surrounding the sample, in much the same way that the rotating magnet in a bicycle dynamo induces a voltage in the coils that surround it. These are the electrical signals we wish to detect and it is these that ultimately produce the observed NMR signal. However, magnetisation in the x - y plane corresponds to deviation from the equilibrium spin populations and, just like any other chemical system that is perturbed from its equilibrium state, the system will adjust to re-establish this condition, and so the transverse vector will gradually disappear and simultaneously grow along the z axis. This return to equilibrium is referred to as *relaxation*, and it causes the NMR signal to decay with time, producing the observed FID (Fig. 2.11). The process of relaxation has wide-ranging implications for the practice of NMR and this important area is also addressed in this introductory chapter.

2.2.3. Chemical shifts and couplings

So far we have only considered the rotating frame representation of a collection of like spins, involving a single vector that is stationary in the rotating frame since the reference frequency ν_0 exactly matches the Larmor frequency of the spins (the rf is said to be *on-resonance* for these spins). Now consider a sample containing two groups of chemically distinct but uncoupled spins, A and X, with a chemical shifts of ν_A and ν_X respectively, which differ by ν Hz. Following excitation with a single 90°_x pulse, both vectors start in the x - y plane along the y axis of the rotating frame. Choosing the reference frequency to be

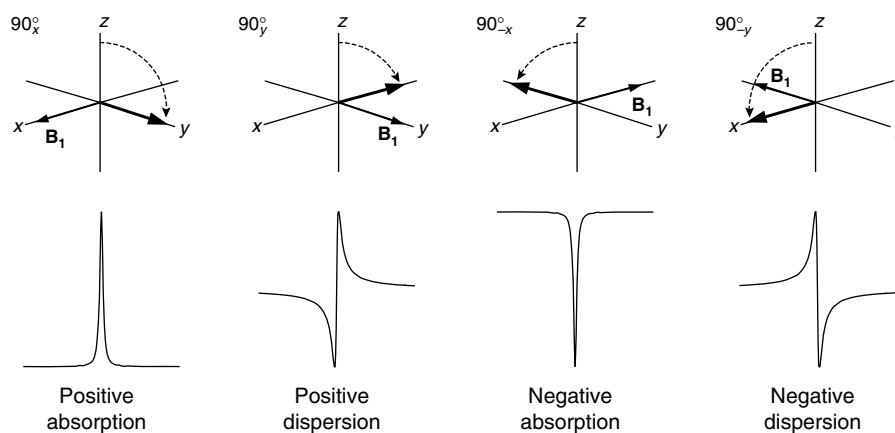


Figure 2.10. Excitation with pulses of varying rf phase. The differing initial positions of the excited vectors produce NMR with similarly altered phases (here the $+y$ axis is arbitrarily defined as representing the positive absorption display).

on-resonance for the A spins ($\nu_0 = \nu_A$) means these remain along the y axis as before (ignoring the effects of relaxation for the present). If the X spins have a greater chemical shift than A ($\nu_X > \nu_A$), then the X vector will be moving faster than the rotating frame reference frequency by the difference ν Hz, so will move *ahead* of A (Fig. 2.12). Conversely, if $\nu_X < \nu_A$ it will be moving more slowly and will lag behind. Three sets of uncoupled spins can be represented by three rotating vectors and so on, such that differences in chemical shifts between spins are simply represented by vectors precessing at different rates in the rotating frame, according to their offsets from the reference frequency ν_0 . By using the rotating frame to represent these events, we need only consider the chemical shift *differences* between the spins of interest, which will be in the kilohertz range, rather than the absolute frequencies, which are of the order of many megahertz. As we shall discover in Section 3.2, this is exactly analogous to the operation of the detection system of an NMR spectrometer, in which a reference frequency is subtracted from the acquired data to produce signals in the kHz region suitable for digitisation. Thus, the ‘trick’ of using the rotating frame of reference in fact equates directly to a real physical process within the instrument.

When considering the effects of scalar coupling on a resonance, it is convenient to remove the effects of chemical shift altogether by choosing the reference frequency of the rotating frame to be the chemical shift of the multiplet of interest. This again helps clarify our perception of events by simplifying the rotation of the vectors in the picture. In the case of a doublet, the two lines are represented by two vectors precessing at $+J/2$ and $-J/2$ Hz, whilst for a triplet, the central line remains static and the outer two move at $+J$ and $-J$ Hz (Fig. 2.13). In many NMR experiments, it is desirable to control the orientation of multiplet vectors with respect to one another, and, as we shall see, a particularly important relationship is when two vectors are *anti-phase* to one another, that is, sitting in opposite directions. This can be achieved simply by choosing an appropriate delay period in which the vectors evolve, which is $1/2J$ for a doublet but $1/4J$ for the triplet.

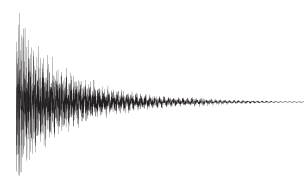


Figure 2.11. The detected NMR response, a Free Induction Decay (FID). The signal fades as the nuclear spins relax back towards thermal equilibrium.

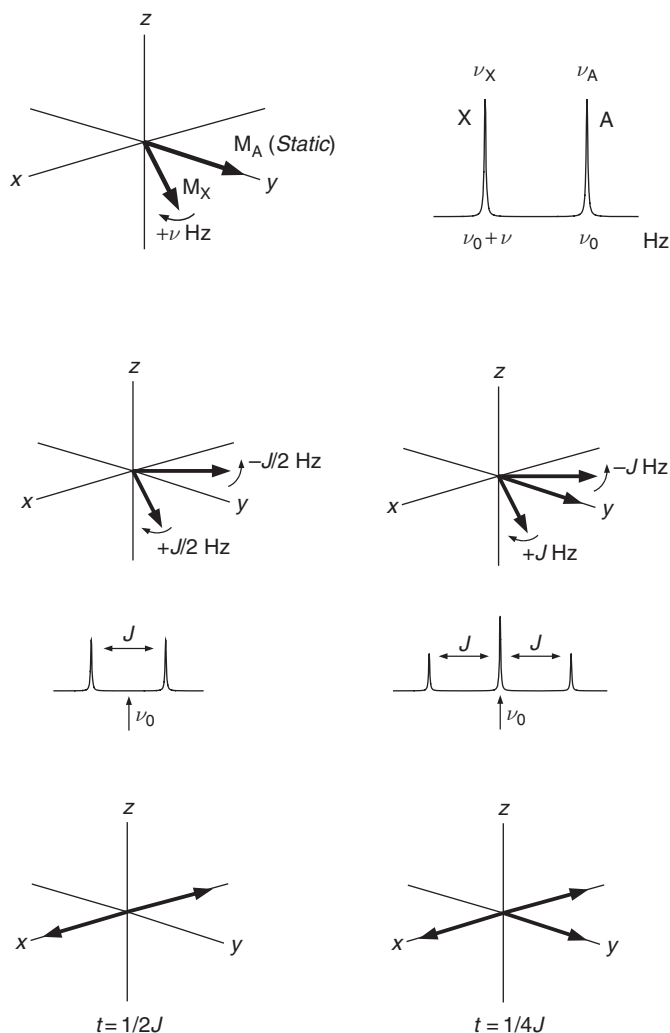


Figure 2.12. Chemical shifts in the rotating frame. Vectors evolve according to their offsets from the reference (transmitter) frequency ν_0 . Here, this is on-resonance for spins A ($\nu_0 = \nu_A$) whilst spins X move ahead at a rate of $+\nu$ Hz (equal to $\nu_X - \nu_0$).

Figure 2.13. Scalar couplings in the rotating frame. Multiplet components evolve according to their coupling constants. The vectors have an anti-phase disposition after an evolution period of $1/2J$ and $1/4J$ s for doublets and triplets respectively.

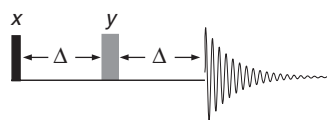


Figure 2.14. The basic spin-echo pulse sequence.

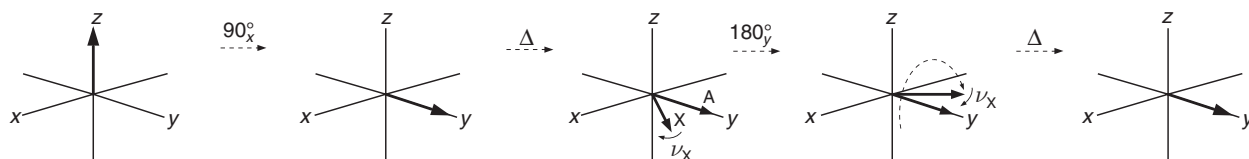
2.2.4. Spin-echoes

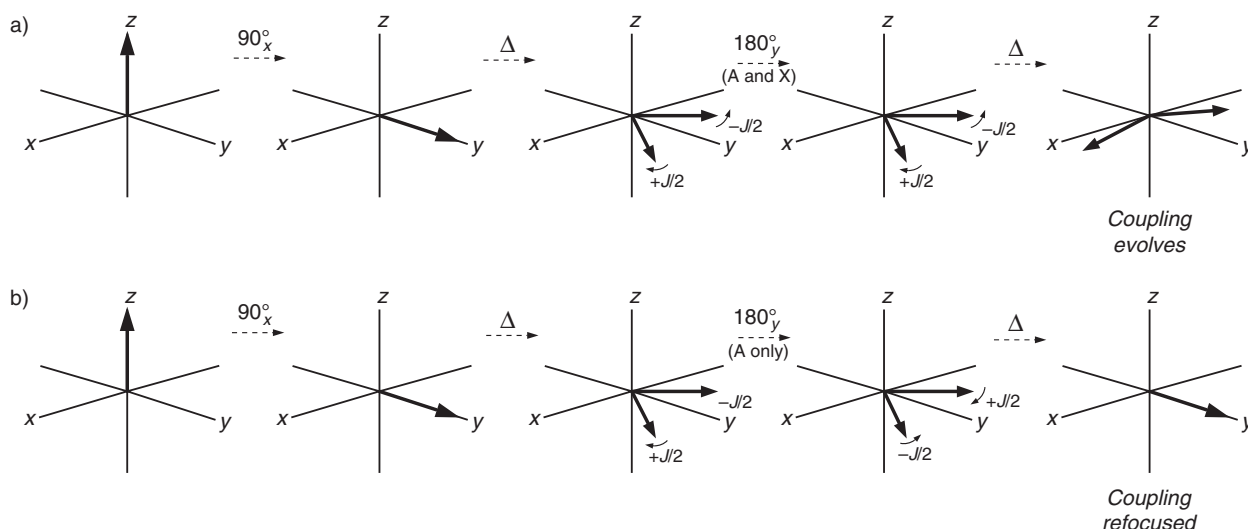
Having seen how to represent chemical shifts and J -couplings with the vector model, we are now in a position to see how we can manipulate the effects of these properties in simple multipulse experiments. The idea here is to provide a simple introduction to using the vector model to understand what is happening during a pulse sequence. In many experiments, there exist time delays in which magnetisation is simply allowed to precess under the influence of chemical shifts and couplings, usually with the goal of producing a defined state of magnetisation before further pulses are applied or data are acquired. To illustrate these points, we consider one of the fundamental building blocks of numerous NMR experiments, the spin-echo.

Consider first two groups of chemically distinct protons, A and X, that share a mutual coupling J_{AX} , which will be subject to the simple two-pulse sequence in Fig. 2.14. For simplicity we shall consider the effect of chemical shifts and couplings separately, starting with the chemical shifts and again assuming the reference frequency to be that of the A spins (Fig. 2.15). The initial 90°_x creates transverse A and x -magnetisation, after which the X vector precesses during the first time interval, Δ . The following 180°_y pulse (note this is now along the y axis) rotates the x -magnetisation through 180° about the y axis, and so places it back in the x - y plane, but now lagging *behind* the A vector. A spin magnetisation remains along the y axis so is invariant to this pulse. During the second time period, Δ , the x -magnetisation will precess through the same angle as in the first period and at the end of the sequence finishes where it began and *at the same position as the A vector*. Thus, after the time period 2Δ , no phase difference has accrued between the A and X vectors despite their different shifts, and it were as if the A and X spins had the same chemical shift throughout the 2Δ period. We say *the spin-echo has refocused the chemical shifts*, the dephasing and rephasing stages giving rise to the echo terminology.

Consider now the effect on the coupling between the two spins with reference to the multiplet of spin A, safe in the knowledge that we can ignore the effects of chemical shifts. Again, during the first period Δ , the doublet components will move in opposite directions, and then have their positions interchanged by the application of the 180°_y pulse. At this point, it would be obvious to assume that the two halves of the doublets would simply refocus as in the case of the chemical shift differences above, but we have to consider the effect of the 180° pulse on the J -coupled partner also, in other words, the effect on the X spins. To appreciate what is happening, we need to remind ourselves of what it is that gives rise to two halves of a doublet. These result from spin A being coupled to its partner, X, which can have one of two orientations (α or β) with respect to the magnetic field. When spin X has one orientation, spin A will resonate as the high frequency half of its doublet, whilst with X in the other, A will resonate as the low frequency half. As there are approximately the same number of X spins in α and β orientations, the two halves of the A doublet will be of equal intensity (obviously there are not exactly equal numbers of α and β X spins, otherwise there would be no NMR signal to observe, but the difference is so small as to be negligible for these arguments). The effect of applying the 180° pulse on the X spins is to invert the relative orientations, so that any A spin that was coupled to $X\alpha$ is now coupled to $X\beta$, and *vice versa*. This means the faster moving vector now becomes the slower and *vice versa*, the overall result being represented in Fig. 2.16a. The two halves of the doublet therefore continue to dephase, so that by the end of the 2Δ period, the J -coupling, in contrast to the chemical shifts, have continued to evolve so that homonuclear couplings are not refocused by a spin-echo. The reason for adding the term *homonuclear* to the previous statement is because it does not necessarily apply to the case of heteronuclear spin-echoes, that is, when we are dealing with two different nuclides, such as ^1H and ^{13}C for example. This is because in a heteronuclear system one may choose to apply the 180° pulse on only one channel, thus only one of the two nuclides will experience this pulse and refocusing of the heteronuclear coupling will occur in this case (Fig. 2.16b). If two simultaneous 180° pulses are applied to both nuclei via two different frequency sources, continued defocusing of the heteronuclear coupling occurs exactly as for the homonuclear spin-echo above.

Figure 2.15. Chemical shift evolution is refocused with the spin-echo sequence.





The use of the 180°_y pulse instead of a 180°_x pulse in the above sequences was employed to provide a more convenient picture of events, yet it is important to realise that the refocusing effects on chemical shift and couplings described above would also have occurred with a 180°_x pulse except that the refocused vectors would now lie along the $-y$ axis instead of the $+y$ axis. One further feature of the spin-echo sequence is that it will also refocus the deleterious effects that arise from inhomogeneities in the static magnetic field, as these may be viewed as just another contribution to chemical shift differences throughout the sample. The importance of the spin-echo in modern NMR techniques can hardly be over-emphasised. It allows experiments to be performed without having to worry about chemical shift differences within a sample and the complications these may introduce (phase differences for example). This then allows us to manipulate spins according to their couplings with neighbours, and it is these interactions that are exploited to the full in many of the modern NMR techniques described later.

Figure 2.16. The influence of spin-echoes on scalar coupling as illustrated for two coupled spins A and X. (a) A homonuclear spin-echo (in which both spins experience a 180° pulse) allows the coupling to evolve throughout the sequence. (b) A heteronuclear spin-echo (in which only one spin experiences a 180° pulse) causes the coupling to refocus. If both heteronuclear spins experience 180° pulses, the heteronuclear coupling evolves as in (a) (see text).

2.3. TIME AND FREQUENCY DOMAINS

It was shown in Section 2.2 that the emitted rf signal from excited nuclear spins (the FID) is detected as a time-dependent oscillating voltage that steadily decays as a result of spin relaxation. In this form, the data are of little use to us because it is a time domain representation of the nuclear precession frequencies within the sample. What we actually want is a display of the frequency components that make up the FID as it is these we relate to transition energies and ultimately chemical environments. In other words, we need to transfer our *time domain* data into the *frequency domain*.

The time and frequency domains are related by a simple function, one being the inverse of the other (Fig. 2.17). The complicating factor is that a genuine FID is usually composed of potentially hundreds of components of differing frequencies and amplitude, in addition to noise and other possible artefacts, and in such cases, the extraction of frequencies by direct inspection is impossible. By far the most widely used method to produce the frequency domain spectrum is the mathematical procedure of Fourier transformation, which has the general form:

$$f(\omega) = \int_{-\infty}^{+\infty} f(t)e^{i\omega t} dt \quad (2.6)$$

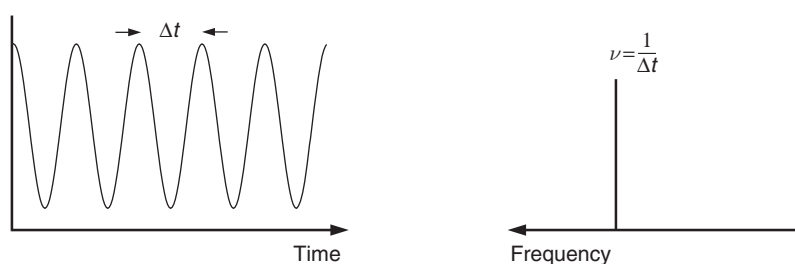
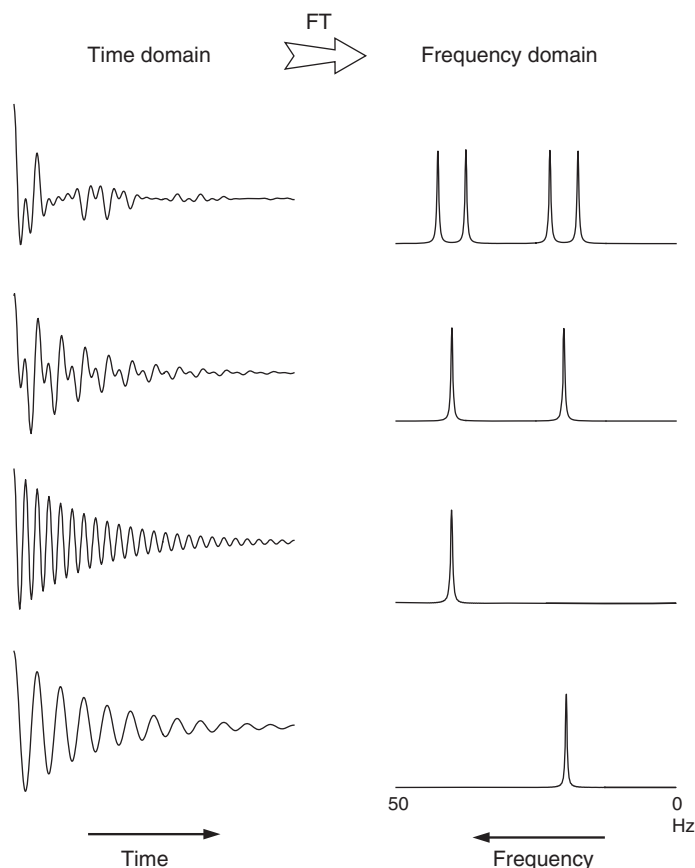


Figure 2.17. Time and frequency domains share a simple inverse relationship.

Figure 2.18. Fourier transformation of time domain FIDs produces the corresponding frequency domain spectra.



where $f(\omega)$ and $f(t)$ represent the frequency and time domain data respectively. In the very early days of pulse-FT NMR, the transform was often the rate-limiting step in producing a spectrum, although with today's computers and the use of a fast FT procedure (the Cooley–Tukey algorithm) the time requirements are of little consequence. Figure 2.18 demonstrates this procedure for very simple spectra. Clearly even for these rather simple spectra of only a few lines, the corresponding FID rapidly becomes too complex for direct interpretation, whereas this is impossible for a genuine FID of any complexity (see Fig. 2.11 for example).

The details of the FT itself are usually of little consequence to anyone using NMR, although there is one notable feature to be aware of. The term $e^{i\omega t}$ can equally be written $\cos \omega t + i \sin \omega t$, and in this form, it is apparent that the transformation actually results in *two* frequency domain spectra that differ only in their signal *phases*. The two are cosine and sine functions so are 90° out of phase relative to one another and are termed the 'real' and 'imaginary' parts of the spectrum (because the function contains complex numbers). Generally, we are presented with only the 'real' part of the data (although the 'imaginary' part can usually be displayed), and with appropriate phase adjustment, we choose this to contain the desired pure absorption mode data and the imaginary part to contain the dispersion mode representation. The significance of this phase relationship will be pursued in Chapters 3 and 5.

2.4. SPIN RELAXATION

The action of an rf pulse on a sample at thermal equilibrium perturbs the nuclear spins from this restful state. Following this perturbation, one would intuitively expect the system to re-establish the equilibrium condition and lose the excess energy imparted into the system by the applied pulse. A number of questions then arise; where does this energy go, how does it get there (or in other words what mechanisms are in place to transfer the energy), and how long does all this take? While some appreciation of all these points is desirable, it is the last of the three that has greatest bearing on the day-to-day practice of NMR. The lifetimes of excited nuclear spins are extremely long when compared to, say, the excited electronic states of optical spectroscopy. These may be a few seconds or even

minutes for nuclear spins as opposed to less than a picosecond for electrons, a consequence of the low transition energies associated with nuclear resonance. These extended lifetimes are crucial to the success of NMR spectroscopy as an analytical tool in chemistry. Not only do these mean that NMR resonances are rather narrow relative to those of rotational, vibrational or electronic transitions (as a consequence of the Heisenberg Uncertainty Principle), but it also provides time to manipulate the spin systems after their initial excitation, performing a variety of spin gymnastics and so modifying the information available in the resulting spectra. This is the world of multipulse NMR experiments, into which we shall enter shortly, and knowledge of relaxation rates has considerable bearing on the design of these experiments, on how they should be implemented and on the choice of experimental parameters for optimum results. Even in the simplest possible case of a single-pulse experiment, relaxation rates influence both achievable resolution and sensitivity (as mentioned in Chapter 1, the earliest attempts to observe the NMR phenomenon probably failed because of a lack of understanding at that time of the spin relaxation properties of the samples used).

Relaxation rates of nuclear spins can also be related to aspects of molecular structure and behaviour in favourable circumstances, in particular internal molecular motions. It is true to say, however, that the relationship between relaxation rates and structural features is not as well defined as those of the chemical shift and spin–spin coupling constants and is not used on a routine basis. The problem of reliable interpretation of relaxation data arises largely from the numerous extraneous effects that influence experimental results, meaning empirical correlations for using such data are not generally available, and this aspect of NMR will not be further pursued.

2.4.1. Longitudinal relaxation: establishing equilibrium

Immediately after pulse excitation of nuclear spins, the bulk magnetisation vector is moved away from the thermal equilibrium $+z$ axis, which corresponds to a change in the spin populations. The recovery of the magnetisation along the z axis, termed *longitudinal relaxation*, therefore corresponds to the equilibrium populations being re-established, and hence to an overall loss of energy of the spins (Fig. 2.19). The energy lost by the spins is transferred into the surroundings in the form of heat, although the energies involved are so small that temperature changes in the bulk sample are undetectable. This gives rise to the original term for this process as *spin-lattice relaxation*, which originated in the early days of solid-state NMR where the excess energy was described as dissipating into the surrounding rigid lattice.

The Bloch theory of NMR assumes that the recovery of the $+z$ -magnetisation, M_z , follows exponential behaviour, described by:

$$\frac{dM_z}{dt} = \frac{(M_0 - M_z)}{T_1} \quad (2.7)$$

where M_0 is the magnetisation at thermal equilibrium and T_1 is the (first-order) time constant for this process. Although exponential recovery was proposed as a hypothesis, it turns out to be an accurate model for the relaxation of spin- $1/2$ nuclei in most cases. Starting from the position of no z -magnetisation (for example, immediately after the sample has been placed in the magnet or after a 90° pulse) the longitudinal magnetisation at time t will be

$$M_z = M_0(1 - e^{-t/T_1}) \quad (2.8)$$

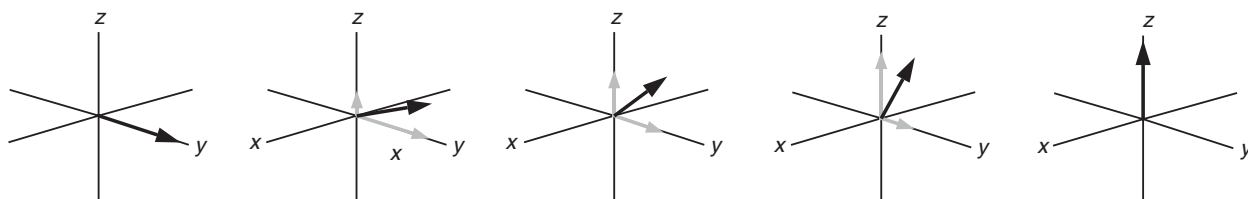
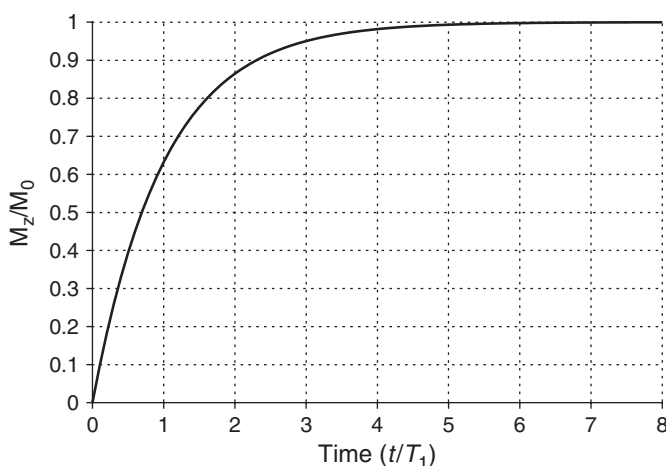


Figure 2.19. Longitudinal relaxation. The recovery of a magnetisation vector (shown on resonance in the rotating frame) diminishes the transverse (x – y) and re-establishes the longitudinal (z) components.

Figure 2.20. The exponential growth of longitudinal magnetisation is dictated by the time constant T_1 and is essentially complete after a period of $5T_1$.



as illustrated in Fig. 2.20. It should be stressed that T_1 is usually referred to as the longitudinal relaxation time throughout the NMR community (and, following convention, throughout the remainder of this book), whereas, in fact, it is a *time constant* rather than a direct measure of the *time* required for recovery. Similarly, when referring to the rate at which magnetisation recovers, $1/T_1$ represents the *rate constant* (s^{-1}) for this process.

For medium-sized organic molecules (those with a mass of a few hundred), proton T_1 s tend to fall in the range 0.5–5 s, whereas carbon T_1 s tend to range from a few seconds to many tens of seconds. For spins to relax fully after a 90° pulse, it is necessary to wait a period of at least $5T_1$ (at which point magnetisation has recovered by 99.33%) and thus it may be necessary to wait many minutes for full recovery. This is rarely the most time efficient way to collect NMR spectra, and Section 4.1 describes the correct approach. The reason such long periods are required lies not in the fact that there is nowhere for the excess energy to go, since the energies involved are so small they can be readily taken up in the thermal energy of the sample, but rather that there is no efficient means for transferring this energy. The time required for *spontaneous* emission in NMR is so long (roughly equivalent to the age of the Universe!) that this has a negligible effect on the spin populations, so *stimulated* emission must be operative for relaxation to occur. Recall that the fundamental requirement for inducing nuclear spin transitions, and hence restoring equilibrium populations in this case, is a magnetic field oscillating at the Larmor frequency of the spins, and the long relaxation times suggests such suitable fields are not in great abundance. These fields can arise from a variety of sources with the oscillations required to induce relaxation coming from local molecular motions. Although the details of the various relaxation mechanisms can become rather complex, a qualitative appreciation of these, as in Section 2.5, is important for understanding many features of NMR spectra. At a practical level, some knowledge of T_1 s in particular is crucial to the optimum execution of almost every NMR experiment, and the simple sequence below offers both a gentle introduction to multipulse NMR techniques and presenting a means of deducing this important parameter.

2.4.2. Measuring T_1 with the inversion recovery sequence

There are a number of different experiments devised for the determination of the longitudinal relaxation times of nuclear spins, and only the most commonly applied method, inversion recovery, will be considered here. The full procedure is described first, followed by the ‘quick-and-dirty’ approach that is handy for experimental set-up.

In essence, all one needs to do to determine T_1 s is to perturb a spin system from thermal equilibrium and then devise some means of following its recovery as a function of time. The inversion recovery experiment is a simple two-pulse sequence (Fig. 2.21) that, as the name implies, creates the initial population disturbance by *inverting* the spin populations through the application a 180° pulse. The magnetisation vector, initially aligned with the $-z$ axis, will gradually shrink back toward the x - y plane, pass through this and eventually make a full recovery along the $+z$ axis at a rate dictated by the quantity of interest, T_1 . Since magnetisation

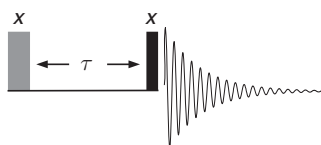
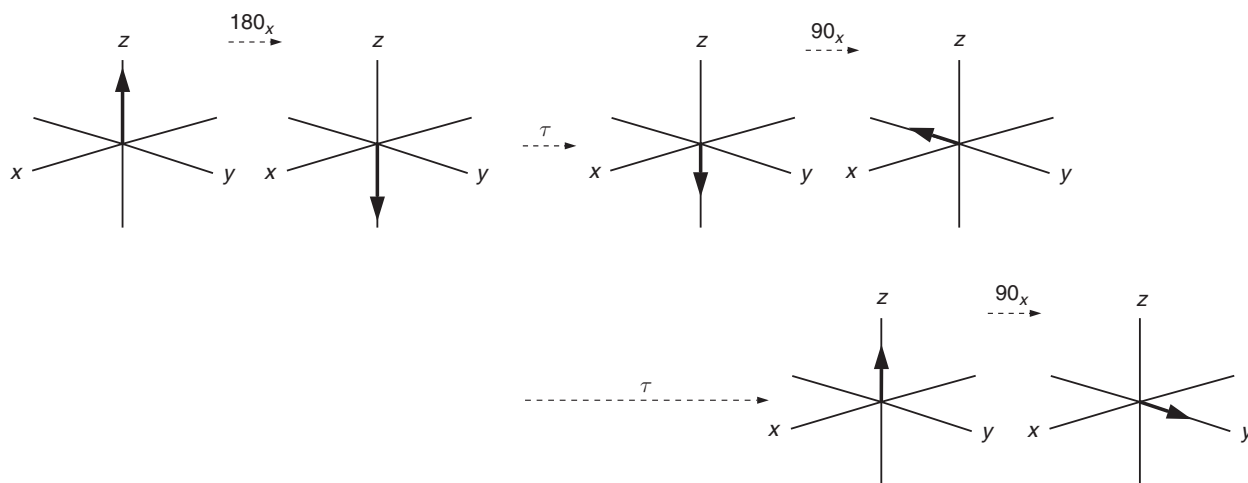


Figure 2.21. The inversion recovery sequence.



along the z axis is unobservable, the recovery is monitored by placing the vector back in the x - y plane with a 90° pulse after a suitable period, τ , following the initial inversion (Fig. 2.22).

If τ is zero, the magnetisation vector terminates with full intensity along the $-y$ axis producing an inverted spectrum using conventional spectrum phasing, that is, defining the $+y$ axis to represent positive absorption. Repeating the experiment with increasing values of τ allows one to follow the relaxation of the spins in question (Fig. 2.23). Finally, when τ is sufficiently long ($\tau_\infty > 5T_1$), complete relaxation will occur between the two pulses and the maximum positive signal is recorded. The intensity of the detected magnetisation, M_t , follows:

$$M_t = M_0(1 - 2e^{-\tau/T_1}) \quad (2.9)$$

where M_0 corresponds to equilibrium magnetisation, such as that recorded at τ_∞ . Note here the additional factor of two relative to Eq. (2.8) as the recovery starts from inverted magnetisation. The relaxation time is determined by fitting the signal intensities to this equation, algorithms for which are found in many NMR software packages. The alternative traditional method of extracting T_1 from such an equation is to analyse a semi-logarithmic plot of $\ln(M_0 - M_t)$ versus τ whose slope is $1/T_1$. The most likely causes of error in the use of the inversion recovery method are inaccurate recording of M_0 if full equilibration is not achieved and inaccuracies in the 180° pulse causing imperfect initial inversion. The scaling factor (2 in Eq. (2.9)) can be made variable in fitting routines to allow for incomplete inversion.

Figure 2.22. The inversion recovery process. With short recovery periods, the vector finishes along the $-y$ axis, so the spectrum is inverted whilst with longer periods a conventional spectrum of scaled intensity is obtained.

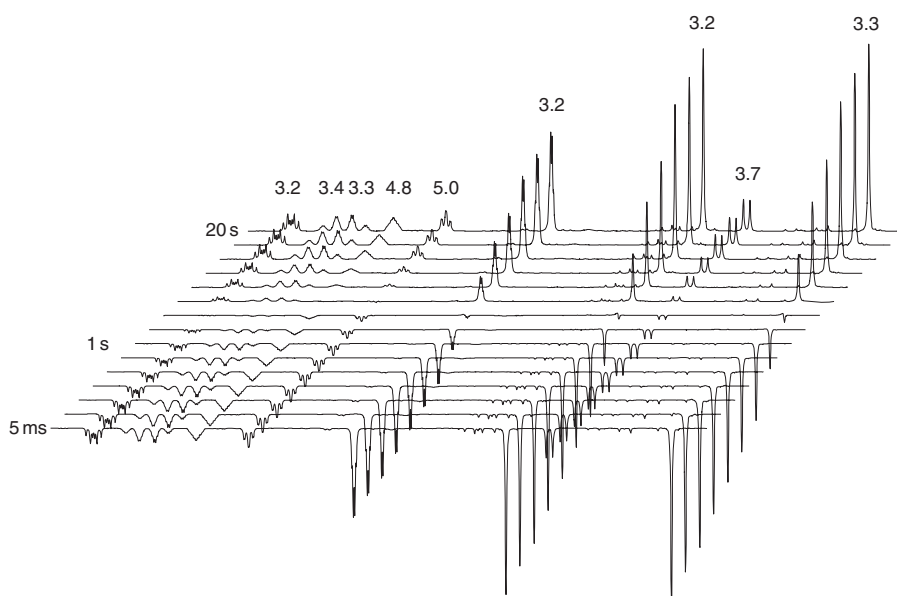
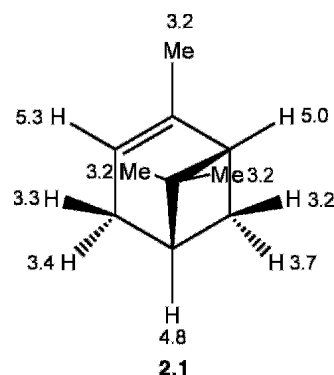


Figure 2.23. The inversion recovery experiment performed on α -pinene **2.1** in non-degassed CDCl_3 (^1H spectra, aliphatic region only shown). Recovery delays in the range 5 ms to 20 s were used and the calculated T_1 s shown for each resonance obtained from fitting peak intensities as described in the text.

The quick T_1 estimation

In many practical cases, it is sufficient to have just an estimation of relaxation times in order to calculate the optimum experimental timings for the sample at hand. In these instances, the procedure described above is overly elaborate and since our molecules are likely to contain nuclei exhibiting a range of T_1 s, accurate numbers will be of little use in experiment set-up. This ‘quick-and-dirty’ method is sufficient to provide estimates of T_1 and again makes use of the inversion-recovery sequence. Ideally the sample in question will be sufficiently strong to allow rather few scans per τ value, making the whole procedure quick to perform. The basis of the method is the disappearance of signals when the longitudinal magnetisation passes through the x - y plane on its recovery (at time τ_{null}), because at this point the population difference is zero ($M_z = 0$). From the above equation, it can be shown that:

$$\tau_{\text{null}} = T_1 \ln 2 \quad (2.10)$$

hence

$$T_1 = \frac{\tau_{\text{null}}}{\ln 2} = 1.443 \tau_{\text{null}} \quad (2.11)$$

Thus, the procedure is to run an experiment with $\tau = 0$ and adjust (phase) the spectrum to be negative absorption. After having waited $>5T_1$, repeat the experiment with an incremented τ using the same phase adjustments, until the signal passes through the null condition (Fig. 2.23), thus defining τ_{null} , which may be different for each resonance in the spectrum. Errors may be introduced from inaccurate 180° pulses, from off-resonance effects (see Section 3.2) and from waiting for insufficient periods between acquisitions, so the fact that these values are estimates cannot be overemphasised.

One great problem with these methods is the need to know something about the T_1 s in the sample even before these measurements. Between each new τ value, one must wait for the system to come to equilibrium, and if signal averaging is required, one would also have to wait this long between each repetition. Unfortunately, it is the weak samples that require signal averaging that will benefit most from a properly executed experiment. To avoid this, it is wise to develop a feel for the relaxation properties of the types of nuclei and compounds you commonly study so that when you are faced with new material you will have some ‘ball park’ figures to provide guidance. Influences on the magnitude of T_1 are considered in Section 2.5.

2.4.3. Transverse Relaxation: loss of magnetisation in the x - y plane

Referring back to the situation immediately following a 90° pulse in which the transverse magnetisation is on-resonance in the rotating frame, there exists another way in which observable magnetisation can be lost. Recall that the bulk magnetisation vector results from the addition of many microscopic vectors for the individual nuclei that are said to possess phase coherence following the pulse. In a sample of like spins, one would anticipate that these would remain static in the rotating frame, perfectly aligned along the y axis (ignoring the effects of longitudinal relaxation). However, this only holds if the magnetic field experienced by each spin in the sample is *exactly* the same. If this is not the case, some spins will experience a slightly greater local field than the mean causing them to have a higher frequency and to creep ahead, whereas others will experience a slightly smaller field and start to lag behind. This results in a fanning-out of the individual magnetisation vectors, which ultimately leads to no *net* magnetisation in the transverse plane (Fig. 2.24). This is another form of relaxation referred to as *transverse relaxation*, which is again assumed to occur with an exponential decay now characterised by the time constant T_2 .

Magnetic field differences in the sample can be considered to arise from two distinct sources. The first is simply from static magnetic field inhomogeneity throughout the sample volume, which is really an instrumental imperfection and it is this one aims to minimise for

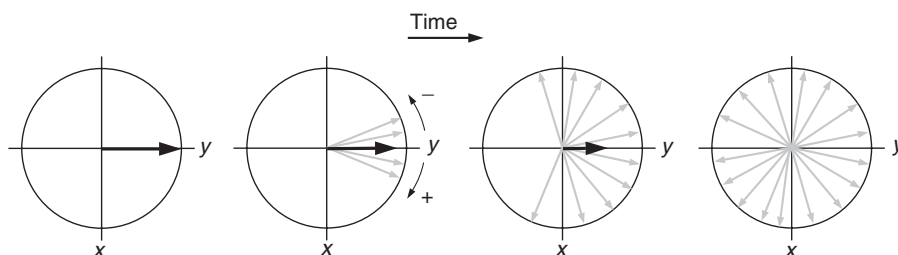


Figure 2.24. Transverse relaxation. Local field differences within the sample cause spins to precess with slightly differing frequencies, eventually leading to zero net transverse magnetisation.

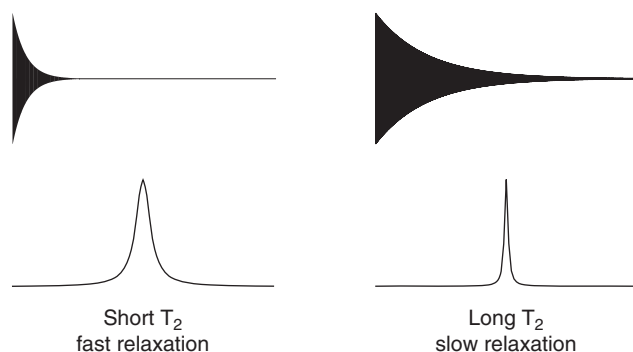


Figure 2.25. Rapidly relaxing spins produce fast decaying FID and broad resonances, whilst those that relax slowly produce longer FIDs and narrower resonances.

each sample when optimising or ‘shimming’ the static magnetic field. The second is from the local magnetic fields arising from intramolecular and intermolecular interactions in the sample, which represent ‘genuine’ or ‘natural’ transverse relaxation processes. The relaxation time constant for the two sources combined is designated T_2^* such that:

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_{2(\Delta B_0)}} \quad (2.12)$$

where T_2 refers to contribution from genuine relaxation processes and T_2 to that from field inhomogeneity. The decay of transverse magnetisation is manifested in the observed FID. Moreover, the widths of NMR resonances are inversely proportional to T_2^* since a short T_2^* corresponds to a faster blurring of the transverse magnetisation, which in turn corresponds to a greater frequency difference between the vectors and thus a greater spread (broader line) in the frequency dimension (Fig. 2.25). For (single) exponential relaxation, the line-shape is Lorentzian with a half-height linewidth, $\Delta\nu_{1/2}$ (Fig. 2.26) of

$$\Delta\nu_{1/2} = \frac{1}{\pi T_2^*} \quad (2.13)$$

For most spin- $1/2$ nuclei in small, rapidly tumbling molecules in low-viscosity solutions, it is field homogeneity that provides the dominant contribution to observed linewidths, and it is rarely possible to obtain genuine T_2 measurements directly from these. However, nuclei with spin $>1/2$ (quadrupolar nuclei) may be relaxed very efficiently by interactions with local electric field gradients and so have broad lines and short T_2 s that can be determined directly from linewidths.

Generally speaking, relaxation mechanisms that operate to restore longitudinal magnetisation also act to destroy transverse magnetisation, and since there clearly can be no magnetisation remaining in the x - y plane when it has all returned to the $+z$ axis, T_2 can never be longer than T_1 . However, additional mechanisms may also operate to reduce T_2 , so that it may be shorter. Again, for most spin- $1/2$ nuclei in small, rapidly tumbling molecules, T_1 and T_2 have the same value, whilst for large molecules that tumble slowly in solution or for solids T_2 is often very much shorter than T_1 (see Section 2.5). Whereas longitudinal relaxation causes a loss of energy from the spins, transverse relaxation occurs by a mutual swapping of energy *between spins*, for example, one spin being excited to the β state while another simultaneously drops to the α state – a so-called flip-flop process. This gives rise to the original term of *spin-spin relaxation*, which is still in widespread use. Longitudinal relaxation is thus an enthalpic process whereas transverse relaxation is entropic. Although the measurement of T_2 has far less significance in routine spectroscopy, methods for this are described below for completeness, and an alternative practical use of these is also presented.

2.4.4. Measuring T_2 with a spin-echo sequence

The measurement of the natural transverse relaxation time T_2 could in principle be obtained if the contribution from magnetic field inhomogeneity was removed. This can be achieved, as has been suggested already, by use of a spin-echo sequence. Consider again a sample of like spins and imagine the sample to be composed of microscopically small regions such that within each region the field is perfectly homogeneous. Magnetisation vectors within any given region will precess at the same frequency, and these are sometimes referred to as *isochromats* (meaning ‘of the same colour’ or frequency). In the basic two-pulse echo sequence (Fig. 2.27a), some components move ahead of the mean whilst others lag behind during the time period, τ (Fig. 2.28). The 180° pulse rotates the vectors towards the $-y$ axis, and following a further period τ , the faster moving vectors coincide with the slower ones

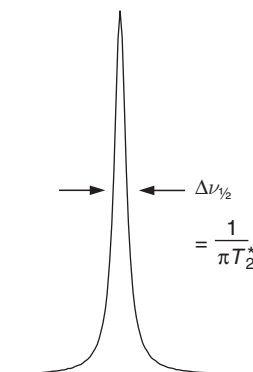


Figure 2.26. The definition of the half-height linewidth of a resonance.

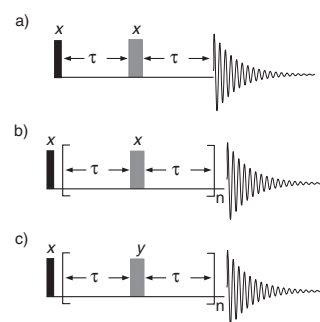


Figure 2.27. Spin-echo sequences for measuring T_2 relaxation times. (a) A basic spin-echo, (b) the Carr–Purcell sequence and (c) the Carr–Purcell–Meiboom–Gill (CPMG) sequence.

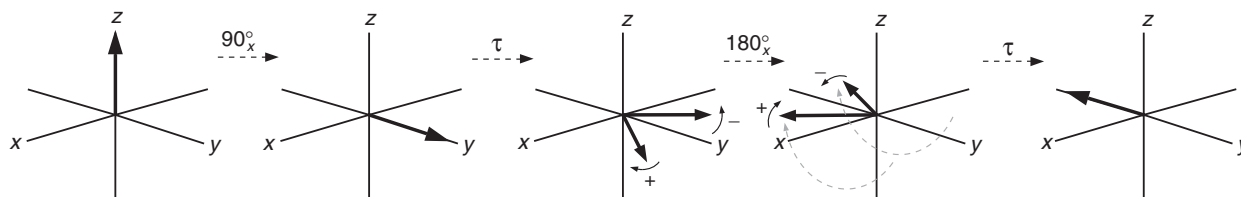


Figure 2.28. The spin-echo refocuses magnetisation vectors dephased by field inhomogeneity.

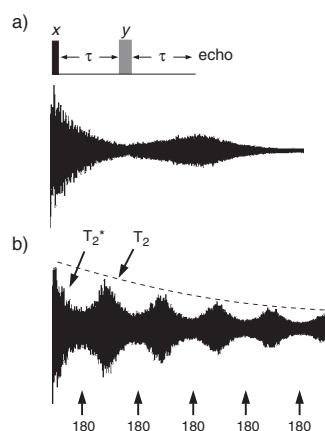


Figure 2.29. Experimental observation of spin-echoes. (a) Signal acquisition was started immediately after a 90° excitation pulse and a 180° pulse applied to refocus field inhomogeneity losses and produce the observed echo. (b) A train of spin-echoes reveals the true T_2 relaxation of magnetisation (dashed line).

along the $-y$ axis. Thus, the echo has refocused the blurring in the x - y plane caused by field inhomogeneities. If one were to start acquiring data immediately after the 90° pulse, one would see the FID away initially but then reappear after a time 2τ as the echo forms (Fig. 2.29a). However, during the 2τ time period, some loss of phase coherence by natural transverse relaxation also occurs, and this is not refocused by the spin-echo since, in effect, there is no phase memory associated with this process to be undone. This means that at the time of the echo, the intensity of the observed magnetisation will have decayed according to the *natural* T_2 time constant, independent of field inhomogeneity. This can clearly be seen in a train of spin-echoes applied during the acquisition of an FID (Fig. 2.29b).

A logical experiment for determining T_2 would be to repeat the sequence with increasing τ and measure the amplitude of the echo versus time, by analogy with the inversion recovery method above. However, some care is required when using such an approach as the formation of the echo depends on the isochromats experiencing exactly the same field throughout the duration of the pulse sequence. If any given spin diffuses into a neighbouring region during the sequence, it will experience a slightly different field from that where it began, and thus will not be fully refocused. As τ increases, such diffusion losses become more severe and the experimental relaxation data less reliable (although this method does provide the basis for measuring molecular diffusion in solution by NMR; see Chapter 9).

A better approach to determining T_2 , which minimises the effect of diffusion, is to repeat the echo sequence within a single experiment using a short τ to form multiple echoes, the decay of which follows the time constant T_2 . This is the Carr–Purcell sequence (Fig. 2.27b), which causes echoes to form alternately along the $-y$ and $+y$ axes following each refocusing pulse. Losses occur from diffusion between the echo peaks, or in other words in the time period 2τ , so if this is kept short relative to the rate of diffusion (typically $\tau < 100$ ms), such losses become negligible. The intensity of the echo at longer time periods is attenuated by repeating the $-\tau-180-\tau-$ sequence many times prior to acquisition. The problem with this method is the fact that any errors in the length of the 180° pulse will be cumulative leading to imperfect refocusing as the experiment proceeds. A better implementation of this scheme is the Carr–Purcell–Meiboom–Gill (CPMG) sequence (Fig. 2.27c) in which 180°_y (as opposed to 180°_x) pulses cause refocusing to take place in the $+y$ hemisphere for every echo. Here, errors in pulse lengths are not cumulative but are small and constant on every odd-numbered echo but will cancel on each even-numbered echo (Fig. 2.30).

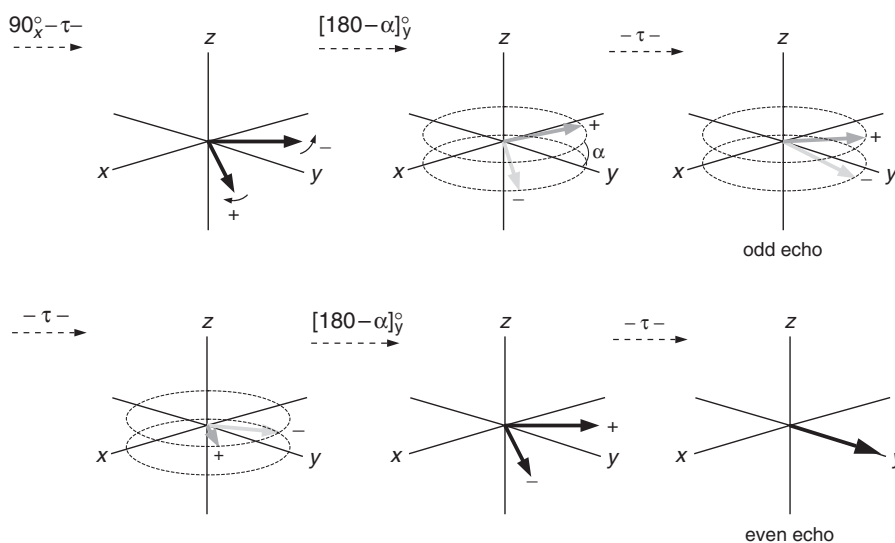


Figure 2.30. The operation of the CPMG sequence in the presence of pulse imperfections. The 180° pulse is assumed to be too short by α° meaning vectors will fall above (dark grey) or below (light grey) the x - y plane following a single 180° pulse and so reduce the intensity of “odd” echoes. By repeating the sequence, the errors are cancelled by the imperfect second 180° pulse so “even” echoes can be used to accurately map T_2 relaxation.

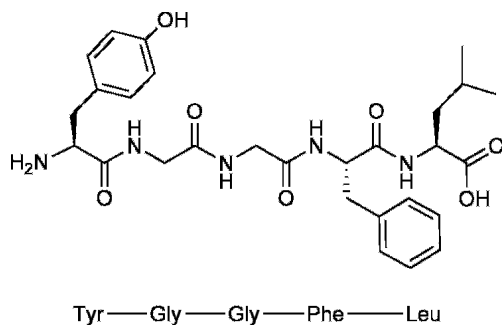
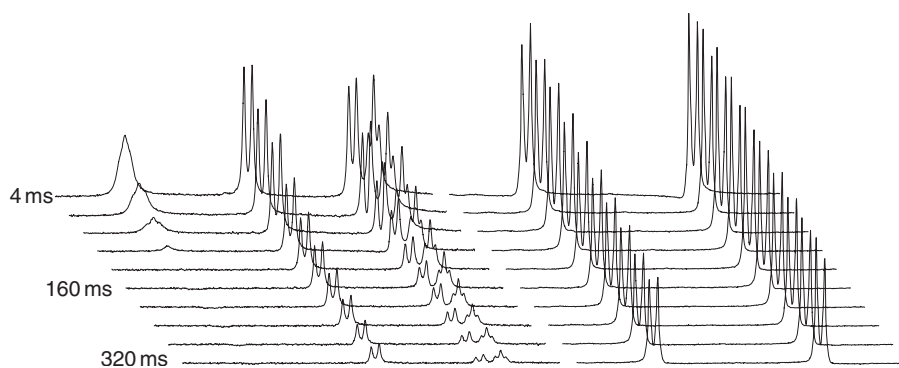
**2.2**

Figure 2.31. The CPMG sequence performed on the pentapeptide Leu-enkephalin **2.2** in DMSO. The faster decay of the amide protons (left) relative to the aromatic protons of the tyrosine residue (right) results from the amide protons coupling to quadrupolar ^{14}N (Section 2.5). The very fast decay of the highest frequency amide proton occurs because this is in rapid chemical exchange with dissolved water, broadening the resonance significantly. The numbers show the total T_2 relaxation period $2\tau n$.

T_2 may then be extracted by performing a series of experiments with increasing $2\tau n$ (by increasing n) and acquiring data following the last even echo peak in each case. Application of the CPMG sequence is shown in Fig. 2.31 for a sample with differing resonance linewidths and illustrates the faster disappearance of the broader resonances, that is, those with shorter T_2 . In reality, the determination of T_2 by any of these methods is still not straightforward. The most significant problem is likely to be from homonuclear couplings that are not refocused by the spin-echo and hence will impose additional phase modulations on the detected signals. As a result, studies involving T_2 measurements are even less widespread than those involving T_1 . Fortunately, from the point of view of performing practical day-to-day spectroscopy, exact T_2 values are not important and the value of T_2^* (which may be calculated from linewidths as described above) has far greater significance. It is this value that determines the rate of decay of transverse magnetisation, so it effectively defines how long a multipulse experiment can be before the system has decayed to such an extent that there is no longer any signal left to detect.

T_2 spectrum editing

One interesting use of these echo techniques lies in the exploitation of gross differences in transverse relaxation times of different species. Larger molecules typically display broader resonances than smaller ones since they possess shorter T_2 spin relaxation times. If the differences in these times are sufficiently large, resonances of the faster relaxing species can be preferentially reduced in intensity with the CPMG echo sequence, whilst the resonances of smaller, slower relaxing molecules decrease by a lesser amount (Fig. 2.32). This therefore provides a means, albeit a rather crude one, of editing a spectrum according to molecular size, retaining the resonances of the smaller components at the expense of the more massive ones. This approach has been widely used in the study of biofluids to suppress background contributions from very large macromolecules such as lipids and proteins.

The selective reduction of a solvent water resonance can also be achieved in a similar way if the transverse relaxation time of the water protons can be reduced (i.e. the resonance broadened) such that this becomes very much shorter than that of the solutes under investigation. This can be achieved by the addition of suitable paramagnetic relaxation agents (about which the water molecules form a hydration sphere) or by reagents that promote chemical exchange. Ammonium chloride and hydroxylamine have been used to great effect in this way [4,5], as illustrated for the proton spectrum of the reduced arginine

Figure 2.32. The T_2 filter. The broad resonances of polystyrene ($M_r = 50,000$) in (a) have been suppressed in (b) through T_2 -based editing with the CPMG sequence, leaving only the resonances of the smaller camphor molecule. The τ delay was 1.5 ms, and the echo was repeated 150 times to produce a total relaxation delay period $2\tau n$ of 450 ms.

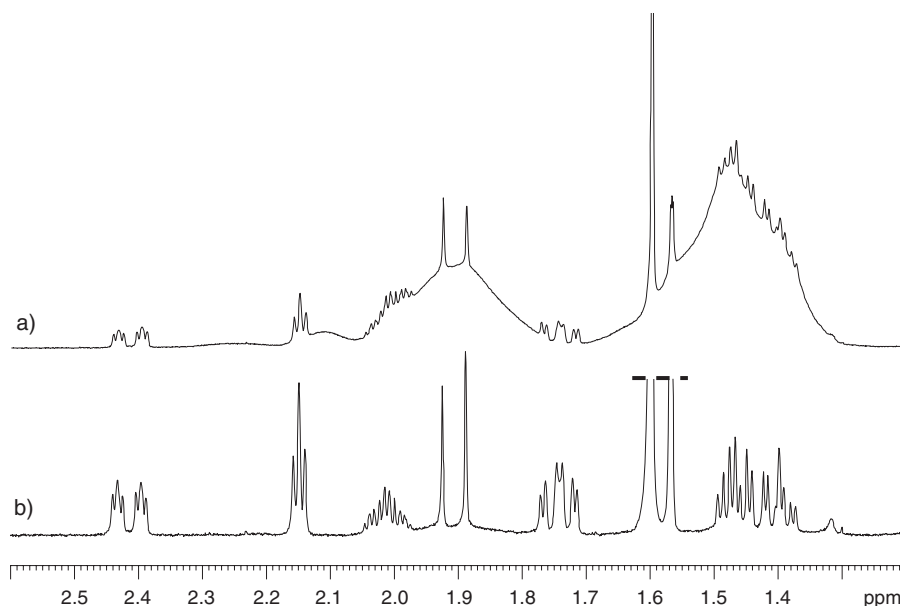
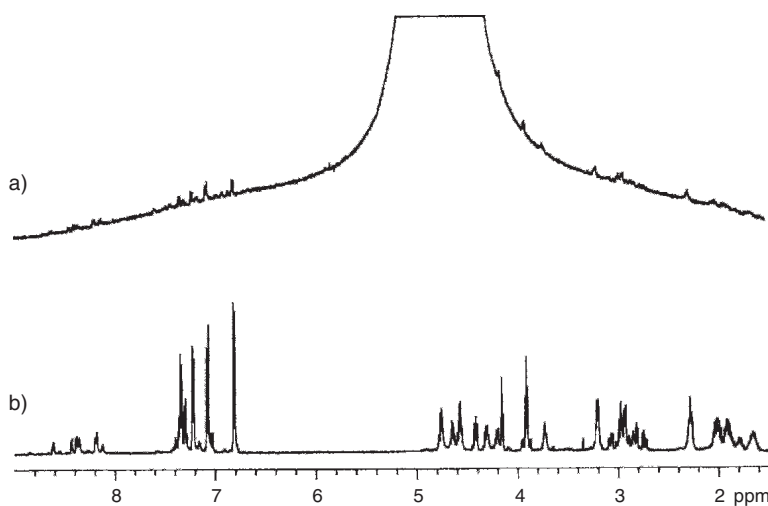


Figure 2.33. Solvent attenuation with the WATR method. (a) The 1D proton spectrum of 8 mM reduced arginine vasopressin in 90% H_2O /10% D_2O , pH = 2.75 containing 0.2 M NH_2OH . (b) The same sample recorded with the CPMG sequence using a total relaxation delay period of 235 ms (reproduced with permission from [6], Copyright © 1991 John Wiley & Sons Limited).



vasopressin peptide in 90% H_2O [6] (Fig. 2.33). This method of solvent suppression has been termed WATR (water attenuation by transverse relaxation). Whilst capable of providing impressive results, it does have limited application; more general solvent suppression procedures are described in Chapter 10.

2.5. MECHANISMS FOR RELAXATION

Nuclear spin relaxation is not a spontaneous process, it requires stimulation by a suitable fluctuating field to induce the necessary spin transitions, and there are four principle mechanisms that are able to do this – the dipole–dipole, chemical shift anisotropy (CSA), spin rotation and quadrupolar mechanisms. Which of these is the dominant process can directly influence the appearance of an NMR spectrum, and it is these factors we consider here. The emphasis is not so much on the explicit details of the underlying mechanisms, which can be found in physical NMR texts [7,8], but on the manner in which the spectra are affected by these mechanisms and how, as a result, different experimental conditions influence the observed spectrum.

2.5.1. The path to relaxation

The fundamental requirement for longitudinal relaxation of a spin- $1/2$ nucleus is a time-dependent magnetic field fluctuating at the Larmor frequency of the nuclear spin. Only through this can a change of spin state be induced or, in other words, can relaxation occur. Local magnetic fields arise from a number of sources, described below, whilst their time

dependence originates in the motions of the molecule (vibration, rotation, diffusion, etc.). In fact, only the chaotic tumbling of a molecule occurs at a rate that is appropriate for nuclear spin relaxation, others being either too fast or too slow. This random motion occurs with a spread of frequencies according to the molecular collisions, associations and so on experienced by the molecule, but is characterised by a rotational *correlation time*, τ_c , the average time taken for the molecule to rotate through one radian. Short correlation times therefore correspond to rapid tumbling and *vice versa*. The frequency distribution of the fluctuating magnetic fields associated with this motion is termed the *spectral density*, $J(\omega)$, and may be viewed as being proportional to the probability of finding a component of the motion at a given frequency, ω (in rad s^{-1}). Only when a suitable component exists at the spin Larmor frequency, can longitudinal relaxation occur. The spectral density function has the general form

$$J(\omega) = 2 \frac{\tau_c}{1 + \omega^2 \tau_c^2} \quad (2.14)$$

and is represented schematically in Fig. 2.34a for fast, intermediate and slow molecular tumbling rates (note the conventional use of the logarithmic scale). As each curve represents a probability, the area under each remains constant. For the Larmor frequency ω_0 indicated in Fig. 2.34a, the corresponding graph of T_1 against molecular tumbling rates is also given (Fig. 2.34b). Fast molecular motion has only a relatively small component at the Larmor frequency, so relaxation is slow (T_1 is long). This is the region occupied by small molecules in low viscosity solvents, known as the *extreme narrowing limit*. As the tumbling rates decrease, the spectral density at ω_0 initially increases but then falls away once more for slow tumbling so the T_1 curve has a minimum at intermediate rates. Thus, for small rapidly tumbling molecules, faster motion corresponds to slower relaxation and hence narrower linewidths, since longitudinal and transverse relaxation rates are identical ($T_2 = T_1$) under these conditions. A reduction in tumbling rate, such as by an increase in solvent viscosity or reduction in sample temperature, reduces the relaxation times and broadens the NMR resonance. The point at which the minimum is encountered and the slow motion regime approached is field dependent because ω_0 itself is field dependent (Fig. 2.34b). Behaviour in the slow motion regime is slightly more complex. The energy-conserving flip-flop processes that lead to transverse relaxation are also stimulated by very low frequency

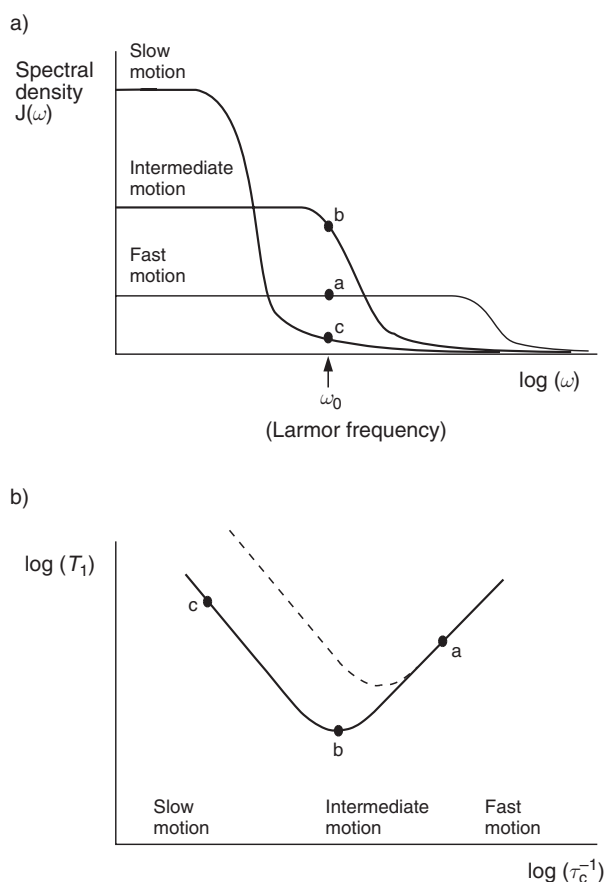
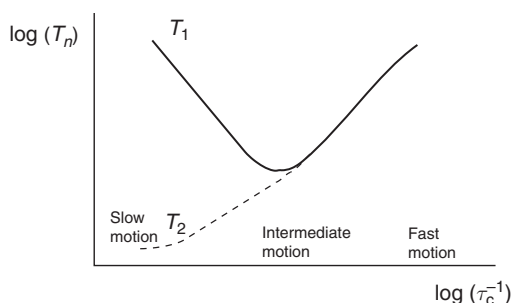


Figure 2.34. (a) A schematic representation of the spectral density as a function of frequency shown for molecules undergoing fast, intermediate and slow tumbling. For spins with a Larmor frequency ω_0 , the corresponding T_1 curve is shown in (b) as a function of molecular tumbling rates (inverse correlation times, τ_c). The T_1 curve is field dependent because ω_0 is field dependent and the minimum occurs for faster motion at higher fields (dashed curve in (b)).

Figure 2.35. A schematic illustration of the dependence of T_1 and T_2 on molecular tumbling rates. T_1 relaxation is insensitive to very slow motions whilst T_2 relaxation may still be stimulated by them.



fluctuations, and the T_2 curve differs markedly from that for T_1 (Fig. 2.35). Thus, for slowly tumbling molecules such as polymers and biological macromolecules, T_1 relaxation times can again be quite long but linewidths become rather broad as a result of short T_2 s.

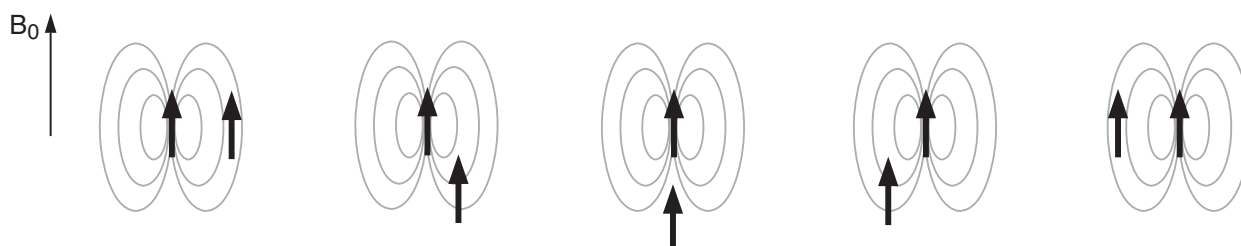
Molecular motion is therefore fundamental to the process of relaxation, but it remains to be seen how the fields required for this arise and how these mechanisms influence observed spectra.

2.5.2. Dipole–dipole relaxation

The most important relaxation mechanism for many spin- $\frac{1}{2}$ nuclei arises from the dipolar interaction between spins. This is also the source of the tremendously important NOE and further discussions on this mechanism can be found in Chapter 8, so are kept deliberately brief here. Dipolar interactions can be visualised using the ‘bar magnet’ analogy for a spin- $\frac{1}{2}$ nucleus in which each is said to possess a magnetic North and South pole. As two such dipoles approach, their associated magnetic fields interact; they attract or repel depending on their relative orientations. Now suppose these dipoles were two neighbouring nuclei in a molecule that is tumbling in solution. The orientation of each nucleus with respect to the static magnetic field does not vary as the molecule tumbles just as a compass needle maintains its direction as a compass is turned. However, their relative positions in space will alter and the local field experienced at one nucleus as a result of its neighbour will fluctuate as the molecule tumbles (Fig. 2.36). Tumbling at an appropriate rate can therefore induce relaxation.

This mechanism is often the dominant relaxation process for protons that rely on their neighbours as a source of magnetic dipoles. As such, protons that lack near neighbours relax more slowly (notice how the methine protons in α -pinene (Fig. 2.23) all have longer T_1 s than the methylene groups). The most obvious consequence of this is lower than expected integrals in routine proton spectra due to the partial saturation of the slower relaxing spins that are unable to recover sufficiently between each pulse-acquire sequence. If T_1 data are available, then protons with long relaxation times can be predicted to be remote from others in the molecule. Carbon-13 nuclei are also relaxed primarily by dipolar interactions, either with their directly bound protons or, in the absence of these, by more distant ones. In very large molecules and at high field, the CSA mechanism described below can also play a role, especially for sp and sp^2 centres. Likewise, this can be significant for spin- $\frac{1}{2}$ nuclei that exhibit large chemical shift ranges. Dipolar relaxation can also arise from the interaction of a nuclear spin with an unpaired electron, the magnetic moment of which is over 600 times that of the proton and so provides a very efficient relaxation source. This is sometimes referred to as the *paramagnetic relaxation* mechanism. Even the presence of dissolved oxygen, which is itself paramagnetic, can contribute to spin relaxation and the deliberate addition of relaxation agents containing paramagnetic species is sometimes used to

Figure 2.36. Dipole–dipole relaxation. The magnitude of the direct through space magnetic interaction between two spins is modulated by molecular tumbling and so induces spin transitions and hence relaxation.



reduce relaxation times and so speed data acquisition (Chapter 4). The most common reagents are chromium(III) acetylacetonate, $\text{Cr}(\text{acac})_3$, for organic solvents and manganese(II) chloride or gadolinium(III) chloride for aqueous solutions.

2.5.3. Chemical shift anisotropy relaxation

The electron distribution in chemical bonds is inherently unsymmetrical or *anisotropic*, and as a result, the local field experienced by a nucleus, and hence its chemical shift, will depend on the orientation of the bond relative to the applied static field. In solution, the rapid tumbling of a molecule averages this chemical shift anisotropy (CSA) such that one observes only a single frequency for each chemically distinct site, sometimes referred to as the isotropic chemical shift. Nevertheless, this fluctuating field can stimulate relaxation if sufficiently strong. This is generally the case for nuclei that exhibit a large chemical shift range since these possess the greatest shift anisotropy, for example, ^{19}F , ^{31}P and, in particular, many metals.

The characteristic feature of CSA relaxation is its dependence on the *square* of the applied field, meaning it has greater significance at higher B_0 . For example, the selenium-77 longitudinal relaxation rate in the selenone **2.3** was shown to be linearly dependent on B_0^2 , indicating CSA to be a significant relaxation mechanism [9] (Table 2.2), whilst no proton-selenium NOE could be detected, demonstrating the ^1H - ^{77}Se dipole-dipole mechanism to be ineffectual. Nuclei whose relaxation is dominated by the CSA mechanism may show significantly larger linewidths at higher fields if they possess a large shift anisotropy, and any potential benefits of greater dispersion and sensitivity may be lost by line broadening. For this reason, the study of some metal nuclei may be more successful at lower fields. Reducing the correlation time, by warming the sample for example, may attenuate the broadening effect, although this approach clearly has rather limited application. In some cases, enhanced CSA relaxation at higher fields can be advantageous. A moderately enhanced relaxation rate, such as in the ^{77}Se example above, allows for more rapid data collection (Section 4.1), thus providing an improvement in sensitivity (per unit time) above that expected from the increase in magnetic field alone.

The CSA mechanism can also have a perhaps unexpected influence on the spectra of nuclei that are scalar spin-coupled to the CSA-relaxed spin. If CSA causes very rapid relaxation, the satellites arising from coupling to this spin will broaden and may even disappear altogether. Thus, whilst the coupling may be apparent at low field, it may vanish at higher values. This effect can be seen in the proton spectra of the platinum complex **2.4** recorded at 80 and 400 MHz [10] (Fig. 2.37). The increased linewidth of the satellites relative to the parent line at higher field scales with the square of the applied field, as expected for the CSA mechanism. To understand why this occurs, consider the origin of the Pt satellites themselves. These doublet components arise from the spin- $1/2$ ^{195}Pt nuclei existing in one of two states, α and β , which result in a frequency difference of $J_{\text{Pt-H}}$ Hz for the corresponding proton signals. The CSA relaxation induces rapid transitions of the platinum spins between these states causing the doublet components to repeatedly switch positions. As this exchange rate (i.e. relaxation rate) increases, the satellites first broaden and will eventually merge into the parent line, as for any dynamic process. This rapid and repeated change of spin states has a direct analogy with conventional spin decoupling (Chapter 4).

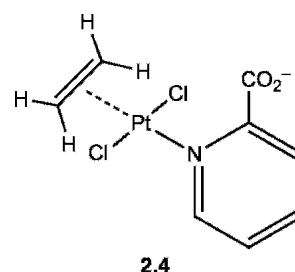
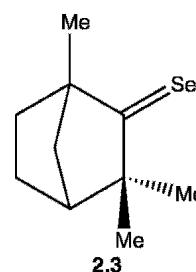


Table 2.2. The ^{77}Se longitudinal relaxation times as a function of B_0 and the corresponding dependence of the relaxation rate on the square of the applied field (adapted with permission from reference [9], Copyright 1984, Elsevier)

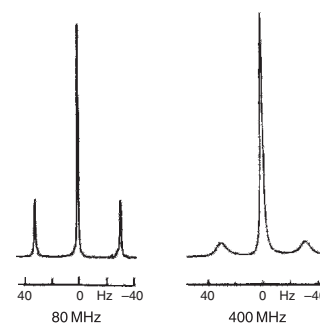
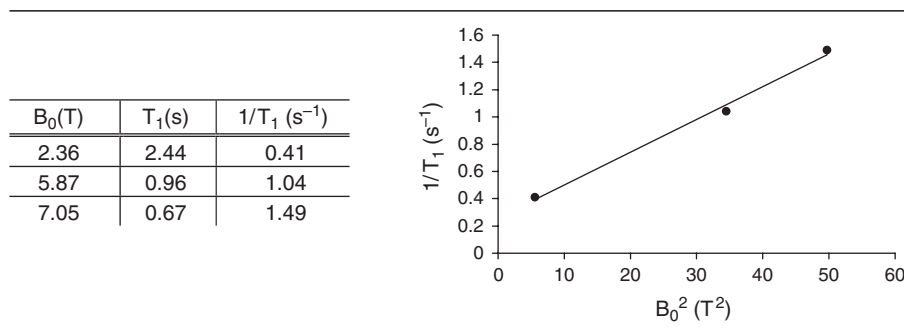


Figure 2.37. The ethene proton resonance of the platinum complex **2.4** in CDCl_3 at 80 and 400 MHz showing broadening of the ^{195}Pt satellites at higher field (reproduced with permission from [10], Copyright 1982, Elsevier).

2.5.4. Spin-rotation relaxation

Molecules or groups that rotate very rapidly have associated with them a *molecular* magnetic moment generated by the rotating electronic and nuclear charges. The field due to this fluctuates as the molecule or group changes its rotational state as a result of, for example, molecular collisions, and this provides a further mechanism for nuclear relaxation. This is most effective for small, symmetrical molecules or for freely rotating methyl groups, and its efficiency *increases* as tumbling rates *increase*. This is in contrast to the previously described mechanisms. Thus, heating a sample enhances spin-rotation relaxation, this temperature dependence being characteristic of this mechanism and allowing its presence to be established.

2.5.5. Quadrupolar relaxation

The quadrupolar relaxation mechanism is only directly relevant for those nuclei that have a nuclear spin quantum number, I , greater than $\frac{1}{2}$ (quadrupolar nuclei) and is often the dominant relaxation process for these. This can also be a very efficient mechanism, and the linewidths of many such nuclei can be hundreds or even thousands of hertz wide. The properties of selected nuclei with $I > \frac{1}{2}$ are summarised in Table 2.3. Whilst the direct observation of these nuclei may not be routine for many organic chemists, their observation can, at times, prove very enlightening for specific problems, and the indirect effects they have on the spectra of spin- $\frac{1}{2}$ nuclei should not be overlooked.

Quadrupolar nuclei possess an electric quadrupole moment in addition to a magnetic dipole moment. This results from the charge distribution of the nucleus deviating from the usual spherical symmetry associated with spin- $\frac{1}{2}$ nuclei and becoming ellipsoidal in shape. This can be viewed as arising from two back-to-back *electric* dipoles (Fig. 2.38). As such, the quadrupole moment is influenced by electric field *gradients* about the nucleus, but not by symmetric electric fields. The gradient is modulated as the molecule tumbles in solution, and again if this occurs at the appropriate frequency, it can induce flipping of nuclear spin states and thus stimulate relaxation. This is analogous to the relaxation of nuclear dipoles by time-dependent local magnetic fields, but the quadrupolar relaxation mechanism is the only one that depends on electric rather than magnetic interactions.

The relaxation rates of a quadrupolar nucleus are dictated by two new factors not previously considered. The first is the magnitude of the quadrupole moment itself (Table 2.3). Larger values contribute to more efficient spin relaxation and hence broader linewidths, whereas smaller values typically produce sharper lines. Thus, those nuclei with smaller quadrupole moments are usually more favoured for NMR observation. As before, for the mechanism to be effective, molecular tumbling must occur at an appropriate

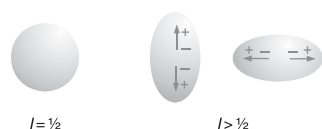


Figure 2.38. Quadrupolar nuclei lack the spherical charge distribution of spin- $\frac{1}{2}$ nuclei, having an ellipsoidal shape that may be viewed as arising from pairs of electric dipoles. Thus, quadrupolar nuclei interact with electric field gradients.

Table 2.3. Properties of selected quadrupolar nuclei. The observation of such nuclei is generally most favourable for those of low quadrupole moment and high natural abundance that exist in more highly symmetric environments; those listed in *italics* are considered the least favoured of the available isotopes. The NMR frequencies are quoted for a 400 MHz instrument (9.4 T magnet) and sensitivities are relative to ^1H and take account of both the intrinsic sensitivity of the nucleus and its natural abundance

Isotope	Spin (I)	Natural abundance (%)	Quadrupole moment (10^{-28} m^2)	NMR frequency (MHz)	Relative sensitivity
^2H	1	0.015	2.8×10^{-3}	61.4	1.45×10^{-6}
^6Li	1	7.42	-8.0×10^{-4}	58.9	6.31×10^{-4}
^7Li	$3/2$	92.58	-4×10^{-2}	155.5	0.27
^{10}B	3	19.58	8.5×10^{-2}	43.0	3.93×10^{-3}
^{11}B	$3/2$	80.42	4.1×10^{-2}	128.3	0.13
^{14}N	1	99.63	1.0×10^{-2}	28.9	1.01×10^{-3}
^{17}O	$5/2$	0.037	-2.6×10^{-2}	54.2	1.08×10^{-5}
^{23}Na	$3/2$	100	0.10	105.8	9.27×10^{-2}
^{27}Al	$5/2$	100	0.15	104.2	0.21
^{33}S	$3/2$	0.76	-5.5×10^{-2}	30.7	1.72×10^{-5}
^{35}Cl	$3/2$	75.73	-0.1	39.2	3.55×10^{-3}
^{37}Cl	$3/2$	24.47	-7.9×10^{-2}	32.6	6.63×10^{-4}
^{59}Co	$7/2$	100	0.38	94.5	0.28

frequency, so again fast molecular tumbling reduces the effectiveness, leading to longer relaxation times and sharper lines. High temperatures or lower viscosity solvents are thus more likely to produce narrow linewidths. The ultimate in low viscosity solvents are supercritical fluids that have viscosities more like those of a gas yet solubilising properties more like liquids. These have indeed been used in the study of quadrupolar nuclei [11], but since they are only supercritical at very high pressures they demand the use of single-crystal sapphire NMR tubes, so their use cannot be considered routine. The second new factor is the magnitude of the electric field gradient. In highly symmetrical environments, such as tetrahedral or octahedral symmetries, the field gradient is, in principle, zero and the quadrupolar mechanism is suppressed. In reality, local distortions still arise, if only momentarily, introducing an element of asymmetry and hence enhanced relaxation and line broadening. Nevertheless, a higher degree of electrical symmetry can be correlated with narrower resonances. Thus, for example, the ^{14}N linewidth of $\text{N}(\text{Me})_4^+$ is less than 1 Hz whereas that for NMe_3 is nearer to 80 Hz. Linewidth changes in ^{11}B spectra ($I = 3/2$) have been used in the identification of tetrahedral boronic acid complexes at the active site of β -lactamases [12], enzymes responsible for the destruction of β -lactam antibiotics such as penicillins, and part of the defence mechanism of bacteria. Boronic acids, such as 3-dansylamidophenylboronic acid **2.5**, are known to be reversible inhibitors of active site serine β -lactamases, and the complexes so formed display significant changes in the ^{11}B chemical shift of the boronic acid together with a reduction in linewidth relative to the free acid (Fig. 2.39). This reduction is attributed to the boron nucleus taking up a more symmetrical tetrahedral environment as it becomes bound by the enzyme's active site serine. This shift and line narrowing can be mimicked by placing the boronic acid in alkaline solution in which the $\text{R-B}(\text{OH})_3^-$ ion predominates.

The broad resonances of many quadrupolar nuclei means field inhomogeneity makes a negligible contribution to linewidths, so the methods described previously for measuring relaxation times are no longer necessary. For small molecules at least, T_2 and T_1 are identical and can be determined directly from the half-height linewidth. The broad resonances together with the sometimes low intrinsic sensitivity and low natural abundance of quadrupolar nuclei are the principal reasons for their relatively low popularity for NMR studies relative to spin- $1/2$ nuclei. The very fast relaxation of certain quadrupolar nuclei can also make their direct observation difficult with conventional high-resolution spectrometers; see Section 4.5.

Scalar coupling to quadrupolar nuclei

Probably of more relevance to the practising organic chemist is the influence quadrupolar nuclei have on the spectra of spin- $1/2$ nuclei, by virtue of their mutual scalar coupling. Coupling to a quadrupolar nucleus of spin I produces, in theory, $2I + 1$ lines, so, for example, the carbon resonance of CDCl_3 appears as a 1:1:1 triplet (^2H has $I = 1$) by virtue of the 32 Hz ^{13}C - ^2H coupling. However, more generally, if the relaxation of the quadrupolar nucleus is rapid relative to the magnitude of the coupling, the splitting can be lost, in much the same way that coupling to a nucleus experiencing rapid CSA relaxation is lost. The carbon resonance of CDCl_3 is only a triplet because deuterium has a relatively small quadrupole moment making its coupling apparent whereas all coupling to the chlorine nuclei (^{35}Cl and ^{37}Cl have $I = 3/2$) is quenched by the very rapid relaxation of these spins (Fig. 2.40). Similarly, the proton resonance of CHCl_3 is a sharp singlet despite the presence of the neighbouring chlorine atoms. A common example of line broadening brought about by a quadrupolar nucleus is seen for the resonances of amino and amido protons due to the adjacent ^{14}N (Fig. 2.31).

The appearance of the spin- $1/2$ nucleus spectrum is therefore also influenced by the factors described above, which dictate the rate of quadrupolar relaxation. Couplings to quadrupolar nuclei that exist in a highly symmetrical environment are likely to be seen because of the slower relaxation the nuclei experience. For this reason, the proton spectrum of $^{14}\text{NH}_4^+$ is an unusually sharp 1:1:1 triplet (Fig. 2.41, ^{14}N has $I = 1$) and the fluorine spectrum of $^{11}\text{BF}_4^-$ is a sharp 1:1:1:1 quartet (Fig. 2.42, ^{11}B has $I = 3/2$). Increasing sample temperature results in slower relaxation of the quadrupolar nucleus, so there is also a greater chance of the coupling being observable. In contrast, reducing the temperature increases relaxation rates and collapses coupling fine structure. This is contrary to the usual behaviour associated with dynamic systems where heating typically leads to simplification of spectra by virtue of resonance coalescence. The likelihood of coupling fine structure being lost is also increased as the magnitude of the coupling constant decreases. In general then, the observation of scalar coupling to a quadrupolar nucleus is the exception rather than the rule.

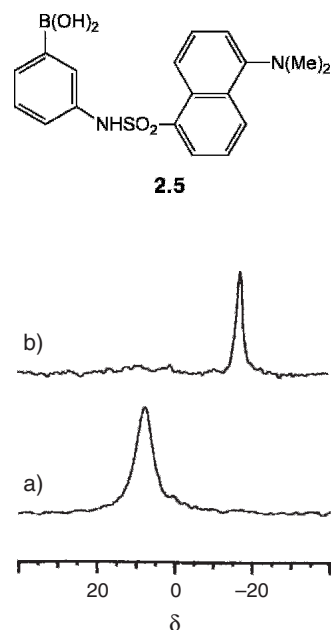


Figure 2.39. The ^{11}B NMR spectrum (128 MHz) of dansylamidophenylboronic acid **2.5** (a) as the free trigonal boronic acid ($\Delta\nu_{1/2} = 580$ Hz) and (b) as the tetrahedral complex with the active site serine of the P99 β -lactamase from *Enterobacter cloacae* ($\Delta\nu_{1/2} = 160$ Hz). Spectra are referenced to external trimethylborate (adapted with permission from [12], Copyright Royal Society of Chemistry).

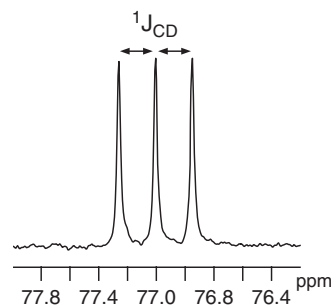


Figure 2.40. The carbon-13 spectrum of CDCl_3 reveals coupling to deuterium but not to chlorine-35 or chlorine-37.

Figure 2.41. The ^1H spectrum of ammonium nitrate in DMSO (a) before and (b) after acidification. In (b), the NH_4^+ ion dominates and the induced symmetry reduces the ^{14}N quadrupolar relaxation rate and reveals the ^1H - ^{14}N one-bond coupling constant (51 Hz).

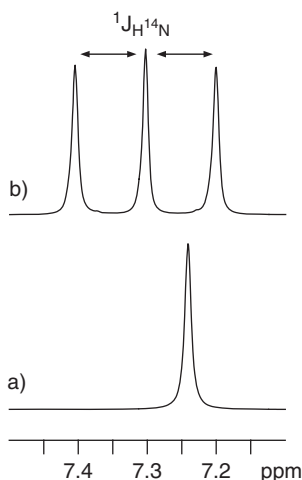
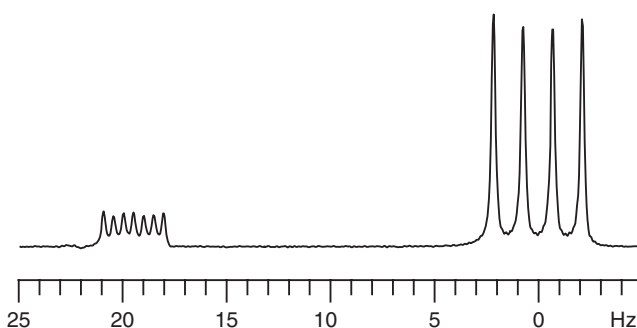


Figure 2.42. The ^{19}F spectrum of sodium borofluoride in D_2O . The $^{11}\text{BF}_4^-$ ion produces the even quartet (^{11}B , $I = 3/2$, 80% abundance, $J = 1.4\text{ Hz}$) and the $^{10}\text{BF}_4^-$ ion produces the even septet (^{10}B , $I = 3$, 20% abundance, $J = 0.5\text{ Hz}$). The frequency difference between the two is due to the $^{10}\text{B}/^{11}\text{B}$ isotope shift.



REFERENCES

- [1] R.K. Harris and E.D. Becker, *J. Magn. Reson.*, 2002, **156**, 323–326.
- [2] R.K. Harris, E.D. Becker, S.M. Cabral de Menezes, R. Goodfellow and P. Granger, *Pure Appl. Chem.*, 2001, **73**, 1795–1818.
- [3] R.K. Harris, J. Kowalewski and S. Cabral de Menezes, *Magn. Reson. Chem.*, 1998, **36**, 145–149.
- [4] D.L. Rabenstein, S. Fan and T.K. Nakashima, *J. Magn. Reson.*, 1985, **64**, 541–546.
- [5] D.L. Rabenstein, G.S. Srivasta and R.W.K. Lee, *J. Magn. Reson.*, 1987, **71**, 175–179.
- [6] C.K. Larive and D.L. Rabenstein, *Magn. Reson. Chem.*, 1991, **29**, 409–417.
- [7] R.K. Harris, *Nuclear Magnetic Resonance Spectroscopy*, Longman, Harlow, 1986.
- [8] M. Levitt, *Spin Dynamics*, Wiley, New York, 2001.
- [9] T.C. Wong, T.T. Ang, F.S. Guziec and C.A. Moustakis, *J. Magn. Reson.*, 1984, **57**, 463–470.
- [10] I.M. Ismail, S.J.S. Kerrison and P.J. Sadler, *Polyhedron*, 1982, **1**, 57–59.
- [11] A.P. Waugh and G.A. Lawless, in *Advanced Applications of NMR to Organometallic Chemistry* (Eds.: M. Gielen, R. Willem, and B. Wrackmeyer), Wiley, Chichester, 1996.
- [12] J.E. Baldwin, T.D.W. Claridge, A.E. Derome, B.D. Smith, M. Twyman and S.G. Waley, *J. Chem. Soc.-Chem. Commun.* 1991, 573–574.

Chapter 3

Practical aspects of high-resolution NMR

NMR spectrometers are expensive instruments, representing one of the largest financial investments a chemical laboratory is likely to make, and to get the best results from these, they must be operated and maintained in the appropriate manner. This chapter explores some of the fundamental experimental aspects of relevance to high-resolution, solution-state NMR spectroscopy, from instrumental procedures through to the preparation of samples for analysis. Later sections also deal with the basics of calibrating a spectrometer and assessing its performance.

3.1. AN OVERVIEW OF THE NMR SPECTROMETER

A schematic illustration of a modern NMR spectrometer is presented in Fig. 3.1. The fundamental requirement for high-resolution NMR spectroscopy is an intense static magnetic field that is provided, nowadays exclusively, by superconducting solenoid magnets composed of niobium–tin alloy wire. These are able to produce the stable and persistent magnetic fields demanded by NMR spectroscopy of up to 22.3 T with current technology, corresponding to proton frequencies of 950 MHz. The drive for ever-increasing magnetic fields, encouraged by the demands for greater signal dispersion and instrument sensitivity, has continued since the potential of NMR spectroscopy as an analytical tool was first realised over 60 years ago (Fig. 3.2) and still represents an area of intense competition between magnet manufacturers. At the time of writing it appears that proton observation frequencies beyond the 1 GHz ceiling will demand the use of newer high-temperature superconducting wires to carry the huge currents required, which not only presents substantial technical challenges for the manufacturer but will likely further increase magnet costs for the end user. However, these very high-field magnets are currently of most interest to areas such as structural biology whilst in the area of organic chemistry the need for such extreme signal dispersion may be less important, although enhancements in sensitivity remain welcome and valuable developments. In recent years, there have been a number of advances in probe technologies that provide the chemist with very significant sensitivity increases without the need for higher field instrumentation, and these will also be reviewed later in this chapter.

Figure 3.1. A schematic illustration of the modern NMR spectrometer. The greyed sections are essential components of the spectrometer, whereas the others may be considered optional features (although pulsed field gradients are essentially standard on research instruments).

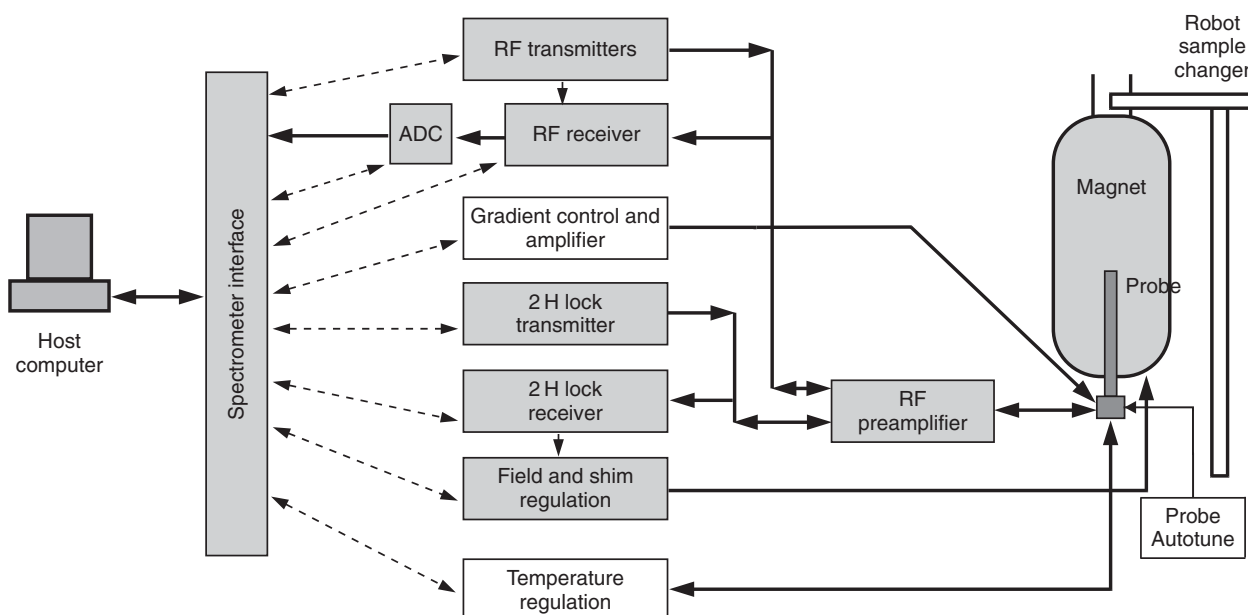
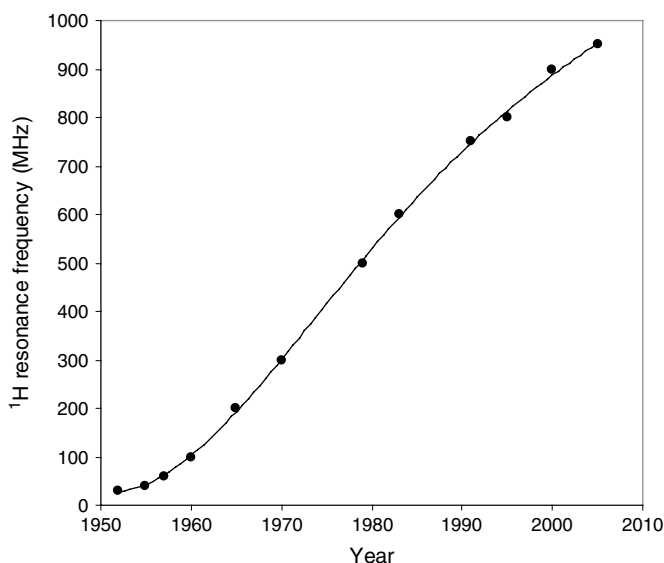


Figure 3.2. The increase in proton resonance frequency since the introduction of NMR spectroscopy as an analytical method (adapted with permission from reference [1] and extended Copyright 1995, Elsevier).



The magnet solenoid operates in a bath of liquid helium (at 4 K), surrounded by a radiation shield and cooled by a bath of liquid nitrogen (at 77 K), itself surrounded by a high vacuum. This whole assembly is an extremely efficient system, and once energised, the magnet operates for many years free from any external power source. It requires only periodic refilling of the liquid cryogenics, typically on a weekly or bi-weekly basis for the nitrogen, but only every 2–12 months for helium depending on magnet age and construction. The central bore of the magnet dewar is itself at ambient temperature, and this houses a collection of electrical coils, known as the shim coils, which generate their own small magnetic fields and are used to trim the main static field and remove residual inhomogeneities. This process of optimising the magnetic field homogeneity, known as *shimming*, is required for each sample to be analysed, and it is discussed in Section 3.4.5. Within the shim coils at the exact centre of the magnetic field sits the head of the probe, the heart of any NMR spectrometer. This houses the rf coil(s) and associated circuitry that act as antennae, transmitting and receiving the electromagnetic radiation. These coils may be further surrounded by pulsed field gradient coils that serve to *destroy* field homogeneity in a controlled fashion (this may seem a rather bizarre thing to do, but it turns out to have rather favourable effects in numerous experiments). The sample, sitting in a cylindrical glass tube, is held in a turbine or ‘spinner’ and descends into the probe head on a column of air or nitrogen. For routine 1D experiments, it is still common practice to spin the sample at 10–20 Hz whilst in the probe to average to zero field inhomogeneities in the transverse (*x*–*y*) plane and so improve signal resolution. Sample spinning is now rarely used for multidimensional NMR experiments on modern instruments, as it can induce additional signal modulations and associated undesirable artefacts. This requires that acceptable resolution can be obtained on a static sample, and while this is perfectly feasible with modern shim technology, older instruments may still demand spinning for all work.

Probes come in various sizes of diameter and length, depending on the magnet construction, but are more commonly referred to by the diameter of the sample tube they are designed to hold. The most widely used tube diameter is still 5 mm, other available sizes have included 1, 1.7, 2.5, 3, 8 and 10 mm (Section 3.4). Probes may be dedicated to observing one frequency (*selective* probes), may be tuneable over a very wide frequency range (*broadband* probes) or may tune to predefined frequency ranges, for example 4- or quad-nucleus probes. In all cases, they will also be capable of observing the deuterium frequency simultaneously to provide a signal for field regulation (the ‘lock’ signal). A second (outer) coil is often incorporated to allow the simultaneous application of pulses on one or more additional nuclei.

In many locations, it is advantageous to mount the whole of the magnet assembly on a vibration damping system as floor vibrations (which may arise from a whole host of sources including natural floor resonances, air conditioners, movement in the laboratory and so on) can have deleterious effects on spectra, notably around the base of resonances (Fig. 3.3). Whilst such artefacts have lesser significance to routine 1D observations, they may severely interfere with the detection of signals present at low levels, for example those in hetero-nuclear correlation or nuclear Overhauser effect experiments.

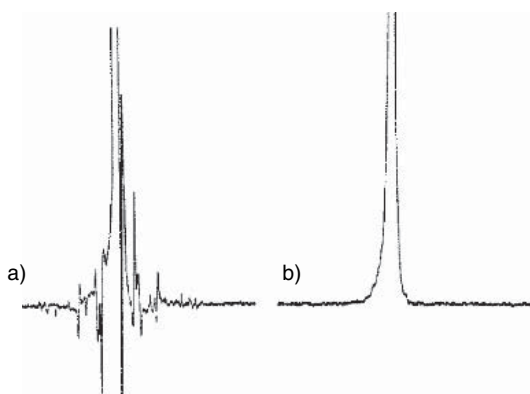


Figure 3.3. Floor vibrations can introduce unwelcome artefacts around the base of a resonance (a), which can be largely suppressed by mounting the magnet assembly on an anti-vibration stand (b) (figure courtesy of Bruker BioSpin).

Within the spectrometer cabinet sit the rf transmitters and the detection system for the observation channel, additional transmitter channels¹, the lock channel and the PFG transmitter. Most spectrometers come in either a two-channel or three-channel configuration, plus the lock channel. The spectrometer is controlled via the host computer (for which there has been a move towards Windows- or Linux-based PC systems in recent years), which is linked to the spectrometer via a suitable interface such as Ethernet. The electrical analogue NMR signals are converted to the digital format required by the host computer via the analogue-to-digital converter (ADC), the characteristics of which can have important implications for the acquisition of NMR data (Section 3.2). The computer also processes the acquired data, although this may also be performed ‘off-line’ with one of the many available NMR software packages.

Various optional peripherals may also be added to the instrument, such as variable temperature units that allow sample temperature regulation within the probe, robotic sample changers and so on. The coupling of NMR with other analytical techniques such as HPLC has become an established method through the development of flow probes and gained popularity in certain areas. The need for this will obviously depend on the type of samples handled and the nature of the experiments employed.

With the hype surrounding the competition between instrument manufacturers to produce ever-increasing magnetic fields, it is all too easy for one to become convinced that an instrument operating at the highest available field strength is essential in the modern laboratory. Whilst the study of biological macromolecules no doubt benefits from the greater sensitivity and dispersion available, problematic small or mid-sized molecules are often better tackled through the use of the appropriate modern techniques. Signal dispersion limitations are generally less severe and may often be overcome by using suitably chosen higher dimensional experiments. Sensitivity limitations, which are usually due to a lack of material rather than solubility or aggregation problems, may be tackled by utilising smaller probe geometries or cryogenically cooled probe heads (Section 3.4.2). So, for example, if one has insufficient material to collect a carbon-13 spectrum, one could consider employing a proton-detected heteronuclear correlation experiment to determine these shifts indirectly. Beyond such considerations, there are genuine physical reasons largely relating to the nature of nuclear spin relaxation, which mean that certain experiments on small molecules are likely to work less well at very high magnetic fields. In particular, this relates to the nuclear Overhauser effect (Chapter 8), one of the principal NMR methods in structure elucidation. For many cases commonly encountered in the chemical laboratory, a lower field instrument of modern specification is sufficient to enable the chemist to unleash an array of modern pulse NMR experiments on the samples of interest and subsequently solve the problem in hand. A better understanding of these modern NMR methods should aid in the selection of the appropriate experiments.

3.2. DATA ACQUISITION AND PROCESSING

This section examines some of the spectrometer procedures that relate to the collection, digitisation and computational manipulation of NMR data, including some of the fundamental parameters that define the way in which data are acquired. Such technicalities may

¹ Reference to the “decoupler channel(s)” is often used when referring to these additional channels, but this should not be taken too literally as they may only be used for the application of only a few pulses rather than a true decoupling sequence. This nomenclature stems from the early developments of NMR spectrometers when the additional channel was only capable of providing “noise decoupling”, usually of protons.

not seem relevant to anyone who does not consider themselves a spectroscopist, but the importance of understanding a few basic relationships between experimental parameters comes from the need to recognise spectrum artefacts or corrupted data that can result from inappropriate parameter settings and to appreciate the limitations inherent in NMR measurements. Only then can one make full and appropriate use of the spectroscopic information at hand.

3.2.1. Pulse excitation

It is widely appreciated that modern NMR spectrometers use a ‘short pulse’ of rf energy to excite nuclear resonances over a range of frequencies. This pulse is supplied as *monochromatic* radiation from the transmitter; yet, the nuclear spin transitions giving rise to our spectra vary in energy according to their differing Larmor frequencies and so it would appear that the pulse will be unable to excite all resonances in the spectrum simultaneously. However, Heisenberg’s Uncertainty Principle tells us that an excitation pulse of duration Δt has associated with it a frequency uncertainty or spread of around $1/\Delta t$ Hz and so effectively behaves as if it were polychromatic. The duration of the rf pulse Δt is usually referred to as the *pulse width*. A short, high-power pulse provides excitation over a wide frequency window whilst a longer, low-power pulse (which will provide the same net tip angle) is effective over a much smaller region (Fig. 3.4).

Consider a proton spectrum recorded at 400 MHz with the transmitter frequency placed in the centre of the spectral region of interest. A 10 ppm spectral window corresponds to 4000 Hz; therefore, we need to excite over ± 2000 Hz, meaning the pulse duration must be 0.5 ms or less. The observation frequency for carbon-13 on the same instrument would be 100 MHz, so a typical 200 ppm spectral width (SW) corresponds to ± 10 kHz, requiring a pulse of only 100 μ s but with considerably higher power as its energy is now spread over a wider area. If the pulse were to be made very long (say tens of milliseconds) and weak, it would only excite over a rather small frequency range, giving rise to *selective excitation* of only part of the spectrum. As will become apparent in later chapters, the use of selective excitation methods is now commonplace in numerous NMR experiments. Long, low-power pulses that excite only a selected region of a spectrum are commonly referred to as ‘soft pulses’, whereas those that are of short duration and of high power are termed ‘hard pulses’. Unless stated explicitly, all pulses in this book will refer to non-selective hard pulses.

Off-resonance effects

In practice, modern NMR instruments are designed to deliver high-power 90° pulses closer to 10 μ s, rather than the hundreds predicted from the above arguments. This is to suppress the undesirable effects that arise when the pulse rf frequency is *off-resonance*, that is, when the transmitter frequency does not exactly match the nuclear Larmor frequency, a situation of considerable practical significance that has been ignored thus far.

As shown in Chapter 2, spins that are on-resonance have their magnetisation vector driven about the rf B_1 field towards the x - y plane during the pulse. Those spins that are off-resonance will, in addition to this B_1 field, experience a *residual* component ΔB of the *static* B_0 field along the z axis of the rotating frame for which:

$$\frac{\gamma \Delta B}{2\pi} = \gamma \Delta B = \Delta \nu \text{ Hz} \quad (3.1)$$

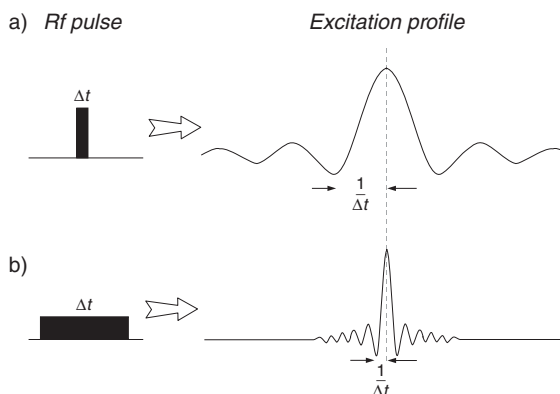


Figure 3.4. A single monochromatic radiofrequency pulse has an effective excitation bandwidth that depends inversely on the duration of the pulse. A short intense pulse is therefore able to excite over a wide frequency window (a), whereas a longer weaker pulse provides a more selective excitation profile (b).

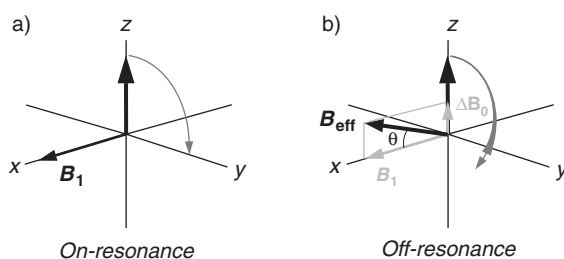


Figure 3.5. Excitation of magnetisation for which the rf is on-resonance (a) results in the rotation of the bulk vector about the applied rf field, B_1 . Those spins which experience off-resonance excitation (b) are instead driven about an effective rf field, B_{eff} , which is tipped out of the x - y plane by an angle θ , which increases as the offset increases.

where $\Delta\nu$ represents the offset from the reference frequency. The vector sum of B_1 and ΔB is the *effective field* B_{eff} experienced by an off-resonance spin about which it rotates (or more correctly, nutates) (Fig. 3.5). This is greater than B_1 itself and is tilted away from the x - y plane by an angle θ , where

$$\tan \theta = \frac{\Delta B}{B_1} \quad (3.2)$$

For those spins further from resonance, the angle θ becomes greater and the net rotation towards the x - y plane diminishes until, in the limit, θ becomes 90° . In this case, the bulk magnetisation vector simply remains along the $+z$ axis and thus experiences no excitation at all. In other words, the nuclei resonate outside the *excitation bandwidth* of the pulse. Since an off-resonance vector is driven away from the y axis during the pulse it also acquires a (frequency-dependent) phase difference relative to the on-resonance vector (Fig. 3.6). This is usually small and an approximately linear function of frequency, so can be corrected by phase adjustment of the final spectrum (Section 3.2.8).

How deleterious off-resonance effects are in a pulse experiment depends to some extent on the pulse tip angle employed. A 90° excitation pulse ideally transfers magnetisation from the $+z$ axis into the x - y plane, but when off-resonance the tilt of the effective field will act to place the vector above this plane. However, the greater B_{eff} will mean the magnetisation vector follows a longer trajectory, and this increased net flip angle offers some compensation, and hence a 90° pulse is fairly tolerant to off-resonance effects as judged by the elimination of z -magnetisation (Fig. 3.7a). In contrast, a 180° inversion pulse ideally generates pure $-z$ -magnetisation leaving none in the transverse plane, but now the increased effective flip angle is detrimental, tending to move the vector further from the South Pole. Thus, 180° pulses do not perform well when applied off-resonance (Fig. 3.7b), and can be a source of poor experimental performance. In practice, it is relatively easy to provide sufficiently short pulses (ca. $10\mu\text{s}$) to ensure that excitation and inversion is reasonably uniform over the relatively small frequency ranges encountered in ^1H NMR spectroscopy. However, for nuclei that display a much greater frequency dispersion, such as ^{13}C or ^{19}F , this is often not the case, and resonance distortion and/or attenuation can occur, and spurious signals may arise in multipulse experiments as a result. Experimental approaches to overcoming these limitations include the use of clusters of pulses, known as *composite pulses*, or of frequency-swept *adiabatic pulses*, both of which aim to compensate for these (and other) defects; see Chapter 10 for further discussion.

Figure 3.6. Experimental excitation profiles for a 90° pulse as a function of resonance offset relative to the applied rf field strength γB_1 . Greater offsets introduce larger phase errors and reduce the amplitude of the resultant transverse magnetisation.

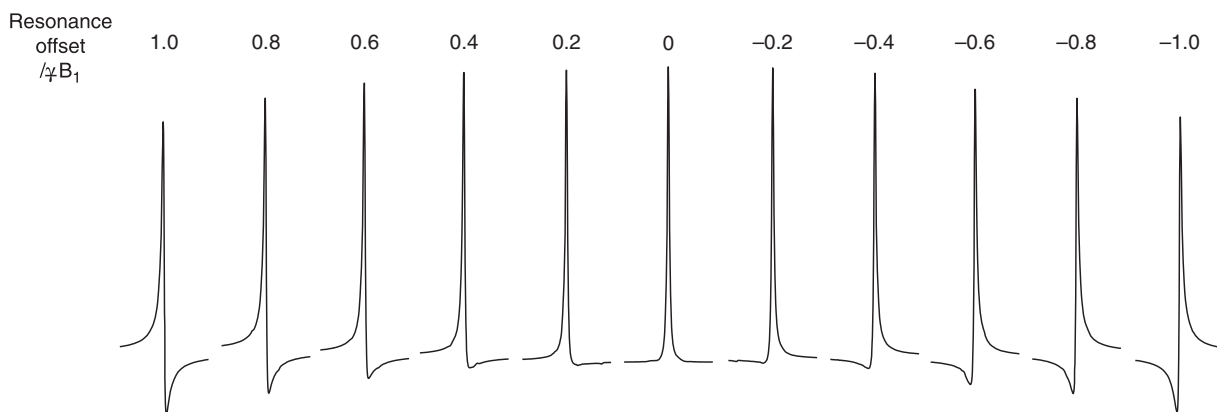
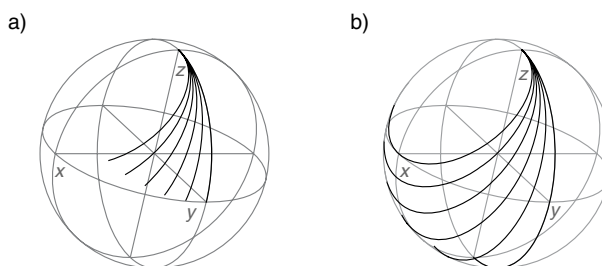


Figure 3.7. Excitation trajectories as a function of resonance offset for (a) a 90° pulse and (b) a 180° pulse. The offset moves from zero (on-resonance) to $+\gamma B_1$ Hz in steps of $0.2\gamma B_1$ (as in Fig. 3.6). The 90° pulse has a degree of “offset-compensation” as judged by its ability to generate transverse magnetisation over a wide frequency bandwidth. In contrast, the 180° pulse performs rather poorly away from resonance, leaving the vector far from the target South Pole and with a considerable transverse component.



3.2.2. Signal detection

Before proceeding to consider how one collects the weak NMR signals, we briefly consider the detection process that occurs within the NMR receiver. The energy emitted by the excited spins produces tiny analogue electrical signals that must be converted into a series of binary numbers to be handled by the computer. This *digitisation* process (Section 3.2.3) must occur for all the frequencies in the spectrum. As chemists we are really only interested in knowing the chemical shifts *differences* between nuclei rather than their *absolute* Larmor frequencies since it is from these differences that one infers differences in chemical environments. As we already know, these frequencies are typically tens or hundreds of megahertz whilst chemical shift ranges only cover some kilohertz. For example, a proton spectrum recorded at 200 MHz corresponds to a frequency range of only 2–3 kHz, and even for carbon (50 MHz on the same instrument), the frequency range is only around 10 kHz. Therefore, rather than digitising signals over many megahertz and retaining only the few kilohertz of interest, a more sensible approach is to subtract a reference frequency from the detected signal *before* digitisation, leaving only the frequency window of interest (Fig. 3.8). The resultant signals are now in the *audio* range, which, for humans at least, corresponds to frequencies less than 20 kHz (so it is also possible to hear NMR resonances).

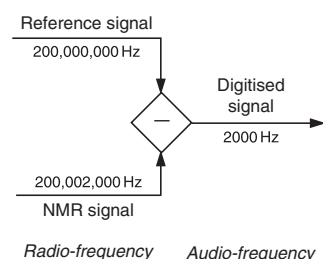


Figure 3.8. The NMR detection process. A fixed reference frequency is subtracted from the detected NMR signal so that only the frequency *differences* between resonances are digitised and recorded.

The reference frequency is usually chosen to be that of the original pulse used to excite the spin system and is supplied as a continuous reference signal from the transmitter (Fig. 3.9). This detection process is exactly analogous to the use of the rotating-frame representation introduced in Chapter 2 where the rotating frame reference frequency is also that of the rf pulse. If you felt a little uneasy about the seemingly unjustified use of the rotating frame formalism previously, then perhaps the realisation that there is a genuine experimental parallel within all spectrometers will help ease your concerns. The digitised FID you see therefore contains only the audio frequencies that remain after subtracting the reference, and it is these that produce the resonances observed in the final spectrum following Fourier transformation.

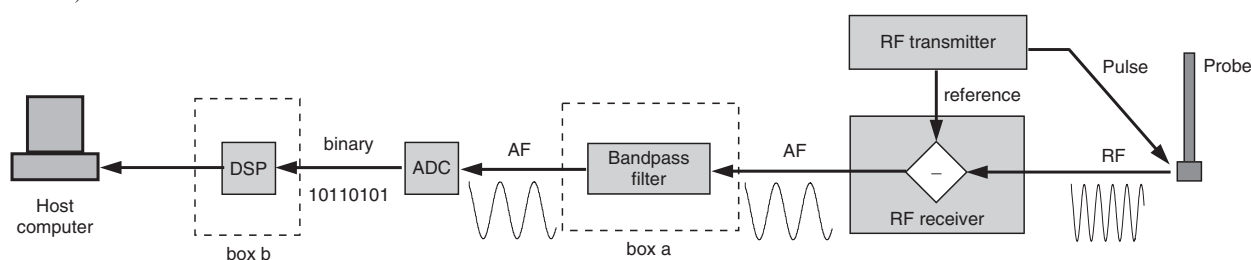
3.2.3. Sampling the FID

The Nyquist condition

To determine the frequency of an NMR signal correctly, it must be digitised at the appropriate rate. The *Sampling* or *Nyquist Theorem* tells us that to characterise a regular, oscillating signal correctly, it must be defined by at least two data points per wavelength. In other words, to characterise a signal of frequency F Hz, we must sample at a rate of at least $2F$; this is also known as the *Nyquist condition*. In NMR parlance, the highest recognised frequency is termed the *spectral width*, SW (Fig. 3.10). The time interval between sampled data points is referred to as the *dwell time*, DW, as given by:

$$DW = \frac{1}{2SW} \quad (3.3)$$

Figure 3.9. Schematic illustration of NMR data collection. Pulsed rf excitation stimulates the NMR response in the probe head, which is then amplified and detected in the receiver. The receiver reference frequency (that of the pulse rf) is subtracted to leave only audio frequencies (AF) that are digitised by the analogue-to-digital converter (ADC) and subsequently stored within the computer. The functions of the boxed sections are described later in the text (DSP: digital signal processor).



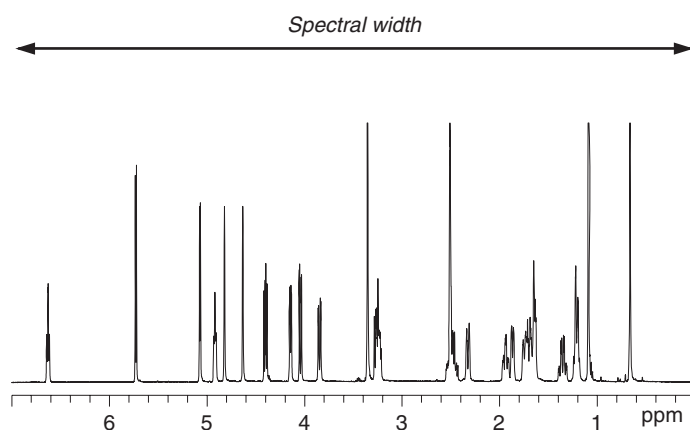


Figure 3.10. The spectral width defines the size of the observed frequency window. Only within this window are the line frequencies correctly characterised.

Signals with frequencies less than or equal to the spectral width will be characterised correctly as they will be sampled at two or more points per wavelength, whereas those with higher frequencies will be incorrectly determined and in fact will appear in the spectrum at frequencies that are lower than their true values. To understand why this occurs, consider the sampling process for three frequencies, two within and the third outside the spectral width (Fig. 3.11). The sampled data points of the highest frequency clearly match those of the lower of the three frequencies, and this signal will therefore appear with an incorrect frequency within the spectral window. Resonances that appear at incorrect frequencies, because in reality they exist outside the spectral width, are said to be *aliased* or *folded* back into the spectrum. Their location when corrupted in this manner is dependent on which of the two commonly employed *quadrature detection* schemes are in use, as explained in Section 3.2.4. On modern NMR spectrometers that employ digital signal detection (see Section 3.2.6), digital filters are able to completely eliminate signals that fall outside the defined spectral width and folded or aliased signals are therefore not observed. This filtering applies only for the directly sampled (observed) dimension of an NMR experiment, so aliasing of peaks may still be observed in the indirectly detected dimensions of a 2D experiment (see Section 5.3.1).

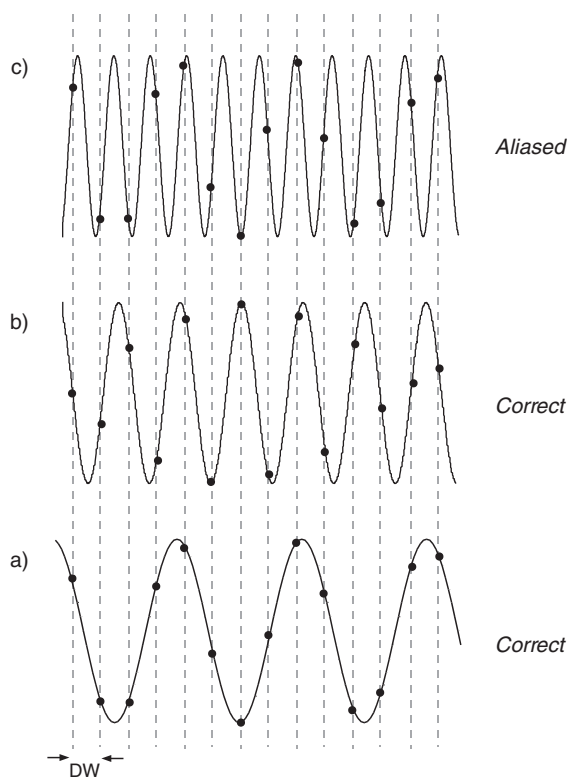


Figure 3.11. The Nyquist condition. To correctly characterise the frequency of a NMR signal, it must be sampled at least twice per wavelength, it is then said to fall within the spectral width. The sampling of signals (a) and (b) meet this criterion. Signals with frequencies too high to meet this condition are aliased back within the spectral width and so appear with the wrong frequency. The sampling pattern of signal (c) matches that of (a) and hence it is incorrectly recorded as having the lower frequency.

Filtering noise

One particularly insidious effect of aliasing or folding is that not only will NMR resonances fold into the spectral window but noise will as well. This can seriously compromise sensitivity, since noise can extend over an essentially infinite frequency range (so-called white noise) all of which, potentially, can fold into the spectrum and swamp the NMR resonances. It is therefore essential to prevent this by filtering out all signals above a certain frequency threshold with an audio *bandpass filter* after detection but prior to digitisation (Fig. 3.9, box a). The effect of the noise filter is demonstrated in Fig. 3.12, and the gain in signal-to-noise ratio in the spectrum employing this is clearly evident in Fig. 3.13. The cut-off bandwidth of the filter is variable, to permit the use of different spectral widths, and is usually automatically set by the spectrometer software to be a little greater than the spectral window. This is necessary because analogue filters are not perfect and do not provide an ideal sharp frequency cut-off, but instead tend to fall away steadily as a function of frequency, as is apparent in Fig. 3.12. A practical consequence is that these filters can attenuate the intensity of signals falling at the extreme ends of the spectrum, so may interfere with accurate signal intensity measurement and it is therefore wise to ensure that resonances do not fall at the edges. Analogue filters can also introduce a variety of distortions to the spectrum, and the use of digital filtration methods having superior characteristics is now a standard feature on modern NMR spectrometers. Digital signal processing is considered in Section 3.2.6.

Acquisition times and digital resolution

The total sampling period of the FID, known as the *acquisition time*, is ultimately dictated by the frequency resolution required in the final spectrum. From the Heisenberg's Uncertainty Principle, the resolution of two lines separated by $\Delta\nu$ Hz requires data collection for at least $1/\Delta\nu$ s. If one samples the FID for a time that is too short, then the frequency differences cannot be resolved and fine structure is lost (the minimum linewidth that can be resolved is given approximately by $0.6/AQ$). Likewise, if the signal decays rapidly then one is unable to sample it for a long period of time and again can resolve no fine structure. In

Figure 3.12. Eliminating noise with analogue filters. Spectra were recorded over a spectral width of 5 kHz with no sample in the probe. The analogue filter window was set to (a) 6 kHz, (b) 3 kHz and (c) 1 kHz and the associated noise attenuation is apparent, although the cut-off point is ill defined.

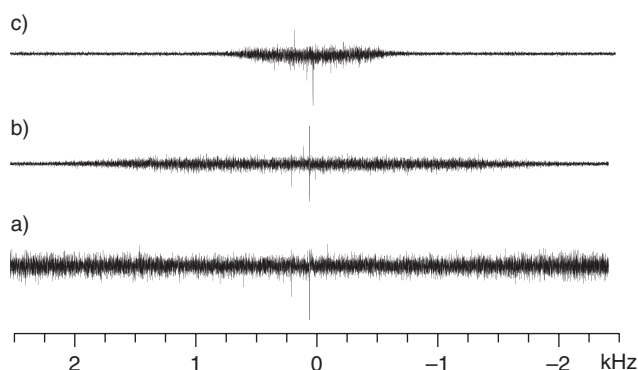
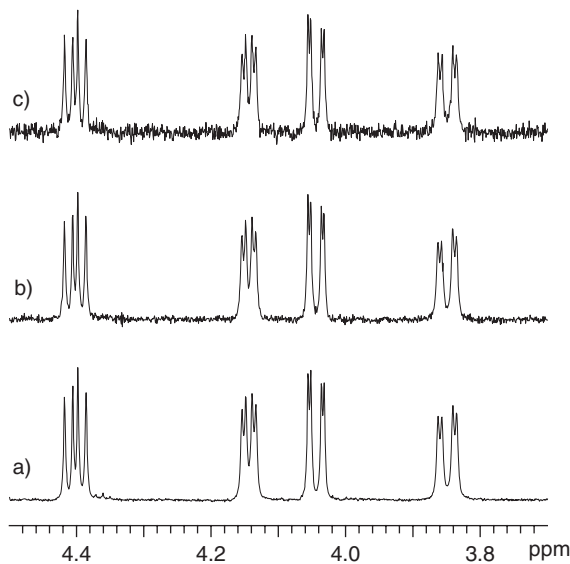


Figure 3.13. The effect of analogue filters on signal-to-noise. Spectrum (a) was recorded with the correct filter width (1.25 times the spectral width), and (b) and (c) with it increased 10- and 100-fold respectively.



this case, the rapid decay implies a large linewidth arising from natural (transverse) relaxation processes, or from poor field homogeneity, and one will be unable to recover resolution by extending the acquisition time. Thus, when selecting the appropriate acquisition time one needs to consider the likely frequency differences that need to be resolved and the relaxation times of the observed spins.

The acquisition time is defined by the digitisation rate, which is dictated by the spectral width and defines the sampling dwell time DW, and on how many data points are sampled in total. If the FID contains TD time-domain data points then:

$$AQ = DW \cdot TD = \frac{TD}{2 \cdot SW} \quad (3.4)$$

When dealing with the final spectrum, we are concerned with frequency and not time, and what one really needs is some measure of how well the resonances in the spectrum are digitised. The figure of interest is the frequency between adjacent data points in the spectrum, the *digital resolution*, DR (quoted in hertz per data point or simply Hz/point). It should be stressed that this is not what spectroscopists speak of when they refer to the 'resolution' achieved on a spectrometer as this relates to the homogeneity of the magnetic field. Digital resolution relates only to the frequency window to data point ratio, which is small for a well-digitised spectrum but large when poorly digitised.

Following the FT, two data sets are generated representing the 'real' and 'imaginary' spectra (Section 2.3), so the real part with which one usually deals contains half the data points of the original FID (in the absence of further manipulation), and its data size, SI, is therefore TD/2. DR is then:

$$DR = \frac{\text{total frequency window}}{\text{total number of data points}} = \frac{SW}{SI} = \frac{2 \cdot SW}{TD} = \frac{1}{AQ} \quad (3.5)$$

Thus, digital resolution is simply the reciprocal of the acquisition time, so to collect a well-digitised spectrum one must sample the data for a long period of time; clearly this is the same argument presented above.

The effect of inappropriate digital resolution is demonstrated in Fig. 3.14. Clearly with a high value, that is short AQ, the fine structure cannot be resolved. Only with the extended acquisition times can the genuine spectrum be recognised. For proton spectroscopy, one needs to resolve frequency differences of somewhat less than 1 Hz to be able to recognise small couplings, so acquisition times of around 2–4 s are routinely used, corresponding to DRs of around 0.5 to 0.25 Hz/point. This then limits the accuracy with which frequency measurements can be made, including shifts and couplings, (even though peak listings tend to quote resonance frequencies to many decimal places).

The situation is somewhat different for the study of nuclei other than protons however, since they often exhibit rather few couplings (especially in the presence of proton decoupling), and because one is usually more concerned with optimising sensitivity, so does not wish to sample the FID for extended periods and thus sample more noise. Since one does not need to define lineshapes with high accuracy, lower digital resolution suffices to resolve chemical shift differences, and 1–2 Hz/point (0.5–1 s AQ) is adequate in ^{13}C NMR. Exceptions occur in the case of nuclei with high natural abundance that may show homonuclear couplings such as ^{31}P , or when the spectrum is being used to estimate the relative ratios of compounds in solution. This is true, for example, in the use of 'Mosher's acid' derivatives for the determination of enantiomeric excess from ^{19}F NMR spectra, for which the resonances must be well digitised to represent the true intensity of each species. In such cases, the parameters that may be used for the 'routine' observation of ^{19}F are unlikely to be suitable for quantitative measurements. Similarly, the use of limited digital resolution in routine carbon-13 spectra is one reason why signal intensities do not provide a reliable indication of relative concentrations. In contrast, the spectra of rapidly relaxing quadrupolar nuclei contain broad resonances that require only low digital resolution and hence short acquisitions times.

Zero-filling and truncation artefacts

Whilst the NMR response decays throughout the FID, the noise component remains essentially constant and will eventually dominate the tail of the FID. At this point, there is little advantage in continuing acquisition since this only adds noise to the final spectrum. Provided the FID has fallen to zero when acquisition stops, one can artificially improve the digital resolution by appending zeros to the end of the FID. This process is known as *zero-filling*, and it interpolates these added data points in the frequency domain and so enhances the definition of resonance lineshapes (Fig. 3.15).

It has been shown [2] that by doubling the number of data points in the time domain by appending zeros (a single 'zero-fill'), it is possible to improve the frequency resolution in the spectrum. The reason for this gain stems from the fact that information in the FID is split

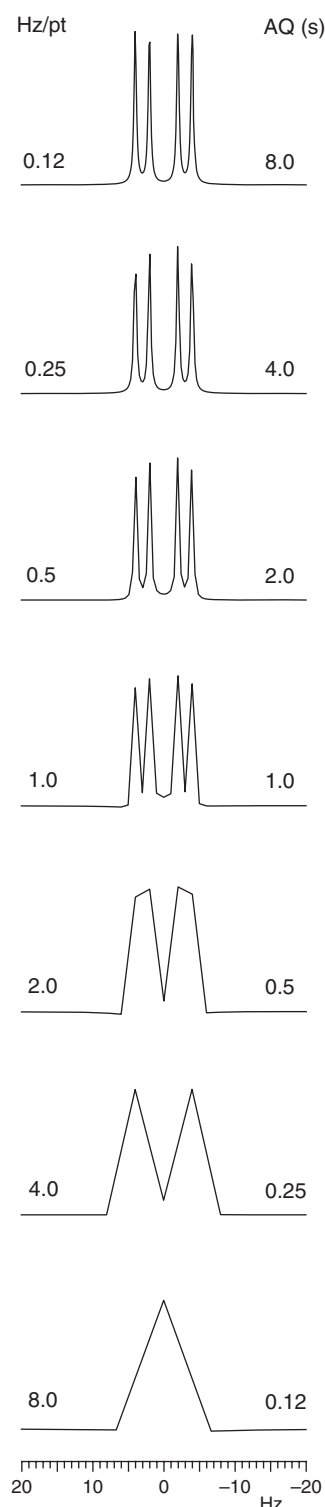


Figure 3.14. The influence of data acquisition times on the ability to resolve fine structure. Longer acquisition times correspond to higher digitisation levels (smaller Hz/pt), which here enable characterisation of the coupling structure within the double-doublet ($J = 6$ and 2 Hz).

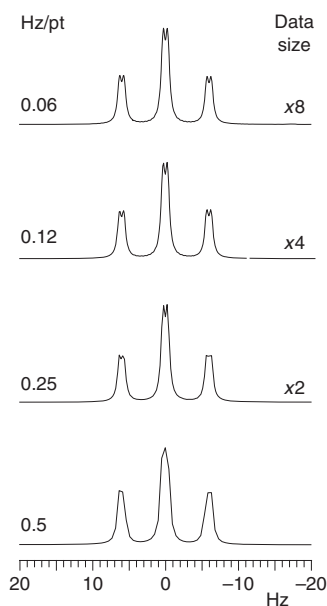


Figure 3.15. Zero-filling can be used to enhance fine structure and improve lineshape definition.

into two parts (real and imaginary) after the transformation, so one effectively loses information when considering only the real (absorption) part. Doubling the data size regains this lost information and is therefore a useful tool in the routine analysis of proton spectra. (One could of course simply double the acquired data points, but this leads to reduced sensitivity and requires more spectrometer time.) Further zero-filling simply increases the digital resolution of the spectrum by interpolating data points, so no new information can be gained and the improvement is purely cosmetic. However, because this leads to a better definition of each line, it can still be extremely useful when analysing multiplet fine structure in detail or when measuring accurate resonance intensities; in the determination of enantiomeric excess via chiral solvating reagents for example. In 2D experiments, acquisition times are necessarily kept rather short, and zero-filling is routinely applied to improve the appearance of the final spectrum. A more sophisticated approach to extending the time-domain signal known as *linear prediction* is described below.

If the FID has not decayed to zero at the end of the acquisition time, the data set is said to be *truncated* and this leads to distortions in the spectrum after zero-filling and FT. The distortions arise from the FT of the sudden step within the FID, the result of which is described by the function $(\sin x)/x$, also known as *sinc x*. Fig. 3.16 shows that this produces undesirable ringing patterns that are symmetrical about the base of the resonance, often referred to as 'sinc wiggles'. To avoid this problem, it is essential to ensure the acquisition time is sufficiently long, to force the FID to decay smoothly to zero with a suitable shaping function (Section 3.2.7) or to artificially extend the FID by LP.

In proton spectroscopy, acquisition times are sufficiently long that truncation artefacts are rarely seen in routine spectra, although they may be apparent around the resonances of small molecules with long relaxation times, solvent lines for example. As stated above, acquisition times for other nuclei are typically kept short and FIDs are usually truncated. This, however, is rarely a problem as it is routine practice to apply a window function to enhance sensitivity of such spectra, which itself also forces the FID to zero so eliminating the truncation effects. Try processing a standard ^{13}C spectrum directly with an FT but without the use of a window function; for some resonances, you are likely to observe negative responses in the spectra that appear to be phasing errors. In fact these distortions arise purely from the low digital resolution used, that is, the short acquisition times, and the corresponding truncation of the FID [3] (Fig. 3.17a). Use of a simple decaying function (Section 3.2.7) removes these effects (Fig. 3.17b) and when used in this way is often referred to as *apodisation*, literally meaning 'removing the feet'. Similar considerations apply to truncated 2D data sets where shaping functions play an essential role.

Linear Prediction

The method of linear prediction (LP) can play many roles in processing of NMR data, [4, 5] from the rectification of corrupted or distorted data, through to the complete generation of frequency-domain data from an FID; an alternative to the FT. Here we consider its most popular usage, known as forward linear prediction, which extends a truncated FID. Rather than simply appending zeros, this method, as the name suggests, predicts the values

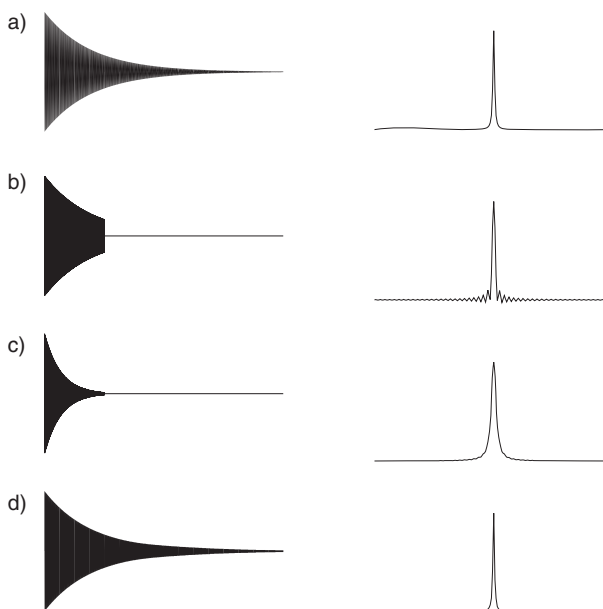


Figure 3.16. Processing a truncated FID. (a) A complete FID and the corresponding resonance, (b) a truncated FID that has been extended by zero-filling produces sinc wiggles in the spectrum, (c) apodisation of the FID in (b) together with zero filling and (d) linear prediction of the FID in (b).

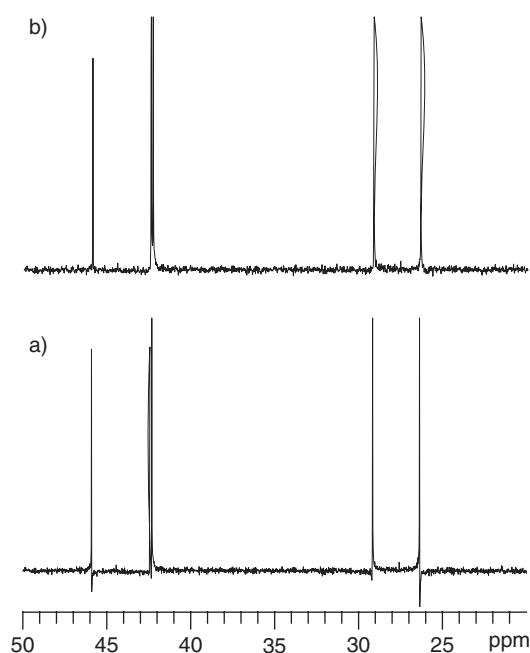


Figure 3.17. Carbon spectra often display distortions when transformed directly (a) which appear to be phase errors but which actually arise from a short acquisition time. Applying a line broadening apodisation function prior to the transform removes these distortions (b, 1 Hz line broadening).

of the missing data points by using the information content of the previous points and so genuinely extends the FID (Fig. 3.16d).

In a time sequence of data points, the value of a single point, d_n , can be estimated from a linear combination of the immediately preceding values:

$$d_n = a_1 d_{n-1} + a_2 d_{n-2} + a_3 d_{n-3} + a_4 d_{n-4} \dots \quad (3.6)$$

where $a_1, a_2, \dots$ and so on represent the LP coefficients. The number of coefficients (referred to as the *order* of the prediction) corresponds to the number of data points used to predict the next value in the series. Provided the coefficients can be determined from the known data, it is then possible to extrapolate beyond the acquired data points. Repetition of this process incorporating the newly predicted points ultimately leads to the extended FID.

This process is clearly superior to zero-filling and produces a much better approximation to the true data than does simply appending zeros. It improves resolution, avoids the need for strong apodisation functions and greatly attenuates truncation errors. Naturally, the method has its limitations, the most severe being the requirement for high signal-to-noise in the FID for accurate estimation of the LP coefficients. Successful execution also requires that the number of points used for the prediction is very much greater than the number of lines that comprise the FID. This may be a problem for 1D data sets with many component signals, and LP of such data is less widely used. The method is far more valuable for the extension of truncated data sets in the indirectly detected dimensions of a 2D or 3D experiment (you may wish to return to these discussions when you are familiar with the 2D approach, see Chapter 5). Here, individual interferograms contain rather few lines (look at a column from a 2D data set) and are thus better suited to prediction, providing sensitivity is adequate. The use of LP in the routine collection of 2D spectra of organic molecules has been subject to detailed investigation [6]. Typically, a twofold to fourfold prediction is used, so for example, 128 t_1 data points may be readily extended to 256 or 512. Apodisation of the data is still likely to be required, although not as severely as for untreated data, and zero-filling may also be applied to further improve digitisation. Figure 3.18 clearly demonstrates the improved resolution attainable through the use of LP in the indirect dimension of

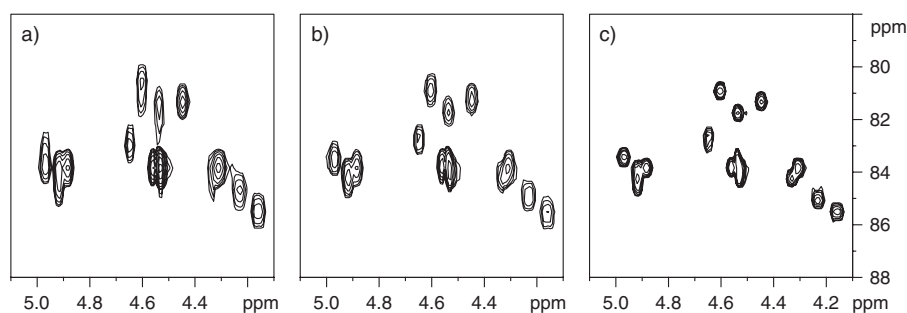


Figure 3.18. Improved peak resolution in a two-dimensional heteronuclear proton-carbon correlation experiment (Chapter 6) through linear prediction (LP). The same raw data was used in each spectrum, with the F_1 (carbon) dimension processed with (a) no data extension, (b) one zero-fill and (c) LP in place of zero-filling.

a 2D heteronuclear correlation experiment. The same principles can be used in *backward* LP. Data points at the start of an FID are sometimes corrupted by ringing in receiver circuitry when detection starts resulting in baseline distortion of the spectrum. Replacing these points with uncorrupted predicted points eliminates the distortion; an example of this is found in Section 4.5.

3.2.4. Quadrature detection

It was described above how during the classical NMR signal detection process a reference frequency equal to that of the excitation pulse is subtracted from the NMR signal to produce an audio frequency signal that is digitised and later subject to FT. The problem with analysing the data produced by this *single-channel detection* is that the FT is intrinsically unable to distinguish frequencies above the reference from those below it, that is, it cannot differentiate positive from negative frequencies. This results in a magnetisation vector moving at $+\nu$ Hz in the rotating frame producing two resonances in the spectrum at $+\nu$ and $-\nu$ Hz after FT (Fig. 3.19). The inevitable confusing overlap in a spectrum acquired with the reference positioned in the centre of the spectral width can be avoided by placing the reference at one edge of the spectrum to ensure all rotating-frame frequencies have the same sign. Whilst this will solve potential resonance overlap problems, it introduces a number of other undesirable factors. Firstly, although there will be no mirror image NMR resonances remaining within the spectral window, noise will still be mirrored about the reference frequency and added to that already present. This leads to a decrease in signal-to-noise by a factor of $\sqrt{2}$, or about 1.4 (not by a factor of 2 because noise is random and does not add coherently). Furthermore, this produces the greatest possible frequency separation between the pulse and the highest frequency resonance, so enhancing undesirable off-resonance effects. The favoured position for the reference is therefore in the centre of the spectrum, and two-channel (*quadrature*) detection is then required to distinguish sign.

To help visualise why single-channel detection cannot discriminate positive and negative frequencies, and how the quadrature method can, consider again a single magnetisation vector in the rotating frame. The use of the single-channel detector equates to being able to observe the precessing magnetisation along only one axis, say the y axis. Figure 3.20 shows that the resultant signal along this axis for a vector moving at $+\nu$ Hz is identical to that moving at $-\nu$ Hz, both giving rise to a cosinusoidal signal, so the two are indistinguishable (Fig. 3.20a). If, however, one were able to make use of two (phase-sensitive) detection channels whose reference signals differ in phase by 90° (hence the term *quadrature*), one would then be able to observe magnetisation simultaneously along both the x and the y axes such that one channel monitors the cosine signal and the other the sine (Fig. 3.20a and b).

Figure 3.19. A single-channel detection scheme is unable to differentiate positive and negative frequencies in the rotating frame. This results in a mirror image spectrum being superimposed on the true one if the transmitter is placed in the centre of the spectrum.

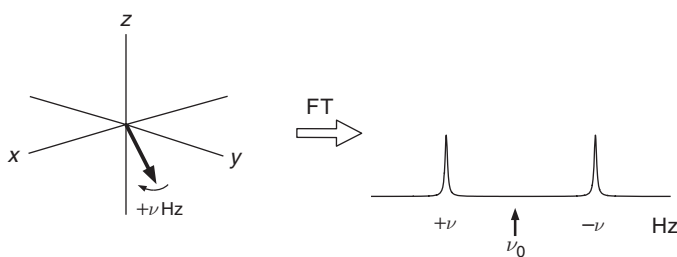
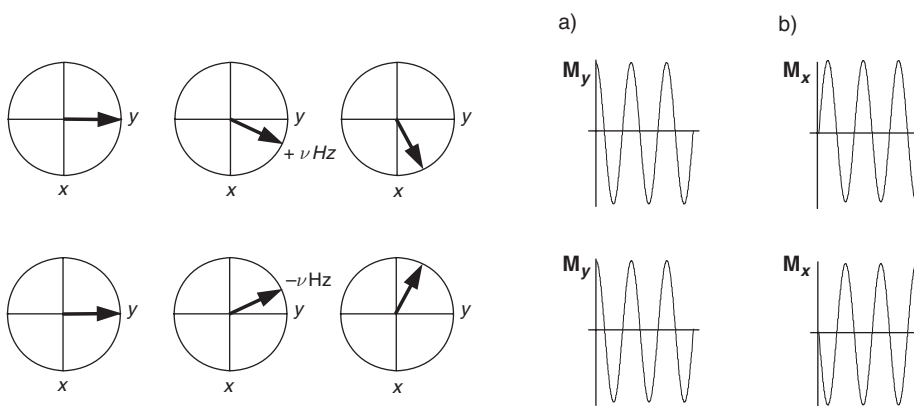


Figure 3.20. A two-channel quadrature detection system monitors magnetisation on two orthogonal axes, providing both (a) cosine- and (b) sine-modulated data, which ultimately allow the sense of precession to be determined (see text).



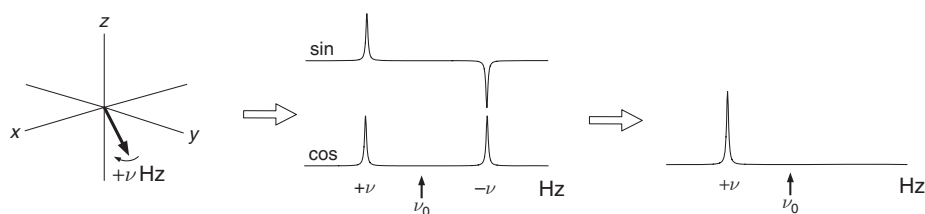


Figure 3.21. A two-channel detection scheme is able to differentiate positive and negative frequencies in the rotating frame, allowing the transmitter to be placed in the centre of the spectrum without the appearance of mirror image signals.

With the additional information provided by the sinusoidal response of the second channel, the sense of rotation can be determined, and vectors moving at $\pm \nu$ Hz can be distinguished (Fig. 3.21). Technically, the FT is then *complex*, with the x and y components being handled separately as the real and the imaginary inputs to the transform, following which the positive and negative frequencies are correctly determined. In the case of the single channel, the data are used as input to a *real* FT.

Simultaneous and sequential sampling

Quadrature detection is universally employed in all modern spectrometers. However, there exist a number of experimental schemes for implementing this, and which of these you are likely to use will be dictated by the spectrometer hardware and perhaps by the age of the instrument (on some modern instruments, the operator can choose between these methods). The differing approaches may be divided into the classical *analogue* scheme described here for which there are two widely used methods, and the more recent *digital quadrature detection* (DQD) scheme that is considered later.

In both analogue implementations, the incoming rf signal from the probe and preamplifier is split into two signals and each fed to separate phase-sensitive detectors whose reference frequencies are identical but differ in phase by 90° (Fig. 3.22). The resulting audio signals are then sampled, digitised and stored for subsequent analysis, and it is in the execution of these that the two methods differ. The first relies on *simultaneous* sampling of the two channels (i.e. the channels are sampled at precisely the same point in time), and the data points for each are stored in separate memory regions (Fig. 3.23a). The two sets of data so generated represent the cosine and sine components required for the sign discrimination and are used as the real and the imaginary inputs to a complex FT routine. As this enables positioning of the transmitter frequency in the centre of the spectrum, a frequency range of only $\pm SW/2$ need be digitised. According to the Nyquist criterion, the sampling rate now becomes equal to SW , that is, $(2 \times SW/2)$ or half of that required for single-channel detection, so the equations given in the previous section are modified slightly for *simultaneous quadrature detection* to give:

$$DW = \frac{1}{SW} \quad \text{and} \quad AQ = DW \cdot \frac{TD}{2} = \frac{TD}{2 \cdot SW} \quad (3.7)$$

also

$$DR = \frac{SW}{SI} = \frac{2 \cdot SW}{TD} \quad \text{and so again} \quad DR = \frac{1}{AQ} \quad (3.8)$$

Notice that in the calculation of the acquisition time, we need only consider *half* the total time-domain data points since now two points are sampled at the same time.

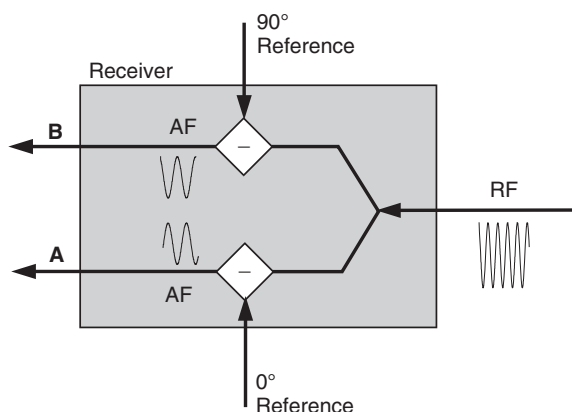


Figure 3.22. Schematic illustration of the experimental implementation of analogue quadrature detection in the NMR receiver. The incoming rf signal is split into two, and the reference signal, differing in phase by 90° in the two channels, subtracted. Channels A and B therefore provide the required sine and cosine components.

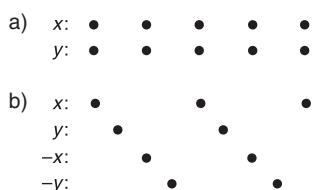


Figure 3.23. Data sampling schemes for the two common quadrature detection methods. (a) Simultaneous sampling: the two quadrature channels (representing x - and y -magnetisation) are sampled at the same point in time. (b) Sequential sampling: the two channels are sampled alternately at twice the rate of method (a), and the phase inverted for alternate pairs of data points (see text).

The second method for quadrature detection actually strives to eliminate the problems of positive and negative frequencies by effectively mimicking a single-channel detection scheme with the transmitter at the edge of the spectrum, so is sometimes referred to as ‘pseudo-quadrature detection’. Although it allows the positioning of the transmitter frequency at the centre of the spectrum, it employs an ingenious sampling scheme to make it appear as if the reference frequency sat at one edge of the spectrum, so that only frequencies of one sign are ever characterised. In this method, data points are sampled *sequentially* at a rate suitable for single-channel detection (i.e. $2 \cdot \text{SW}$ or twice that in the simultaneous method), but for each data point sampled, the reference phase is incremented by 90° (Fig. 3.23b). The effect on a magnetisation vector is that it appears to have advanced in the rotating frame by 90° more between each sampling period than it actually has, so the signal appears to be precessing faster than it really is. As digitisation occurs at a rate of $2 \cdot \text{SW}$ and each 90° phase shift corresponds to an advance of $1/4$ of a cycle, the frequency appears to have increased by $2 \cdot \text{SW}/4$ or $\text{SW}/2$. Since the transmitter is positioned at the centre of the spectrum, the genuine frequency range runs from $+\text{SW}/2$ to $-\text{SW}/2$, meaning the artificial increase of $+\text{SW}/2$ moves the frequency window to $+\text{SW}$ to 0 . Thus, there are no longer negative frequencies to distinguish. Experimentally, the required 90° phase shifts are achieved by alternating between the two phase-sensitive detectors to give the 0° and 90° shifts, and simply inverting the signals from these channels to provide the 180° and 270° shifts respectively (Fig. 3.23b). The sampled data are handled by a single memory region as *real* numbers that are used as the input to a *real* FT. In this *sequential quadrature detection* scheme, the sampling considerations, given in Section 3.2.3, apply as they would for single-channel detection. In Chapter 5, these ideas are extended to quadrature detection in 2D spectroscopy.

For either scheme, the total number of data points digitised, acquisition times and spectral widths are identical, so the resulting spectra are largely equivalent. The most obvious difference is in the appearance of aliased signals, that is, those that violate the Nyquist condition, as described below. Experimentally, flatter baselines are observed for the simultaneous method as a result of the *symmetrical* sampling of the initial data points in the FID, and this is the recommended protocol for an analogue detection scheme.

Aliased signals

In Section 3.2.3 it was shown that a resonance falling outside the spectral window (because it violates the Nyquist condition) will still be detected but will appear at an incorrect frequency and is said to be *aliased* or *folded* back into the spectrum (if digital signal filters are not employed to eliminate this). This can be confusing if one is unable to tell whether the resonance exhibits the correct chemical shift or not. The precise location of the aliased signal in the spectrum depends on the quadrature detection scheme in use and on how far outside the window it truly resonates. With the simultaneous (complex FT) scheme, signals appear to be ‘wrapped around’ the spectral window and appear at the *opposite* end of the spectrum (Fig. 3.24b), whereas with the sequential (real FT) scheme, signals are ‘folded back’ at the *same* end of the spectrum (Fig. 3.24c). If you are interested to know why this difference occurs, see reference [7].

Fortunately, in proton spectroscopy it is generally possible to detect the presence of an aliased peak because of its esoteric phase that remains distorted when all others are correct. In heteronuclear spectra that display only a single resonance, such phase characteristics cannot provide this information as there is no other signal with which to make the comparison. In such cases, it is necessary to widen the spectral window and record any movement of the peak. A signal that is not aliased will be unchanged and will appear with the same chemical shift. In contrast, an aliased resonance will move towards the edge of the spectrum and will appear at a different shift. The situation is even worse if digital filters are employed as any resonance that falls outside the defined spectral window is simply not observed. If you happen to be searching for a resonance of unknown chemical shift, it would be wise to turn off digital filtration to increase the chance of detecting the peak.

Quadrature images

In addition to the corruption of spectra due to signal aliasing, there may also exist unwelcome artefacts that arise from imperfections in the analogue quadrature detection schemes. In these, one requires two signals to be digitised for subsequent storage in two separate memory blocks that differ in phase by 90° but which are otherwise identical. However, experimentally it is rather difficult to ensure the phase difference is *exactly* 90° and that the signal amplitudes are identical in each channel. Such channel imbalance in phase and amplitude leads to spurious images of resonances mirrored about the transmitter

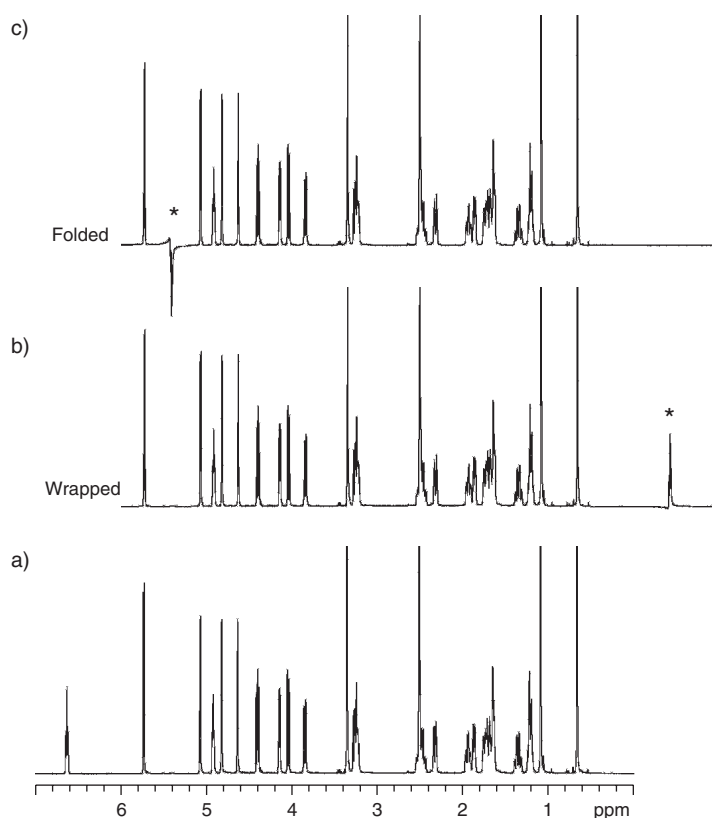


Figure 3.24. Aliasing of resonances. Spectrum (a) displays all resonances at their correct shifts, whilst (b) and (c) result from the spectral window being positioned incorrectly. Spectrum (b) shows how the resonance wraps back into the spectrum at the far end when simultaneous sampling is employed, whereas in (c) it folds back in at the near end with sequential sampling. Typically, the phase of the aliased resonance(s) is also corrupted.

frequency known as *quadrature images* or colloquially *quad images* (Fig. 3.25). Their origin can be realised if one imagines the signal amplitude in one channel to fall to zero. The scheme is then one of single-channel detection, so again positive and negative frequencies cannot be distinguished and thus mirror images appear about the reference (see Fig. 3.19 again). In a correctly balanced receiver, these images are usually less than 1% on a single scan, but even these can be significant if you wish to detect weak resonances in the presence of very strong ones, so some means of compensating the imbalance is required. The traditional approach to this is through *phase cycling* of the rf pulses and the receiver to cancel these artefacts, and the elimination of these images in this way serves as an introduction to the concept of phase cycling and is described in the section that follows. On modern instruments, these images are avoided through the use of digital quadrature detection in place of the analogue schemes described above.

Digital quadrature detection

In the traditional analogue detection scheme illustrated in Fig. 3.22, the two quadrature signals required for frequency discrimination were generated by mixing with analogue reference signals of (ideally) 90° phase difference prior to independently digitising the data from the two channels. In the digital quadrature detection (DQD) scheme, only a single channel is digitised, representing, say, the 0° component. This digital data may then be

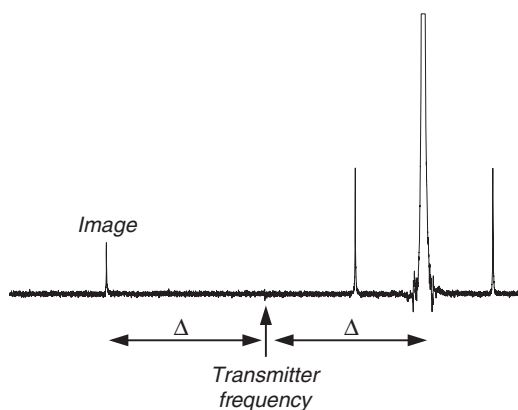
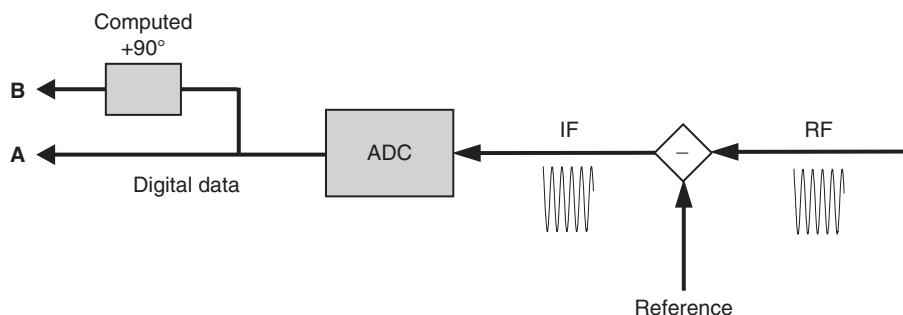


Figure 3.25. Quadrature images are unwanted mirror image artefacts that arise from spectrometer imperfections. Here an image of CHCl_3 can be seen at about one-half the height of the carbon satellites in a single-scan spectrum. Phase cycling is typically employed to suppress these, although digital quadrature detection can eliminate them completely.

Figure 3.26. Schematic illustration of digital quadrature detection. A single channel is digitised as a suitable intermediate frequency input (*if*) and the second channel is generated numerically with an identical amplitude and a precise 90° phase shift.



copied and manipulated numerically to generate a second data set with a mathematically defined 90° phase difference from the originally detected data. This synthesized second channel data has the same amplitude and a precisely defined phase difference, so the imbalance in the quadrature channels that gives rise to quad images no longer occurs in the DQD scheme, and these images are thus eliminated. Since direct digitisation of the rf is not possible (current ADCs do not run fast enough for this) down-mixing of the NMR signal to a suitably low *intermediate frequency* (*if*) must occur prior to digitisation, which nowadays take place with input frequencies of anything from kilohertz up to tens of megahertz, this made possible by developments coming from telecommunications (Fig. 3.26). With digital schemes, it also becomes possible to offset the reference frequency used at the detection stage from that used for the transmitter pulse, and this then eliminates any “zero-frequency” glitch or spike that may be seen at the centre of spectra when analogue receivers are used that produce a residual DC offset of the detected FID.

3.2.5. Phase cycling

As mentioned above, the removal of quadrature images can be achieved through the process of *phase cycling*, an experimental method that lies at the very heart of almost every NMR experiment. This process involves repeating a pulse sequence with identical timings and pulse tip angles but with judicious changes to the *phases* of the rf pulse(s) and to the routing of the data to the computer memory blocks (often loosely referred to as the receiver phase cycle). The aim in all this is for the desired signals to add coherently with time averaging whereas all other signals, whether from unwanted NMR transitions or from instrumental imperfections, cancel at the end of the cycle and do not appear in the resulting spectrum. We shall see the importance of phase cycles throughout the remainder of the book, particularly in the sections on multipulse 1D and 2D NMR, but as an introduction we remain with the idea of analogue quadrature detection and the cancellation of quadrature images.

Recall that the quadrature scheme results in two signals differing in phase by 90° that are stored in two separate memory blocks (here designated 1 and 2) and that phase and amplitude imbalance between these gives rise to the images. One solution to the problem is to ensure that data from each channel, A and B, contributes equally to the two memory blocks 1 and 2. This can be achieved by performing two experiments, the first with a 90°_x pulse the second with 90°_y , and adding the data. To keep the cosine and sine components in separate memory regions for use in the FT routine, appropriate data routing must also be used (Fig. 3.27a and b). This is generally handled internally by the spectrometer, being defined as the ‘receiver phase’ from the operator’s point of view, and taking values of 0, 90° , 180° or 270° or x , y , $-x$ and $-y$. Note, however, that the phase of the receiver *reference* rf does not alter on sequential scans, only the data routing is changed. For any pulse NMR sequence, it is necessary to define the rf phases and the receiver phase to ensure retention of the desired signals and cancellation of artefacts.

By use of the two-step phase cycle, it is therefore possible to compensate for the effects of imbalance in the two receiver channels. It is also possible to remove extraneous signals that may occur, such as from DC offsets in the receiver, by simultaneously inverting the phase of the rf pulse and the receiver; thus, as shown in Fig. 3.27, a steps to c and likewise b becomes d. The NMR signals will follow the phase of the pulse and so will add in the memory whereas offsets or spurious signals will be independent of this and so will cancel. This gives us a second possible two-step phase cycle which, when combined with the first, produces an overall four-step cycle known as CYCLOPS [8] (CYCLically Ordered PhaSe cycle, Table 3.1). This is the standard phase cycle used for one-pulse acquisitions on all spectrometers and is often nested within the phase cycles of 2D experiments again with the aim of removing receiver artefacts.

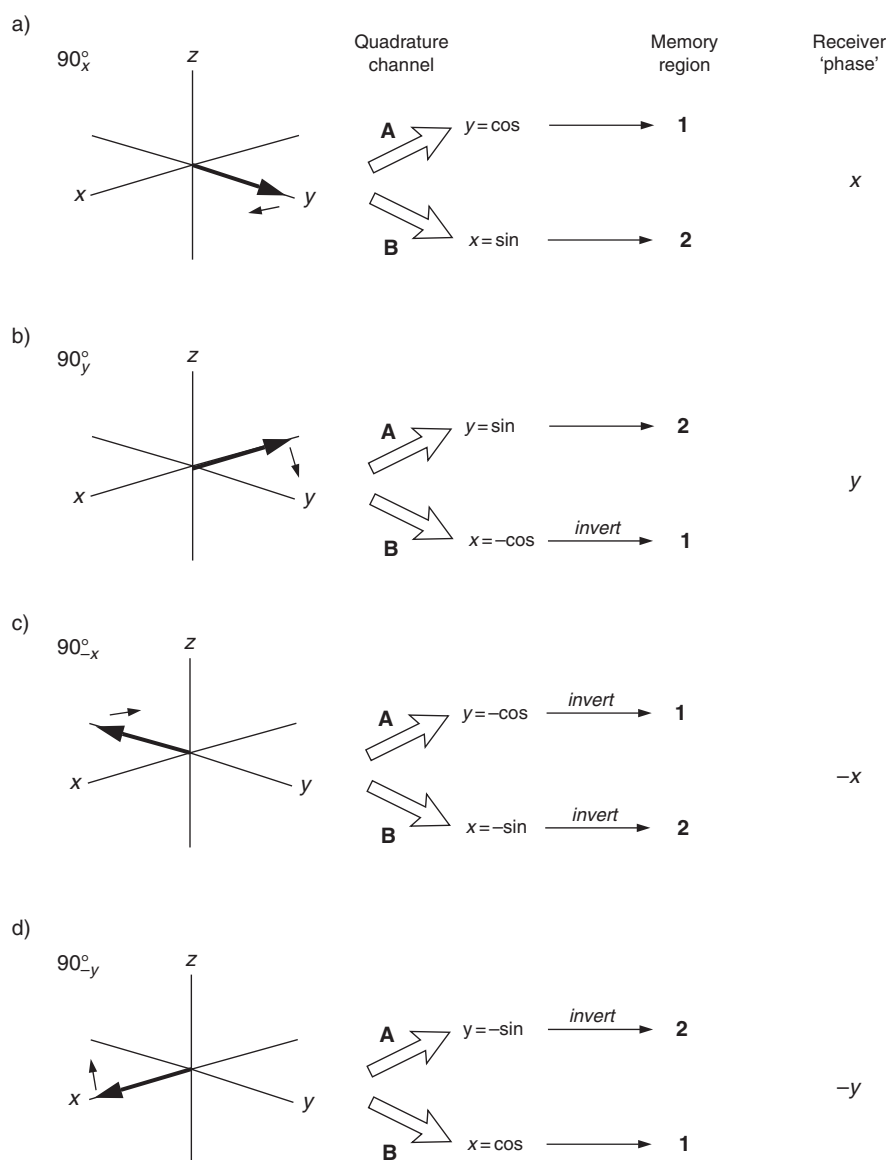


Figure 3.27. Phase cycling. The CYCLOPS scheme cancels unwanted artefacts whilst retaining the desired NMR signals. This four-step phase cycle is explained in the text.

Table 3.1. The four-step CYCLOPS illustrated in Fig. 3.27. This shorthand notation is conventionally used to describe all pulse sequence phase cycles

Scan number	Pulse phase	Receiver phase
1	x	x
2	y	y
3	$-x$	$-x$
4	$-y$	$-y$

3.2.6. Dynamic range and signal averaging

When sampling the FID, the analogue-to-digital converter (ADC or digitiser) limits the frequency range one is able to characterise (i.e. the spectral width) according to how fast it can digitise the incoming signal. In addition to limiting the *frequencies*, ADC performance also limits the *amplitudes* of signals that can be measured. The digitisation process converts the electrical NMR signal into a binary number proportional to the magnitude of the signal. This digital value is defined as a series of computer bits, the number of which describes the *ADC resolution*. Typical digitiser resolutions on modern spectrometers operate with 14 or 16 bits. The 16-bit digitiser is able to represent values in the range $\pm 32,767$, that is, $2^{15}-1$ with one bit reserved to represent the sign of the signal. The ratio between the largest and smallest detectable value (the most and least significant bits), 32,767:1, is the *dynamic*

range of the digitiser. If we assume the receiver amplification (or *gain*) is set such that the largest signal in the FID on each scan fills the digitiser, then the smallest signal that can be recorded has the value 1. Any signal whose amplitude is less than this will not trigger the ADC; the available dynamic range is insufficient. However, noise will also contribute to the detected signal and this may be sufficiently intense to trigger the least significant bit of the digitiser. In this case, the small NMR signal will be recorded as it rides on the noise, and signal averaging therefore leads to summation of this weak signal, meaning even those whose amplitude is below that of the noise may still be detected. However, the digitiser may still limit the detection of smaller signals in the presence of very large ones when the signal-to-noise ratio is high. Figure 3.28 illustrates how a reduction in the available dynamic range limits the observation of smaller signals when thermal noise in the spectrum is low. This situation is most commonly encountered in proton studies, particularly of protonated aqueous solutions where the water resonance may be many thousand times that of the solute. Such intensity differences impede solute signal detection, so many procedures have been developed to selectively reduce the intensity of the H_2O resonance and ease the dynamic range requirements; some of these *solvent suppression* schemes are described in Chapter 10.

If any signal is so large that it cannot fit into the greatest possible value the ADC can record, the signal intensity will not be measured correctly and this results in severe distortion of the spectrum (Fig. 3.29a). The effect can be recognised in the FID as ‘clipping’ of the most intense part of the decay (Fig. 3.29b), which results from setting the receiver gain or amplification too high; this must be set appropriately for each sample studied, either by manual adjustment or more conveniently via the spectrometer’s automated routines.

Signal averaging

The repeated acquisition and summation of a FID leads to an overall increase in the signal-to-noise ratio as the NMR signals add coherently over the total number of scans, NS , whereas the noise, being random, adds according to $\sqrt{NS}$. Thus, the signal-to-noise ratio increases according to $\sqrt{NS}$. In other words, to double the signal-to-noise ratio it is necessary to acquire *four times* as many scans (Fig. 3.30). It is widely believed that continued averaging leads to continuous improvement in the signal-to-noise ratio, although

Figure 3.28. Dynamic range and the detection of small signals in the presence of large ones. As the digitiser resolution and hence its dynamic range are reduced, the carbon-13 satellites of the parent proton resonance become masked by noise until they are barely discernible with only 6-bit resolution (all other acquisition parameters were identical for each spectrum). The increased noise is *digitisation* or *quantisation noise* (see text below).

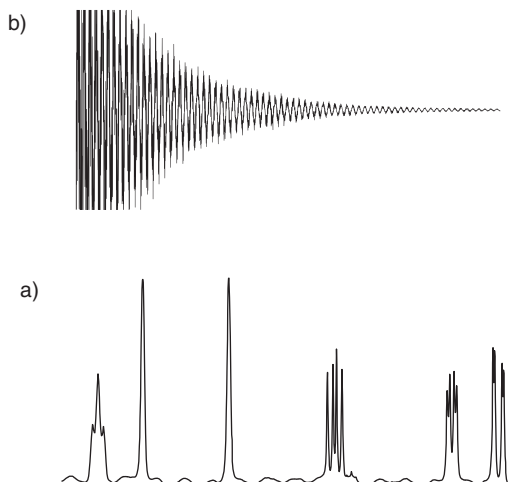
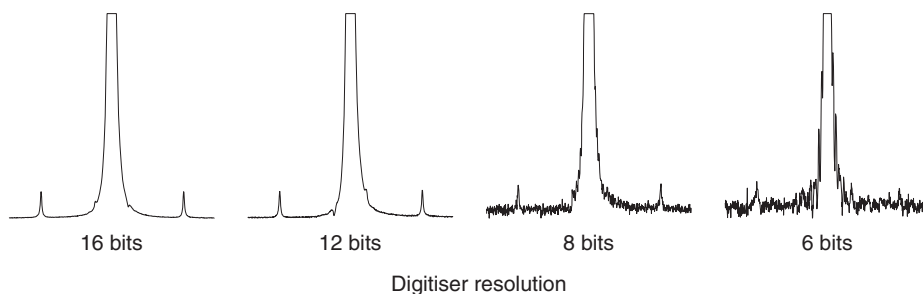


Figure 3.29. Receiver or digitiser overload distorts the spectrum baseline (a). This can also be recognised as a “clipping” of the FID (b).

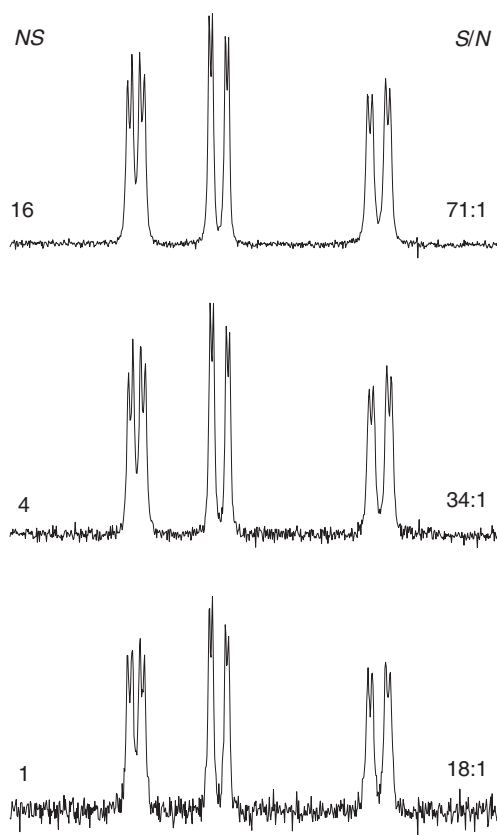


Figure 3.30. Signal averaging produces a net increase in the spectrum signal-to-noise ratio (S/N). This improves as the *square root* of the number of acquired scans (NS) because noise, being random, adds up *more slowly* than the NMR signal.

this is only true up to a point. Each time a scan is repeated, a binary number for each sampled data point is added to the appropriate computer memory location. As more scans are collected, the total in each location will increase as the signal ‘adds up’. This process can only be repeated if the cumulative total fits into the computer word size; if it becomes too large, it cannot be recorded, potentially leading to corruption of the data (although spectrometers generally handle this problem internally by simply dividing the data and reducing the ADC resolution whenever memory overflow is imminent, so preserving the data and allowing the acquisition to continue). The point at which the computer word length can no longer accommodate the signal will be dependent on the number of bits used by the ADC (its resolution) and the number of bits in the computer word. For example, if both the ADC and the word length comprise 16 bits, then any signal that fills the ADC will also fill the memory, so no further scans may be collected. Thus, the word length must be greater than the ADC resolution to allow signal averaging, and the larger the word length or the smaller the ADC resolution, the more scans can be collected. Typical word lengths on modern instruments are 32 bits, so with a 16-bit ADC it is possible to collect around 66,000 scans (2^{32-16}) if the largest signal fills the digitiser (in fact, more scans than this could be collected since the NMR signal also contains noise that does not add coherently, meaning the memory will not fill as rapidly as this simple estimation suggests). Since signal averaging is most necessary when noise levels are relatively high and because longer word lengths are used on modern instruments, the problem of overflow is now rarely encountered.

Oversampling and digital filtering

As per the standard usage on the latest generation spectrometers, it has long been realised that the effective dynamic range can, in certain circumstances, be improved by *oversampling* the FID [9]. In particular, this applies when the *digitisation noise* (or *quantisation noise*) is significant and limits the attainable signal-to-noise level of the smallest signals in the spectrum. This noise arises from the ‘rounding error’ inherent in the digitisation process, in which the analogue NMR signal is characterised in discrete one-bit steps that may not accurately represent the true signal intensity (Fig. 3.31). Although this is not true noise, but arises from systematic errors in the sampling process, it does introduce noise into the NMR spectrum. Figure 3.28 has already shown how larger errors in the digitisation process, caused by fewer ADC bits, increases the noise level. In realistic situations, this noise becomes dominant when the receiver amplification or gain is set to a relatively low level such that the thermal (analogue) noise arising from the probe and

Figure 3.31. Digitisation errors. Discrete digital sampling of an analogue waveform introduces errors in amplitude measurements, indicated by the vertical bars. These errors ultimately contribute to additional noise in the spectrum.

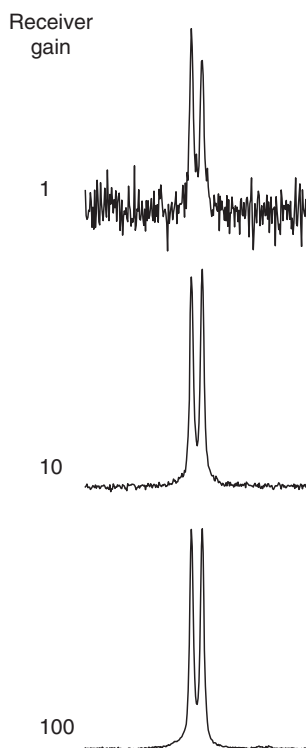
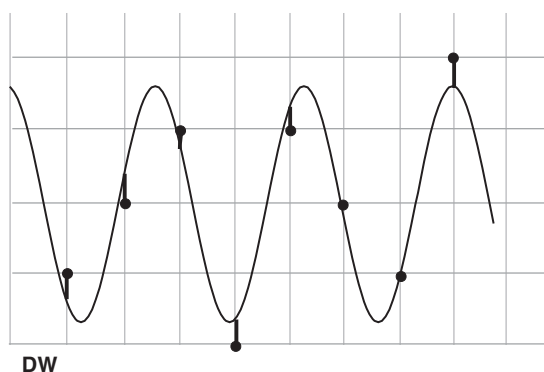


Figure 3.32. Digitisation noise. At high receiver gain settings, the noise in this proton spectrum is vanishingly small, meaning system thermal noise is low. As the gain is reduced, the amplitude of the NMR signal (and thermal noise) is reduced and digitisation noise becomes significant relative to the NMR signal.

amplifiers is negligible (Fig. 3.32). This is most likely to arise in proton spectroscopy when attempting to observe small signals in the presence of far larger ones and is especially significant with the introduction of low noise cryogenic probes. High gain settings are therefore favoured to overcome digitisation noise.

Oversampling, as the name suggests, involves digitising the data at a much faster rate than is required by the Nyquist condition or, equivalently, acquiring the data with a much greater spectral width than would normally be needed. The digitisation noise may then be viewed as being distributed over a far greater frequency range, such that in the region of interest this noise has reduced intensity so leading to a sensitivity improvement (Fig. 3.33). If the rate of sampling has been increased by an oversampling factor of N_{os} , then the *digitisation* noise is reduced by $\sqrt{N_{os}}$. Thus, sampling at four times the Nyquist frequency would theoretically produce a twofold reduction in noise, which in turn equates to an effective gain in ADC resolution of 1 bit. Likewise, oversampling by a factor of 16 corresponds to a fourfold noise reduction (a resolution gain of 2 bits) and a factor of 64 gives an 8-fold reduction or an extra 3 bits. The degree of oversampling that can be achieved is limited by the digitisation speed of the ADC, and typical values of N_{os} for proton observation are 16–32, meaning spectral widths become a few hundred kilohertz and the ADC resolution may be increased by 2–3 bits in favourable circumstances (when thermal noise can be considered insignificant relative to digitisation noise).

To maintain the desired digital resolution in the spectral region of interest when oversampling, data sets would have to be enlarged according to the oversampling factor also, which would demand greater storage capacity and slower data processing. To overcome these limitations, modern spectrometers generally combine oversampling with digital signal processing methods. Since one is really only interested in a relatively small part of our oversampled data set ($1/N_{os}$ of it), the FID is reduced after digitisation to the conventional number of data points by the process of *decimation* (literally ‘removing one-tenth of’) prior to storage. This, in effect, takes a running average of the oversampled data points, leaving one point for every N_{os} sampled, at intervals defined by the Nyquist condition for the desired spectral window (the peculiar distortions seen at the beginning of a digitally processed FID arise from this decimation process). The resulting FID then has the same number of data points as it would have if it had been sampled normally. These digital signal processing steps are generally performed with a dedicated processor after the ADC (Fig. 3.9, box b) and are typically left invisible to the user (short of setting a few software flags perhaps) although they can also be achieved by separate post-processing of the

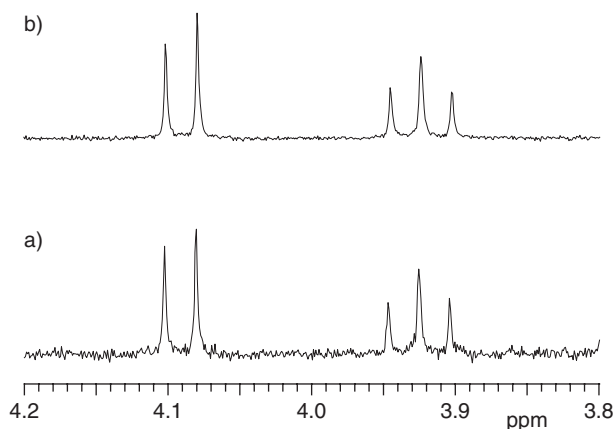


Figure 3.33. Enhanced sensitivity can be realised in favourable cases by oversampling the data and hence reducing digitisation noise. Spectrum (a) shows part of a conventional proton spectrum sampled according to the Nyquist criterion. Oversampling the data by a factor of 24 as in (b) provides a sensitivity gain (all other conditions as for (a)).

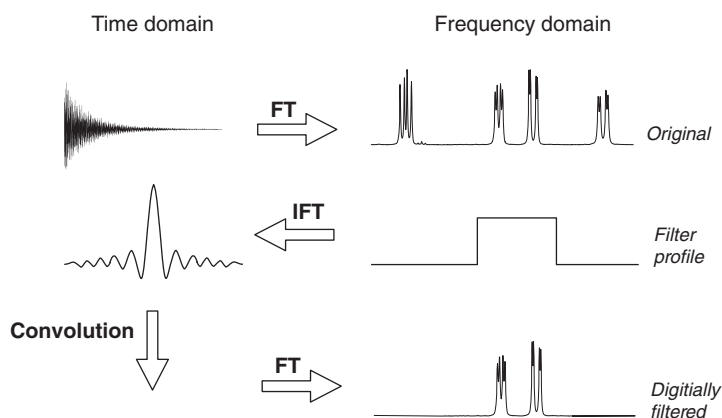


Figure 3.34. The use of a digital filter to observe a selected region of a spectrum. The desired frequency window profile is subject to an inverse Fourier Transformation and the resulting time-domain function convolved with the raw FID. Transformation of the modified data produces a spectrum containing only a subset of all resonances as defined by the digital filter.

original data or even included in the FT routine itself [10]. The use of fast dedicated processors means the calculations can readily be achieved as the data are acquired thus not limiting data collection.

One further advantage of digital processing of the FID is the ability to mathematically define frequency filters that have a far steeper and more complete cut-off than can be achieved by analogue filtration alone, meaning signal aliasing can be eliminated. Spectral widths may then be set to encompass only a sub-section of the whole spectrum, allowing *selective detection* of NMR resonances [11]. The ability to reduce spectral windows in this way without complications from signal aliasing has considerable benefit when acquiring 2D NMR data in particular. The principle behind the filtration is as follows (Fig. 3.34). The digital time-domain filter function is given by the inverse FT of the desired frequency-domain window. This function may then be convolved with the digitised FID such that, following FT, only those signals that fell within the originally defined window remain in the spectrum. One would usually like this window to be rectangular in shape although in practice such a sharp cut-off profile cannot be achieved without introducing distortions. Various alternative functions have been used which approach this ideal, generally with the property that the more coefficients used in the function, the steeper is their frequency cut-off. Since these operate only after the ADC, the analogue filter (Fig. 3.9, box a) is still required to reject broadband noise from outside the oversampled spectral width, but its frequency cut-off is now far removed from the normal spectral window and its performance less critical.

3.2.7. Window functions

There exist various ways in which the acquired data can be manipulated prior to FT in an attempt to enhance its appearance or information content. Most commonly one would either like to improve the signal-to-noise ratio of the spectrum to help reveal resonances above the noise level or to improve the resolution of the spectrum to reveal hidden fine structure. Many mathematical weighting functions, known as *window functions*, have been proposed to achieve the desired result, but a relatively small number have come into widespread use, some of which are illustrated in Fig. 3.35. The general philosophy behind all these functions is the same. In any FID, the NMR signal intensity declines throughout the acquisition time whereas the noise amplitude remains constant, meaning the relative noise level is greater in the latter part. Decreasing the tail of the FID will therefore help reduce the noise amplitude in the transformed spectrum, so enhancing *sensitivity*. Conversely, to increasing the middle and latter part of the FID equates to retarding the decay of the signal, thus narrowing the resonance and so enhancing *resolution*.

One word of warning here before proceeding. The application of window functions has also been referred to in the past as ‘digital processing’ or ‘digital filtering’ of the data. However, this should not be confused with the digital signal processing terminology introduced in previous sections and in widespread use nowadays. The adoption of the terms ‘window’ or ‘weighting’ function is therefore recommended in the context of sensitivity or resolution enhancement.

Sensitivity enhancement

As suggested above, the noise amplitude in a spectrum can be attenuated by de-emphasising the latter part of the FID, and the most common procedure for achieving this is to multiply the raw data by a decaying exponential function (Fig. 3.35a). This process is therefore also referred to as *exponential multiplication*. Because this forces the tail of the

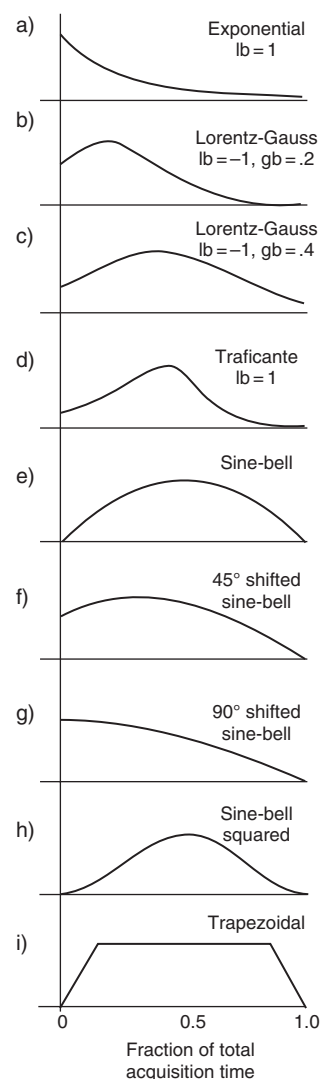
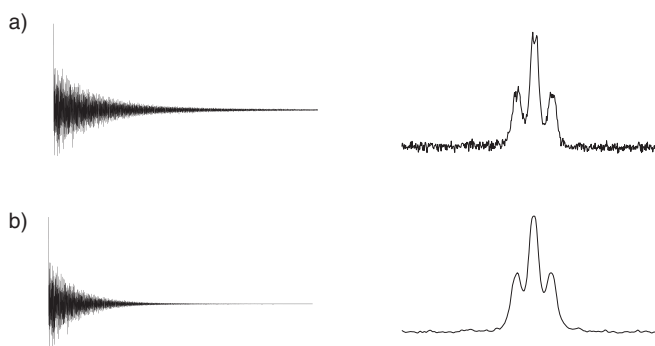


Figure 3.35. Some commonly employed window functions. These are used to modify the acquired FID to enhance sensitivity and/or resolution (lb = line broadening parameter, gb = Gaussian broadening parameter, i.e. the fraction of the acquisition time when the function has its maximum value; see text).

Figure 3.36. Exponential multiplication of the FID can be used to reduce noise in the spectrum. (a) Raw FID and spectrum following Fourier transformation. (b) Results after exponential processing with $lb = 1$ Hz.



FID towards zero, it is also suitable for the apodisation of truncated data sets. However, it also increases the apparent decay rate of the NMR signal, causing lines to broaden, meaning one compromises resolution for the gain in sensitivity (Fig. 3.36). Using too strong a function, that is one that causes the signal to decay too rapidly, can actually lead to a decrease in signal-to-noise ratio in the resulting spectrum because the broadening of the lines causes a reduction of their peak heights. Spectrometer software usually allows one to define the amount of line broadening directly in hertz (here parameter lb), so exponential multiplication is rather straightforward to use and allows the chemist to experiment with different degrees of broadening to attain a suitable result. The optimum balance between reducing noise and excessive line broadening is reached when the decay of the window function matches the natural decay of the NMR signal, in which case it is known as the *matched filter* [12], which results in a doubling of the resonance linewidth. For the exponential function to truly match the FID, the decay of the NMR signal must also be an exponential (meaning the NMR resonances have a Lorentzian lineshape), which on a correctly adjusted spectrometer is usually assumed to be the case (at least for spin- $\frac{1}{2}$ nuclei). Despite providing the maximum gain in sensitivity, the matched condition is often not well suited to routine use in proton spectroscopy as the resulting line broadening and loss of resolution may preclude the separation of closely spaced lines, so less line broadening than this may be more appropriate. Furthermore, different resonances in the spectrum often display different unweighted linewidths, so the matched condition cannot be met for all resonances simultaneously. For routine proton work, it turns out to be convenient to broaden the line by an amount equal to the digital resolution in the spectrum, as this leads to some sensitivity enhancement but to a minimal increase in linewidth. For heteronuclear spectra, resolution is usually a lesser concern and line broadening of a few hertz is commonly employed (comparable to or slightly greater than the digital resolution). This can be considered essential to attenuate the distortions about the base of resonances arising, in part, from truncation of the data set (Section 3.2.3 and Fig. 3.17). For spectra that display resonances that are tens or even hundreds of hertz wide (most notably those of quadrupolar nuclei), the amount of line broadening must be increased accordingly to achieve any appreciable sensitivity gain.

Resolution enhancement

One might suppose that to improve the resolution in spectra, we should apply a function that enhances the latter part of the FID, so increasing the decay time. The problem with this approach is that by doing this one also increases the noise amplitude in the tail of the FID and emphasises any truncation of the signal that may be present, so increasing the potential for undesirable ‘truncation wiggles’. A better approach is to apply a function that initially counteracts the early decay but then forces the tail of the FID to zero to provide the necessary apodisation. The most popular function for achieving this has been the *Lorentz–Gauss transformation* [13] (Fig. 3.35 b and c), sometimes loosely referred to as *Gaussian multiplication* (although this strictly refers to yet another mathematical weighting function) and also known as double-exponential multiplication. This transforms the usual Lorentzian lineshape into a Gaussian lineshape, which has a somewhat narrower profile, especially around the base (Fig. 3.37), and it is this feature that allows the resolution of closely spaced lines.

The shape of the function is altered by two variable parameters that define the degree of line narrowing in the resulting spectra and the point during the acquisition time at which the function reaches its maximum value. These are usually presented to the operator as negative line broadening in hertz (here parameter lb) and as a fraction of the total acquisition time (here parameter gb), respectively. The choice of suitable values for these usually comes down to a case of trial-and-error, and different optimum values may be found for different groups of peaks within the same spectrum. Modern NMR

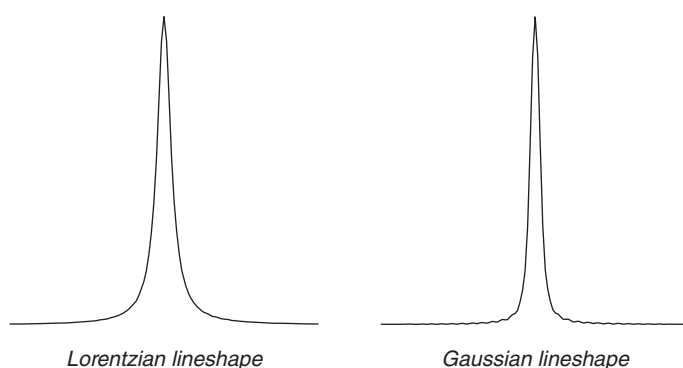


Figure 3.37. A comparison of the Lorentzian and Gaussian lineshapes.

processing packages usually allow an interactive variation of the parameters during which one can observe both the window function itself and the resulting spectrum, so an optimum can rapidly be determined. A more negative line broadening will produce narrower lines whilst positioning the maximum further along the decay will enhance this effect. This will also lead to greater distortion of the resonances about the base and to degradation of sensitivity as the early signals are sacrificed relative to the noise (Fig. 3.38). In any case, some reduction in sensitivity will invariably result, and it is necessary to have reasonable signal-to-noise for acceptable results. It has been suggested [14] that the optimum Gaussian resolution enhancement for routine use on spectra with narrow lines aims for a reduction in linewidth by a factor of 0.66 for which the function maximum should occur at a time of $1/(\Delta\nu_{1/2})$ s, that is, the inverse of the resonance linewidth in the absence of window functions. The appropriate figures for setting in the processing software can then be arrived at by simple arithmetic. For example, a starting linewidth of 1 Hz will require a line narrowing of 0.66 Hz and the maximum to occur after 1 s. With a typical proton acquisition time of around 3 s, the function should therefore reach a maximum at 1/3 of the total acquisition. Gaussian multiplication can be used to good effect when combined with zero-filling, thus ensuring the digital resolution is sufficiently fine to define any newly resolved fine structure.

In more recent years, new window functions have been introduced [15, 16] that are similar to the Lorentz–Gauss window but which aim to improve resolution without a discernible reduction in sensitivity. These so called *TRAF* functions (Fig. 3.35d) generally aim to enhance the middle part of the FID but also use matched filtration of the later part to attenuate noise. They are appearing in NMR software packages and provide an alternative to the established functions. Another function, more commonly used in the processing of 2D data sets, is the *sine-bell* window, which has no adjustable parameters. This comprises one half of a sine wave, starting at zero at the beginning of the FID, reaching a maximum half way through the acquisition and falling back to zero by the end of the decay (Fig. 3.35e). The function always has zero intensity at the end, so eliminates the truncation often encountered in 2D data sets. This tends to be a rather

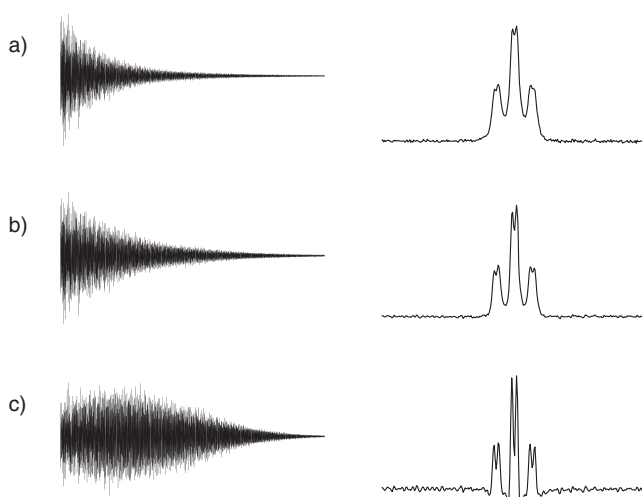


Figure 3.38. The Lorentz–Gauss transformation (“Gaussian multiplication”) can be used to improve resolution. (a) Raw FID and spectrum following Fourier transformation and results after the Lorentz–Gauss transformation with (b) $lb = -1$ Hz, $gb = 0.2$ and (c) $lb = -3$ Hz and $gb = 0.2$.

severe resolution enhancement function that can introduce undesirable lineshape distortions and also produces severe degradation in sensitivity because the early part of the NMR signal is heavily attenuated, and hence this is rarely used in 1D spectra. A variation on this is the *phase-shifted sine-bell* for which the point at which the maximum occurs is a variable (Fig. 3.35f and g) and can be moved towards the start of the FID. Again, this is commonly applied in 2D processing and has the advantage that the user has the opportunity to balance the gain in resolution against the lineshape distortion and sensitivity degradation. A final variant is the (optionally shifted) *squared sine-bell* (Fig. 3.35h), which has similar properties to the sine-bell but tails more gently at the edges, which can invoke subtle differences in 2D spectra. Likewise, the *trapezoidal* function (Fig. 3.35i) is sometimes used in the processing of 2D data.

3.2.8. Phase correction

It has already been mentioned in Section 2.2 that the phase of a spectrum needs correcting following FT because the receiver reference phase does not exactly match the initial phase of the magnetisation vectors. This error is constant for all vectors, and since it is independent of resonance frequencies, it is referred to as the ‘zero-order’ phase correction (Fig. 3.39). Practical limitations also impose the need for a frequency-dependent or ‘first-order’ phase correction. Consider events immediately after the pulse is applied to the sample. A short period of time, the pre-acquisition delay, DE, is required for the spectrometer electronics to recover from the effect of the pulse before an undistorted FID can be collected. This delay is typically tens of microseconds, during which magnetisation vectors will evolve a little according to their chemical shifts so that at the point digitisation begins they no longer have the same phase (Fig. 3.40). Clearly those resonances with the greatest shifts require the largest corrections. If the DE is small relative to the frequency offsets, the phase errors have an approximately linear offset dependency and can be removed. If the delay becomes large, the correction cannot be made without introducing a rolling spectrum baseline. The appearance of these zero- and first-order phase errors is illustrated in the spectra of Fig. 3.41.

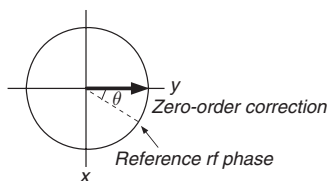


Figure 3.39. Zero-order (frequency-independent) phase errors arise when the phase of the detected NMR signals does not match the phase of the receiver reference rf. All resonances in the spectrum are affected to the same extent.

Typically, both forms of error occur in a spectrum directly after the FT. The procedure for phase correction is essentially the same on all spectrometers. The zero-order correction is used to adjust the phase of one signal in the spectrum to pure absorption mode, as judged ‘by eye’, and the first-order correction is then used to adjust the phase of a signal far away from the first in a similar manner. Ideally, the two chosen resonances should be as far apart in the spectrum as possible to maximise the frequency-dependent effect. Experimentally, this process of phase correction involves mixing of the real and imaginary parts of the spectra produced by the FT process such that the final displayed ‘real’ spectrum is in pure absorption mode whereas the usually unseen ‘imaginary’ spectrum is pure dispersion.

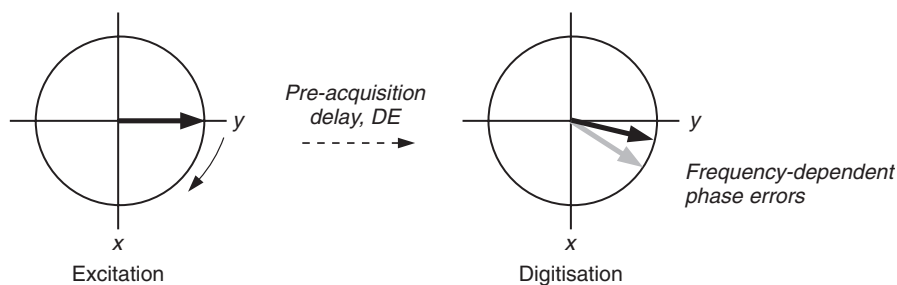
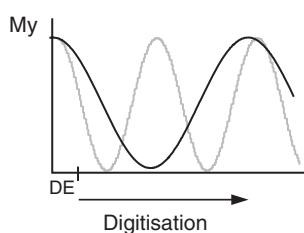


Figure 3.40. First-order (frequency-dependent) phase errors arise from a dephasing of magnetisation vectors during the pre-acquisition delay that follows the excitation pulse. When data collection begins, vectors with different frequencies have developed a significant phase difference, which varies across the spectrum.



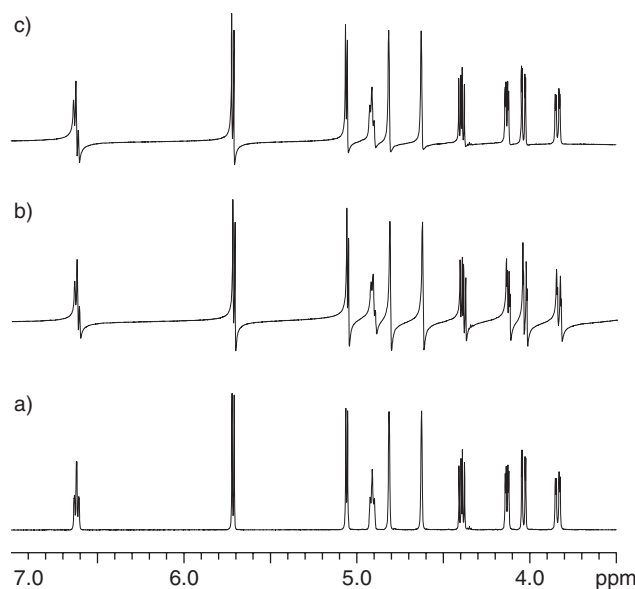


Figure 3.41. Zero- and first-order phase errors in a ^1H spectrum. (a) The correctly phased spectrum, (b) the spectrum in the presence of frequency-independent (zero-order) phase errors and (c) the spectrum in the presence of frequency-dependent (first-order) phase errors.

3.3. PREPARING THE SAMPLE

This section describes some of the most important aspects of sample preparation for NMR analysis, a topic that is all too frequently given insufficient consideration in the research laboratory. Even for routine applications of NMR, it is wise for the chemist to adopt a systematic strategy for sample preparation that will give consistently good results, so saving instrument time and eliminating frustration.

3.3.1. Selecting the solvent

Since all modern NMR spectrometers rely on a deuterium lock system to maintain field stability, a deuterated solvent is invariably required for NMR, and these are now available from many commercial suppliers. In a busy organic chemistry laboratory, the volume of NMR solvents used can be surprisingly large, and the cost of the solvent may play some part in its selection. Generally, chloroform and water are the most widely used solvents and are amongst the cheapest, although dimethylsulfoxide is becoming increasingly favoured, notably in the industrial sector, because of its useful solubilising properties. Acetone, methanol and dichloromethane are also in widespread routine use.

The selection of a suitable solvent for the analyte is based on a number of criteria. The most obvious requirement is that the analyte be soluble in it at the concentration required for the study. This will be dependent on a diverse range of factors including the fundamental sensitivity of the nucleus, the overall sensitivity of the instrument, the nature of the experiment and so on. If experiments are likely to be performed at very low temperatures, then it is also important to know that the solute remains in solution when the temperature is reduced so that a precipitate does not form in the NMR tube and degrade resolution. For experiments at temperatures other than ambient probe temperatures (which may be a few degrees greater than the ambient room temperature), the melting and boiling points of the solvents must also be considered. Table 3.2 summarises some important properties of the most commonly encountered solvents. For work at very high temperatures, dimethylsulphoxide or toluene is generally the solvents of choice, whilst for very low temperatures, dichloromethane, methanol or tetrahydrofuran are most appropriate. Even for experiments performed at ambient temperatures, solvent melting points may be limiting; on a cold day, dimethylsulfoxide solutions can freeze in the laboratory. The viscosity of the solvent will also influence the resolution that can be obtained with the best performance provided by the least viscous solvents, such as acetone (which, for this reason, is used as the solvent for spectrometer resolution tests). The sharpest possible lock signal is also beneficial in experiments based on difference spectroscopy, the most important of these being the NOE difference experiment (Chapter 8).

For proton and carbon spectroscopy, the chemical shifts of the solvent resonances need to be anticipated as these may occur in a particularly unfortunate place and interfere with resonances of interest. In proton spectroscopy, the observed solvent resonance arises from the residual *protonated* species (NMR solvents are typically supplied with deuteration

Table 3.2. Properties of the common deuterated solvents. Proton shifts, δ_H , and carbon shifts, δ_C , are quoted relative to tetramethylsilane (TMS) (proton shifts are those of the residual partially *protonated* solvent). The proton shifts of residual HDO/H₂O vary depending on solution conditions

Solvent	δ_H /ppm	$\delta_{(HDO)}$ /ppm	δ_C /ppm	Melting point/°C	Boiling point/°C
Acetic acid-d ₄	11.65, 2.04	11.5	179.0, 20.0	16	116
Acetone-d ₆	2.05	2.0	206.7, 29.9	−94	57
Acetonitrile-d ₃	1.94	2.1	118.7, 1.4	−45	82
Benzene-d ₆	7.16	0.4	128.4	5	80
Chloroform-d ₁	7.27	1.5	77.2	−64	62
Deuterium oxide-d ₂	4.80	4.8	—	4	101
Dichloromethane-d ₂	5.32	1.5	54.0	−95	40
N,N-dimethyl formamide-d ₇	8.03, 2.92, 2.75	3.5	163.2, 34.9, 29.8	−61	153
Dimethylsulfoxide-d ₆	2.50	3.3	39.5	18	189
Methanol-d ₄	4.87, 3.31	4.9	49.2	−98	65
Pyridine-d ₅	8.74, 7.58, 7.22	5.0	150.4, 135.9, 123.9	−42	114
Tetrahydrofuran-d ₈	3.58, 1.73	2.4	67.6, 25.4	−109	66
Toluene-d ₈	7.09, 7.00, 6.98, 2.09	0.4	137.9, 129.2, 128.3, 125.5, 20.4	−95	111
Trifluoroacetic acid-d ₁	11.30	11.5	164.2, 116.6	−15	75
Trifluoroethanol-d ₃	5.02, 3.88	5.0	126.3, 61.5	−44	75

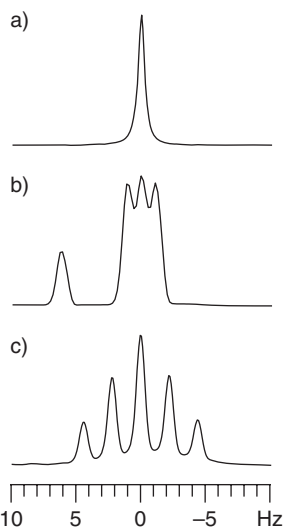


Figure 3.42. Residual protonated resonances of deuterated solvents (a) CHCl₃ in CDCl₃, (b) CHDCl₂ in CD₂Cl₂ and (c) CHD₂COCOD₃ in (CD₃)₂CO. The multiplicity seen in (b) and (c) arises from two-bond (geminal) couplings to spin-1 deuterium producing a 1:1:1 triplet and a 1:2:3:2:1 quintet respectively. The left-hand singlet in (b) is residual CH₂Cl₂ in the solvent, the shift difference arises from the H–D isotope shift of 6 Hz.

levels in excess of 99.5%). For routine studies, where a few milligrams of material may be available, the lower specification solvents should suffice for which the residual protonated resonance is often comparable in magnitude to that of the solute. Solvents with higher levels of deuteration are beneficial for proton spectroscopy when sample quantities are of the order of tens of micrograms or less and are likely to be used in conjunction with microsample techniques (Section 3.3.3). The solvent proton resonance, with the exception of chloroform and water, will comprise a multiplet from coupling to spin-1 deuterium. For example, dichloromethane displays the triplet of CDHCl₂ whilst acetone, dimethylsulphoxide or methanol show a quintuplet from CD₂H (Fig. 3.42). In carbon spectroscopy, the dominant resonance is often that of the deuterated solvent that again will be a multiplet owing to coupling with deuterium. For other common nuclei, interference from the solvent is rarely a consideration, the one notable exception to this being deuterium itself. Unless very large sample quantities are being used (many tens of milligrams) and one can be sure the solvent resonance will not overlap those of the solute, it is usually necessary to record ²H spectra in *protonated* solvent since the huge signals of the deuterated solvent are likely to swamp those of interest. This then precludes the use of the lock system for maintaining field stability. The lack of a suitable lock reference requires a small quantity of the deuterated solvent to be added to the solution to provide an internal chemical shift reference (see below).

Aside from the solvent resonance itself, the other significant interference seen in proton spectra is water, which is present to varying degrees in all solvents and is also often associated with solutes. The water resonance is often rather broad, and its chemical shift can vary according to solution conditions. All solvents are hygroscopic to some degree (including deuterated water) and should be exposed to the atmosphere as little as possible to prevent them from becoming wet. Very hygroscopic solvents such as dimethylsulphoxide, methanol and water are best kept under a dry inert gas atmosphere. Solvents can also be obtained in smaller glass ampoules that are particularly suitable for those that are used only infrequently. Molecular sieves may also be used to keep solvents dry, although some care is required in filtering the prepared solution before analysis to remove fine particulates that may be present. Alternatively, the NMR solution may be passed through activated alumina to remove water, as part of the filtration process.

The solvent may also result in the loss of the resonances of exchangeable protons since these will become replaced by deuterons. In particular, this is likely to occur with deuterated water and methanol. To avoid deuterium replacement altogether, one must consider using H₂O containing 10–20% D₂O (to provide a lock signal) or d₃-methanol (CD₃OH). In these cases, a suitable solvent suppression scheme (Chapter 10) is required to attenuate the large solvent resonance (which may still lead to the loss of the exchangeable protons of the solute by the process known as saturation transfer). Studies in 90% H₂O dominate the NMR of biological macromolecules because solvent exchangeable protons, such as the backbone amide NH protons in peptides and proteins or the imido protons of DNA and RNA base pairs, often play a key role in the structure determination of these molecules [17, 18].

The nature of the solvent can also have a significant influence on the appearance of the spectrum, and substantial changes can sometimes be observed on changing solvents. Whether these changes are beneficial is usually difficult to predict, and a degree of trial

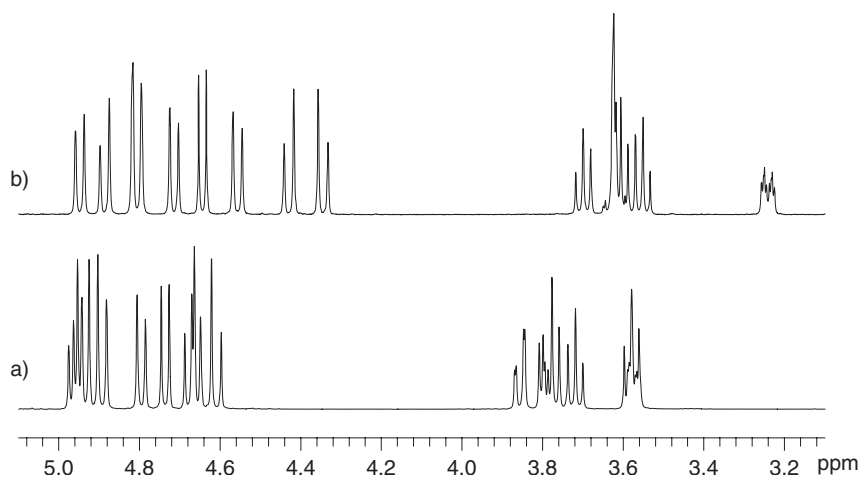
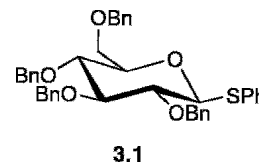


Figure 3.43. Changes in solvent can be used to improve resonance dispersion. The proton spectrum of the sugar **3.1** is shown in (a) CDCl_3 and (b) C_6D_6 . Notice the appearance of the resonance at 3.2 ppm in (b) that was hidden at 3.6 ppm by another resonance in (a).



and error is required. A useful switch of solvent is from a non-aromatic solvent into an aromatic one, for example, chloroform to benzene (Fig. 3.43), making use of the magnetic anisotropy exhibited by the latter. In cases of particular difficulty, where the change from one solvent to another simply produces a different but equally unsuitable spectrum, then titration of the second solvent into the first may provide a suitable compromise between the two extremes. Such changes may prove useful in 1D experiments that use selective excitation of a specific resonance, by revealing a target proton when previously it was hidden, as in the example in Fig. 3.43. The selective removal of a resonance by the addition of another solvent can also prove useful in spectrum interpretation. Adding a drop of D_2O to an organic solution in the NMR tube, mixing thoroughly and leaving the mix to settle removes (or attenuates) acidic exchangeable protons. Acidic protons that are protected from the solvent, such as those in hydrogen bonds, may not fully exchange immediately, but may require many hours to disappear. This can provide a useful probe of H-bonding interactions.

3.3.2. Reference compounds

When preparing a sample it is common practice to add a suitable compound to act as an *internal* chemical shift reference in the spectrum, and the selection of this must be suitable for the analyte and the solvent. In proton and carbon NMR, the reference used in organic solvents is tetramethylsilane (TMS, 0.0 ppm), which has a number of favourable properties; it has a sharp 12-proton singlet resonance that falls conveniently to one end of the spectrum, and it is volatile so can be readily removed and it is chemically inert. In a few cases, this material may be unsuitable, such as in the study of silanes or cyclopropanes. For routine work, it is often not necessary to add any internal reference as the residual lines of the solvent itself can serve this purpose (Table 3.2). For aqueous solutions, the water soluble equivalent of TMS is partially deuterated sodium 3-(trimethylsilyl)propionate- d_4 (TSP- d_4), which is also referenced to 0.0 ppm. A volatile alternative is 1,4-dioxane (^1H 3.75 ppm, ^{13}C 67.5 ppm), which can be removed by lyophilisation. The standard reference materials for some other common nuclides are summarised in Table 3.3. Often these are not added into the solution being studied but are held in an outer, concentric jacket or within a separate axial capillary inside the solution, in which case the reference material may also be in a different solvent to that of the sample.

An alternative to adding additional reference materials is to use a so-called external reference. Here the spectrum of a separate reference substance is acquired before and/or after the sample of interest and the spectrum reference value carried over. Identical field settings should be used for both, which, on some older instruments, requires the same lock solvent, or an additional correction to the spectrum reference frequency must be used to compensate any differences. This restriction does not arise on instruments that use shifting of the lock transmitter frequency to establish the lock condition.

The referencing of more 'exotic' nuclei is generally less clear-cut than for those in common use, and in many cases, it is impractical to add reference materials to precious samples, and it is sometimes even difficult to identify what substance is the 'accepted' reference standard. In such cases, the IUPAC Ξ -scale can be used, which does not require use of a specific reference material. Instead this scheme defines the *reference frequency* for the reference material of each nuclide at a field strength at which the proton signal of TMS resonates at exactly 100.000 MHz. The reference frequencies are scaled appropriately for

Table 3.3. Spectrum reference materials (0 ppm) and reference frequencies (Ξ values) for selected nuclides

Nuclide	Primary reference	Ξ value/MHz	Alternative reference
^1H	Me_4Si	100.000,000	TSP- d_4 (aq)
^2H	Me_4Si	15.351	Trace deuterated solvent
^6Li	LiCl (aq)	14.717	
^7Li	LiCl (aq)	38.866	
^{10}B	$\text{BF}_3\text{O}(\text{Et})_2$	10.746	H_2BO_3 (aq)
^{11}B	$\text{BF}_3\text{O}(\text{Et})_2$	32.089	H_2BO_3 (aq)
^{13}C	Me_4Si	25.145,004	1,4-dioxan @ 67.5 ppm, TSP- d_4 (aq)
^{14}N	CH_3NO_2	7.224	
^{15}N	CH_3NO_2	10.136,767	NH_4NO_3 (aq), NH_3 (liq) ^a
^{17}O	H_2O	13.557	
^{19}F	CFCl_3	94.094,003	
^{29}Si	Me_4Si	19.867,184	
^{31}P	H_3PO_4 (85%)	40.480,747	
^{119}Sn	Me_4Sn	37.290,665	
^{195}Pt	$\text{K}_2[\text{Pt}(\text{CN})_6]$	21.414,376	
^{207}Pb	Me_4Pb	20.920,597	

^a ^{15}N shifts of biomolecular materials are more often quoted relative to external liquid ammonia.

the magnetic field in use and this then defines the absolute frequency at 0.0 ppm for the nuclide in question. The ‘master’ reference sample for these measurements is 1% TMS in CDCl_3 , which is used to determine the absolute frequency of TMS for the instrument in use. The Ξ values for selected nuclei are also summarised in Table 3.3, whilst more extensive tables are available [19, 20].

3.3.3. Tubes and sample volumes

Newcomers to the world of practical NMR often find the cost of NMR tubes surprisingly high. The prices reflect the need to produce tubes that conform to strict requirements of straightness (camber) and concentricity, as deviation from these can produce undesirable artefacts in spectra, usually in the form of ‘spinning-sidebands’. Generally, the higher quality (and more expensive) tubes are required on higher field instruments, and this is particularly so for proton work. Some experimenting may be required to decide on a suitable balance between cost and performance for your particular instruments.

The diameter of the tube is generally determined by the dimensions of the probe to be used, or more precisely the probe’s rf coils. Tube diameters employed in chemical laboratories (Fig. 3.44) include 1, 1.7, 2.5 or 3 mm (for use with the so-called microprobes), 5 mm (the most widely used) or 10 mm (typically used for the observation of low sensitivity nuclei where the solubility of the material to be studied is limiting or for dedicated polymer studies). Eight millimetre tubes have found use in biological macromolecular work, where solubility and aggregation considerations preclude the use of concentrated samples. The benefit of the smaller diameter tubes and probes lies in their greater ‘mass sensitivity’ as will be explained in Section 3.4.2, which describes available probe technologies. As sensitivity is usually of prime importance when performing any NMR experiment, it is

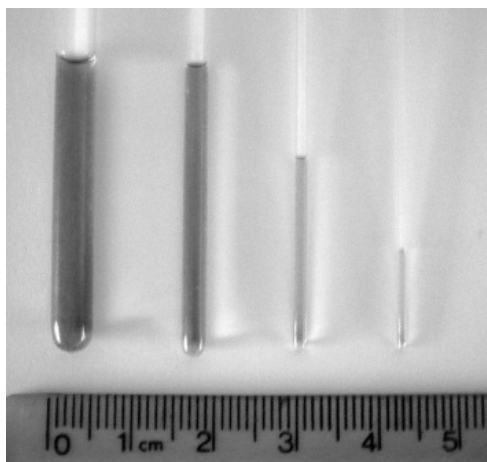


Figure 3.44. NMR tubes of various diameters and their optimum solution volumes (from left to right): 5 mm (500 μL), 3 mm (150 μL), 1.7 mm (30 μL) and 1 mm (5 μL).

Table 3.4. Sample tube diameters and their associated working sample volumes and heights

Tube dimensions (outer diameter)	Solute volume/ μL	Solute column height/mm
10	2500	40
8	1500	40
5	500	40
3	150	40
1.7	30	20
1	5	10

prudent to use the correct sample volume for the probe/tube configuration and not to dilute the sample any more than is necessary. An important factor here is the length of the detection coil within the probe. The sample volume should be sufficient to leave a little solution above and below the coil since magnetic susceptibility differences at the solution/air and solution/glass interfaces lead to local distortions of the magnetic field. For a standard 5 mm tube, the coil length is typically 16 mm and the required volume will be around 500–600 μL whereas for the narrower coils and tubes this may reduce to 100–150 μL for the 3 mm tube, 25–30 μL for the 1.7 mm tube or as little as 5–8 μL for the 1 mm microtube (Table 3.4). For 10 mm tubes, it may be necessary to use a ‘vortex-suppressor’ to prevent whirlpool formation in the tube if the sample is spun. These are usually polytetrafluoroethylene plugs designed to fit tightly within the NMR tube, which are pushed down to sit on the surface of the sample and thus hold the solution in place. Some care is required when using these plugs in variable temperature work due to the thermal expansion or contraction of the plug, and at low temperatures, the plug may even fall into the solution so its use may be inappropriate. Filling the smaller 1 and 1.7 mm tubes requires the use of fine needles with a manual syringe or robotic system, or alternatively can be readily filled by placing the solution in the top tube funnel and centrifuging this down.

For the handling of smaller sample quantities when microprobes are not available, the microtubes of small diameter that are designed for use with microprobes may be employed to concentrate the available material within the detection coil. Thus, using a 3 mm tube in a 5 mm probe has been shown to provide a modest signal-to-noise improvement for conventional probes whereas this approach can provide greater gains when using cryogenically cooled probe heads; see Section 3.4.2. Alternatively, microcells can be obtained that fit within standard 5 mm NMR tubes, but which require only tens of microlitres of solution again allowing greater sample concentration. When using such cells, it is advisable to fill the volume around the outside of the cell with solvent, as this minimises susceptibility differences and improves resolution. Additionally, special tubes and plugs (‘Shigemi tubes’) are available that allow all the sample volumes to be held within the sensitive region of the rf coil (Fig. 3.45). These use glasses that have a magnetic susceptibility matched to that of the solvent and so allow shorter sample heights without the introduction of lineshape defects [21]. Typically, tubes are available individually matched to water and to a small selection of organic solvents and are available in 5 and 3 mm sizes. When using these it is imperative that no air bubbles become trapped between the solvent and the upper plug to avoid lineshape perturbations, and some practise in their use is highly recommended

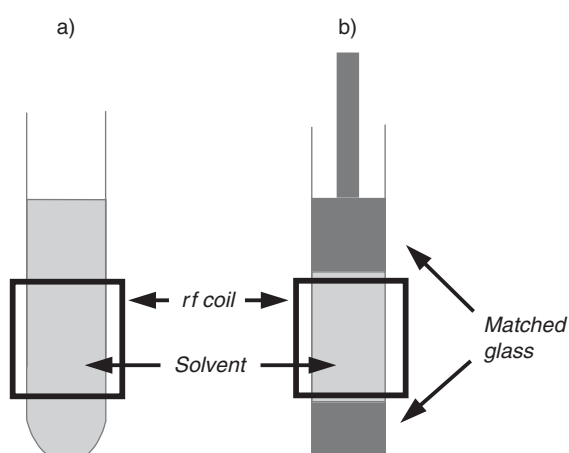


Figure 3.45. Schematic comparison of (a) a conventional NMR tube and (b) a Shigemi matched tube and plug in which the magnetic susceptibility of the glass is matched to that of the solvent. This allows a smaller sample volume and so concentrates more of the analyte within the detection coil.

before embarking on the preparation of your most precious sample. More recently, specialised tubes have been introduced specifically for use with cryogenically cooled probes that have rectangular cross-sections, for example, 3×6 mm (Section 3.4.2). These have been shown to provide enhanced sensitivity when observing samples of high ionic strength (buffered or salty samples) by reducing pick-up of electrical noise arising from the sample solution and so are likely to be of greatest applicability for the study of biomacromolecules or biofluids.

NMR tubes should be kept clean, dry, free from dust and must also be free from scratches on the glass as this distorts the cylinder in which the sample is confined. New tubes may not always be clean when delivered, although this is often assumed to be the case. Organic lubricants used in their manufacture may remain on the glass and become all too apparent when the tube is first used. For routine washing of tubes, rinsing with a suitable organic solvent, such as acetone, or with distilled water a few times is usually sufficient. More stubborn soiling is best tackled by chemical rather than mechanical means and either soaking in detergent or strong mineral acids is recommended. The use of chromic acid must be avoided as this can leave traces of paramagnetic chromium(VI), which will degrade resolution. Tubes should not be dried by subjecting them to high temperatures for extended periods of time as this tends to distort them. A better approach is to keep the tubes under vacuum, at slightly elevated temperatures if possible, or to blow filtered nitrogen into the tubes. If oven drying is used, the tubes must be laid on a flat tray and heated for only 30 min, not placed in a beaker or tube rack as this allows them to bend. If the removal of all traces of protiated water from the tube is important, then it is necessary to rinse the tube with D_2O prior to drying to ensure the exchange of water adsorbed onto the surface of the glass. Unused tubes should be stored with their caps on to prevent dust from entering. Finally, it is also important to avoid contamination of the *outside* of the NMR tube (which includes fingerprint oils) as this leads to the transfer of the contaminants into the probe head. The accumulation of these contributes to degradation of the instrument's performance over time.

3.3.4. Filtering and degassing

For any NMR experiment, it is necessary to achieve a uniform magnetic field throughout the whole of the sample to obtain a high-resolution spectrum, and it is easy to imagine a range of circumstances that detract from this condition. One of the most detrimental is the presence of particulates in the sample since these can distort the local magnetic field, so it is of utmost importance to remove these from an NMR sample prior to analysis. Even very slight changes in the field within the sample can produce a noticeable reduction in resolution. This should not come as a surprise if one recalls that one often wishes to resolve lines whose resonant frequencies are many hundreds of megahertz but which are separated by less than 1 Hz. This corresponds to a resolution of 1 part in 10^9 , roughly equivalent to measuring the distance from the Earth to the Sun to an accuracy of 1 km! Commercial sintered filters can be obtained but require constant washing between samples. A convenient and cheap alternative is to pass the solution through a small cotton wool plug in a Pasteur pipette directly into the NMR tube (it is good practise to rinse the cotton wool with a little of the NMR solvent before this). If cotton wool is not suitable for the sample, a glass wool plug may be used although this does not provide such a fine mesh. If metal ions are likely to be present in solution (paramagnetic ions being the most unwelcome), either from preparation of the sample or from decomposition of the sample itself, then passing the solution through a small column of a metal chelating resin should remove these.

In situations where very high resolution is demanded or if relaxation studies are to be performed, then it may also be necessary to remove all traces of oxygen from the solution. This may include the measurement of nuclear Overhauser enhancements, although for routine NOE measurements, it is usually not necessary to go to the trouble of degassing the sample (see Chapter 8). The need to remove oxygen arises because O_2 is paramagnetic and its presence provides an efficient relaxation pathway that leads to line broadening. Essentially, there are two approaches to degassing a sample. The first involves bubbling an inert gas through the solution to displace oxygen. This is usually oxygen-free nitrogen or argon, which need be passed through the solution in the NMR tube for about 1 minute for organic solvents and double this for aqueous solutions. Great care is required when attempting this, and a fine capillary must be used to introduce the gas *slowly*. It is all too easy to blow the sample clean out of the tube in an instant by introducing gas too quickly, so it is probably wise to get some feel for this before attempting the procedure on your most precious sample. Note that with volatile solvents a significant loss in volume is likely to occur and TMS, if used, may well also be lost.

The second and more thorough approach is the ‘freeze-pump-thaw’ technique. The NMR tube containing the solution is frozen with liquid nitrogen or dry ice and placed under vacuum on a suitable vacuum line. Commercial tube manufacturers produce a variety of specialised tubes and adapters for this purpose, but a simple alternative is to connect a standard tube to a vacuum line via a needle through a rubber septum cap on the tube. The tube is then isolated from the vacuum by means of a stopcock and allowed to thaw, during which the dissolved gases leave the solution. The procedure is then repeated typically at least twice more, this usually being sufficient to fully degas the sample. When using an ordinary vacuum pump, it may be necessary to place a liquid nitrogen trap between the pump and the sample to avoid the possibility of vacuum oils condensing in the sample tube. Furthermore, when freezing aqueous samples, it is easy to crack the tube if this is carried out too fast; holding the sample tube just above the freezing medium whilst tilting it is usually sufficient to avoid such disasters. An alternative approach for aqueous samples containing involatile solutes is to carefully place the sample directly under vacuum without freezing, for example, in a vacuum jar or schlenk, and allowing dissolved gas to bubble out of solution.

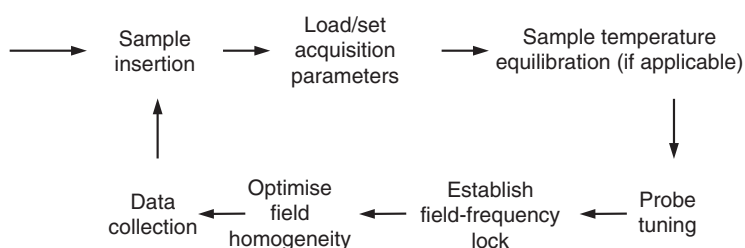
Following the degassing procedure, the tube should be sealed. If the sample is likely to be subject to short-term analysis, say over a few hours, then a standard tight-fitting NMR cap wrapped with a *small* amount of paraffin wax film or, better still, use of a rubber septum, is usually sufficient since diffusion into solution of gases in the tube will be rather slow. For longer duration studies, specialised adapters or screw-top tubes can be purchased or standard tubes can be flame-sealed, for which NMR tubes with restrictions towards the top are available to make the whole process very much easier. When flame sealing, it is highly advisable to cool or freeze the solution and then seal whilst pulling a gentle vacuum. Failure to cool the solution or evacuate the tube may cause the pressure within it to become dangerously high as the sample warms. The seal should be symmetric, otherwise the sample will spin poorly, so practice on discarded tubes is likely to pay dividends.

3.4. PREPARING THE SPECTROMETER

Whenever a new sample is placed within the NMR spectrometer, the instrument must be optimised for this. The precise nature of the adjustments required and the amount of time spent making these will depend on the sample, the spectrometer and the nature of the experiment, but in all cases, the aim will be to achieve optimum resolution and sensitivity and to ensure system reproducibility. The details of the approaches required to make these adjustments depend on the design of spectrometer in question, so no attempt to describe such detail is made here. They all share the same general procedures however, which are summarised in Scheme 3.1 and described below.

3.4.1. The probe

If you are a chemist making use of the NMR facilities available to you, then choosing the appropriate probe for the study in question may not be relevant as you will probably be forced to use that which is available. If, however, you are involved with instrument purchasing or upgrading or you are fortunate enough to have available a variety of probes for a given instrument, then it is important to be able to make the appropriate selection. Since it is the probe that must receive the very weak NMR signals, it is perhaps the most critical part of the NMR spectrometer, and its particular design and construction will influence not only the types of experiments it is able to perform but also its overall performance. There has been considerable progress over the years in the design of probes, resulting in ever-increasing performance and a greater array of available probe technologies, and these will be reviewed in more detail in Section 3.4.2 below.



Scheme 3.1. The typical procedure followed in preparing a spectrometer for data collection. All these steps can be automated, although probe tuning demands additional equipment or suitably equipped auto-tuneable probe heads.

Important considerations when selecting a probe are the frequency range(s) it may be tuned to, since this will dictate the nuclei one is able to observe, and how the rf coil configuration has been optimised. The simplest design is a probe containing a single coil, which is designed for the observation of only one nucleus. In fact, it would be ‘doubly-tuned’ to enable the simultaneous observation of deuterium for the field-frequency regulation via the lock system, although the presence of a deuterium channel is usually implicit when discussing probe configurations. However, many modern NMR experiments require pulses to be applied to two (or more) different nuclides, of which one is most often proton, for which two rf coils are necessary. In this case, two possible configurations are in widespread use depending on whether one wishes to observe the proton or another nuclide, referred to as the X-nucleus. The traditional two-coil design is optimised for the observation of the X-nucleus, with the X-coil as the inner of the two allowing it to sit closer to the sample, so offering the best possible sensitivity for X-observation; it is said to have the greatest ‘filling factor’. This configuration can be described by the shorthand notation $X\{^1\text{H}\}$. Nowadays, multipulse experiments tend to utilise the higher sensitivity offered by proton observation wherever possible and benefit from probes in which the proton coil sits closest to the sample with the X-coil now the outer most; $^1\text{H}\{X\}$. It is this design of probe that is widely referred to as having the *inverse* configuration because of this switch in geometry. In either case, the X-coil circuitry can be designed to operate at only a single frequency or can be tuneable over a wide frequency range, such probes being known as *broadband observe* or *broadband inverse* probes. An alternative popular configuration in organic chemistry is the *quad-nucleus* probe that allows observation of the four most commonly encountered nuclei ^1H , ^{13}C , ^{19}F , and ^{31}P . In studies of biological macromolecules, and less frequently in organic chemistry, *triple-resonance* probes are employed, allowing proton observation and pulsing or decoupling of two other nuclei; $^1\text{H}\{X, Y\}$, the most common configurations being $^1\text{H}\{^{13}\text{C}, ^{15}\text{N}\}$ or $^1\text{H}\{^{13}\text{C}, ^{31}\text{P}\}$. Alternatively, triple-resonance inverse probes that include one BB channel may offer greater flexibility in a chemical laboratory where many nuclei may be of interest with $^1\text{H}\{^{13}\text{C}, \text{BB}\}$ or $^1\text{H}\{^{31}\text{P}, \text{BB}\}$ being most common.

A further feature offered is the addition of magnetic field gradient coils to the probe head. These surround the usual rf coils and are designed to destroy the static magnetic field homogeneity throughout the sample for short periods of time in a very reproducible manner; gradient-selected techniques are introduced in Chapter 5. Whilst offered as optional features, the inclusion of a single-gradient coil in a probe can now be considered as standard.

Flow probes

A fundamentally different approach to sample handling is used in the case of flow probes in which a flow cell sits within the rf coils, and solutions are pumped into the cell for analysis, dispensing with the need for NMR tubes altogether. Traditional designs held a fixed cell assembly meaning these probes could only be used in the flow mode, whereas newer versions of both conventional and cryogenic probes (Section 3.4.2) utilise an interchangeable flow cell that allows this to be placed within probes designed to accommodate conventional tubes, so providing greater flexibility. Flow probes are typically used either as the NMR detector at the output of hyphenated systems including HPLC for mixture analysis (as LC-NMR or LC-NMR-MS configurations), or in systems where direct flow injection is employed for automated sample handling often of very large sample numbers, a notable example being the screening analysis of biofluids. NMR analyses may then be performed in an *on-flow mode* wherein the sample moves continually through the cell, so limiting observation to 1D ^1H (or ^{19}F) spectra only due to the short sample residence time. This mode is typically used for front-line screening of mixtures and has limited applicability to full structural characterisation. Alternative static modes include the *stopped-flow mode* in which the analyte slug is held in the cell for a discrete period by stopping sample elution, so enabling the collection of longer running and 2D NMR spectra for structure identification, or *loop-collection mode* where the analyte slug is diverted to a storage loop during a chromatographic run and later parked within the flow cell for NMR analyses. Some of the main issues that arise with flow-based NMR when compared with tube-based NMR include the potential for cross-contamination between samples, requiring appropriate cleaning cycles between these, sample dilution during the transfer process and the fact that samples are not isolated in convenient storage vessels (tubes) should further analysis be required. Hyphenated methods for on-line separation also tend to be time consuming and technically demanding, and procedures employing off-line chromatography followed by tube-based NMR analysis still appear to be widely employed. Developments such as solid-phase extraction (SPE) for sample concentration combined with high sensitivity microscale or cryogenic probes also provide competitive routes to the analysis of complex mixtures

[22]. Indeed, the 30 μL volume of a 1.7 mm NMR tube matches the elution volume of SPE cartridges, and the most recent 1.7 mm cryogenic probe design (see below) was no doubt developed with this in mind.

LC-NMR coupling technology has been recently reviewed [23], and the interested reader is also referred to the detailed text edited by Albert [24]. The miniaturisation of flow probes to the capillary scale has been developed to enhance signal detection sensitivity, as discussed in Section 3.4.2, and more recently the hyphenation of gas chromatography with microflow cells (GC-NMR) has even been demonstrated [25].

3.4.2. Probe design and sensitivity

There now exists an ever-increasing array of probe designs and dimensions aimed at delivering improved and optimised detection sensitivity for the analytes of interest, and this section sets out some of these key developments and the rationale underlying them. Over the years, a number of factors have been employed that have a progressively improved probe performance and sensitivity in addition to novel probe designs. One such development has been the material used in the construction of the receiver coil itself. Since this coil sits in very close proximity to the sample, this material may distort the magnetic field within this, compromising homogeneity. Modern composite metals are designed so that they do not lead to distortions of the field (they are said to have *zero magnetic susceptibility*), so allowing better lineshapes to be obtained, which ultimately leads to improved signal-to-noise figures. Increasingly, coil materials are also matched to the properties of the cooling gas that flows over the NMR tube, typically either air or pure nitrogen meaning it may be advantageous to define this when ordering probe heads. A second factor lies in the coil dimensions, with modern coils on standard probes tending to be longer than used previously so that a greater sample volume sits within them. This demands a greater volume in which the magnetic field is uniform, so these changes in probe construction have largely followed improvements in room temperature shim systems (see below). Developments in electronics have also contributed to enhanced instrument sensitivity.

Detection sensitivity

In order to appreciate the developments in probe and instrument design, we shall first consider some key factors that contribute to the detection sensitivity of an NMR measurement and hence how this may be improved. As an illustration of the progressive enhancements in instrument sensitivity, Fig. 3.46 shows the specified signal-to-noise ratios of commercial ^1H observe probes at the time of new magnet launch (as judged by the 0.1% ethyl benzene ‘sensitivity test’, Section 3.6.2). Although the data reflect in part increasing performance with higher magnetic fields, the improvement in overall instrument and probe performance that paralleled these developments is illustrated by the comparison of 500 MHz data at the time of launch (1979) with that in 2007, which shows an approximately fivefold improvement in S/N (dashed line in Fig. 3.46). The current S/N specifications for various field strengths (represented by ^1H frequency) are also shown for the most recent

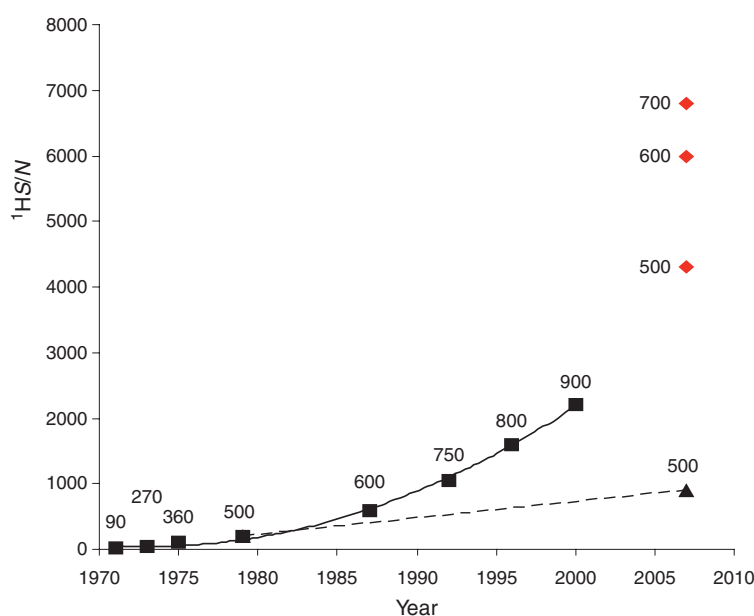


Figure 3.46. The specified ^1H signal-to-noise ratios for ^1H observe probes at the time of magnet launch (indicated by ^1H frequency) as a function of year (squares) and for the more recent cryogenically cooled probes (red diamonds). The current data for a conventional 500 MHz probe is shown by the triangle (data courtesy of Bruker Biospin in 2007).

(2007) cryogenically cooled probes (described below). These data demonstrate that a quarter of century of combined instrument development has led to a tenfold gain in field strength and ~ 400 -fold enhancement of ^1H signal-to-noise. Nevertheless, sensitivity limitations can still present a barrier when compared against other spectrometric methods, and experimental technologies for enhancing this further continue to represent an active research area; see Section 10.8.2 for example.

The signal-to-noise of an NMR measurement depends on many factors, but improving this ultimately comes down to either boosting the signal intensity or reducing the background noise. A general expression for this parameter for a conventional NMR spectrometer operating at ambient temperature may be formulated thus:

$$\frac{S}{N} \propto NAT_s^{-1} B_0^{3/2} \gamma^{5/2} T_2^* (\text{NS})^{1/2} \quad (3.9)$$

where N is the number of molecules in the observed sample volume, A is a term that represents the abundance of the nuclide, T_s is the temperature of the sample and surrounding rf coil, B_0 is the static magnetic field, γ represents the magnetogyric ratio of the nuclide, T_2^* is the effective transverse relaxation time and NS is the total number of accumulated scans. Many of these factors are dictated by the properties of the nuclide involved, including the natural abundance, the magnetogyric ratio and relaxation behaviour so are not dictated by instrument design and are considered further in Chapter 4 where pulse techniques that exploit these parameters are introduced. Suffice it to say that the high magnetogyric ratio, near 100% natural abundance and favourable relaxation properties of the proton, explains its popularity in high-resolution NMR spectroscopy.

The number of observable molecules N is, of course, dictated by the amount of material available but also by how much of this is held within the observe region of the detection coil, the so-called *active volume* (or *observe volume*) of the coil (Fig. 3.47). Any solution that sits outside this region will not induce a response in the coil and so does not contribute to the NMR signal but may be required to avoid lineshape distortions arising from magnetic susceptibility discontinuities too close to the coil. The susceptibility matched ('Shigemi') tubes introduced in Section 3.3.3 enhance sensitivity by concentrating more of the sample within the active volume and represent a cost-effective approach to improving signal-to-noise.

From an instrumental point of view, sensitivity scales as $B_0^{3/2}$ ², and an increase in field strength has traditionally been one of the drivers of enhanced sensitivity, although is becoming more difficult to justify on these grounds alone given the enormous costs associated with the very highest field magnets. Increasingly, advances in probe design are now employed to provide substantial sensitivity gains at more affordable prices, as illustrated below. In addition, improved electronics have reduced system noise, especially that associated with the NMR signal preamplifier stage and digital data handling in the form of oversampling reduces so-called quantisation noise in spectra (Section 3.2.6) adding further enhancement. These advances combined with the aforementioned refinements in probe technology have themselves lead to progressive increases in signal-to-noise figures for conventional 5 mm probes, as illustrated by the data for 500 MHz probes in Fig. 3.46.

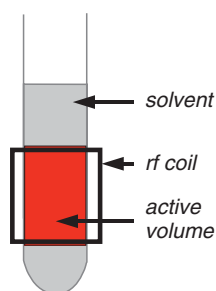


Figure 3.47. The active (observe) volume of an NMR coil is the region of the sample that sits within the coil and so contributes to the detected response.

Mass and concentration sensitivity

When discussing sensitivity in the context of real laboratory samples as opposed to instrument testing, it becomes useful to consider two definitions of this. The classic measurement of instrument sensitivity uses a fixed solution concentration (0.1% ethyl benzene in CDCl_3 , equivalent to 14 mM) under standard conditions and so measures the *concentration sensitivity* of the system.

$$S_c = \frac{\text{signal-to-noise}}{\text{solution concentration}}$$

Concentration sensitivity may be improved by using greater solution volumes within the active volume of the coil. Thus, a wider or longer detection coil will hold more sample and show an enhanced concentration sensitivity, and it follows that the use of wider sample tubes and probes is advantageous when sample solubility is a limiting factor. The use of longer coils in 5 mm probes has contributed to enhanced signal-to-noise performance specifications for certain probe designs. However, such gains may not be realised for *real*

² A more thorough theoretical analysis shows signal-to-noise should scale as $B_0^{7/4}$ [26].

Table 3.5. Comparative ^1H mass sensitivities for various probe configurations discussed in the text (data for entries 1–6 courtesy of Bruker Biospin)

Probe diameter (inverse configuration)	Sample volume (μl)	Relative mass sensitivity
5 mm	500	1
3 mm	150	1.5
1.7 mm	30	2
1 mm	5	4
5 mm Cryogenic	500	4
1.7 mm Cryogenic	30	14
Capillary micro-flow	5	10

mass-limited laboratory samples. Such samples are typically not presented as fixed solution concentrations but are more commonly available as materials of fixed mass and can thus be prepared in any chosen sample volume, to the limit of sample solubility. In such cases, it is more useful to refer to the *mass sensitivity* of the probe or system, a measure of signal-to-noise as a function of sample mass or the number of moles of material.

$$S_M = \frac{\text{signal-to-noise}}{\text{moles of material}}$$

A probe of higher mass sensitivity will provide a greater signal-to-noise ratio in a spectrum for a fixed amount of material, and this better reflects the intrinsic detection sensitivity of the probe design. When making comparisons between the performances of different probes, it is common practice to relate the results to those of a ‘conventional’ 5 mm probe operating at the same field strength, and this approach will be employed in the discussions that follow. The relative mass sensitivities of some commercially available probes are summarised in Table 3.5. Note that these are conservative figures that the manufacturer expects always to meet, and it is not uncommon to exceed these but they nevertheless provide us with an approximate guide to the sensitivity gains afforded by the differing probe configurations.

Microprobes

The mass sensitivity of an rf coil scales inversely with the diameter of the coil, d ($S/N \propto 1/d$), to first approximation, meaning that greater intrinsic sensitivity can be achieved by narrowing the coil. This is the basis for the development of the so-called *microprobes*. This terminology is used somewhat loosely and there exists no formal definition of a microscale probe, but it is generally taken to mean a probe geometry that is small when compared to a ‘standard’ probe. Nowadays, the standard remains a probe designed to accept a 5 mm diameter maximum NMR tube, colloquially referred to as a *5 mm probe*, so commercial microprobes would now include the 3.0, 1.7 and 1.0 mm probes.

The mass sensitivity benefits of a microprobe relative to a larger diameter probe can be exploited for a fixed mass of sample through the enhanced signal-to-noise achievable or through reduced data collection times for data sets of equivalent signal-to-noise ratio, or perhaps as a balance between these. This of course demands that the solute can be dissolved in the reduced sample volumes employed; see below. As a consequence, such probes enable data to be collected on reduced sample masses and so extend the operating range of NMR experiments for mass-limited samples. For example, a microprobe that has a fivefold gain in mass sensitivity relative to a larger probe will provide a fivefold improvement in signal-to-noise ratio, which may be traded for a 25-fold reduction in data collection time for a fixed sample mass. Alternatively, this would allow sample masses to be reduced by a factor of 5 for data of comparable quality to that from the larger probe collected in a similar timeframe. An additional benefit from the reduced solvent volumes of the microprobes is the reduction in background solvent resonances and potentially also those of solvent impurities that can become apparent when dealing with very small sample masses. The smaller sample volumes also mean solvent suppression becomes much easier. The microprobes also provide reduced pulse widths leading to more uniform excitation over wider bandwidths. Indeed, it is generally the case that the length of a 90° pulse width for a given pulse power provides a direct indication of the detection sensitivity of the coil with smaller pulse widths correlating with higher sensitivity, and this itself can be useful way of gauging a probe’s performance. They may also demand lower pulse powers to prevent damage to the more delicate rf coils, so even greater caution is required with such probes.

The availability of commercial tube-based microprobes came about in 1992 with the introduction of the 3 mm inverse probe, although much earlier work had demonstrated the potential for microscale probes [27]. The 3 mm probe with a 150 μL sample volume demonstrated an approximately twofold gain in signal-to-noise in ^1H -detected heteronuclear correlation spectra relative to a 5 mm probe with the same sample mass in 600 μL [28]. Subsequent to this in 1999 [29], a 1.7 mm probe [30] utilising a 30 μL sample demonstrated a further approximately twofold sensitivity gain in 2D correlation experiments relative to the 3 mm probe, and most recently in 2002 [31], a 1 mm probe using only a 5 μL sample volume demonstrated a fivefold mass sensitivity gain in ^1H observation relative to a 5 mm probe. Owing to limitations in sample handling and the delicate nature of 1 mm tubes, it seems likely that this will represent the smallest size for tube-based NMR probes, although smaller dimensions are feasible for microflow probes as described below.

Figure 3.48 compares the performance capabilities for ^1H observation of 5 and 1 mm triple-resonance inverse probes. Data were collected under identical conditions (single scan, 90° pulse excitation) on the same 500 MHz spectrometer for a 50 μg sample of sucrose in D_2O . The relative signal-to-noise of the anomeric resonance (not shown) is 7.8:1 and 39.5:1 for the 5 and 1 mm probes respectively, indicating a fivefold sensitivity gain for the smaller diameter probe. The reduced solvent volume also attenuates the solvent background, and this can also be a significant factor in the selection of smaller diameter tubes for mass-limited samples. Despite the miniscule samples and hence the small volume over which good field homogeneity is required, experience suggests the 1 mm probe resolution tends to be a little poorer than that of the larger geometry probes, and the vendor probe specifications also support this observation. This most probably is due to the proportionately larger volume of glass present from the NMR tube relative to the solution within the coil and with the associated susceptibility discontinuities; susceptibility matched 1 mm NMR tubes may yield still greater performance but are not currently available.

One further approach to miniaturisation in the Varian NanoProbeTM has been the use of small rotors of 40 μL total volume inside the specially constructed probe head that spins the sample at the ‘magic angle’ (54.7° from the axis of the B_0 field) at several kilohertz to eliminate lineshape distortions that would arise from sample discontinuities close to or within the rf coil (magic angle spinning is described in Section 10.7). This allows the whole sample volume to be retained within the coil’s active volume, unlike tube-based approaches where up to 50% of the sample may sit outside of this volume, and so improves sensitivity. The use of MAS also makes the probe suitable for the analysis of heterogeneous samples such as those attached to resins from combinatorial synthesis [32], but it lacks the convenience of tube-based sample handling and appears to be less widely adopted for work with mass-limited samples.

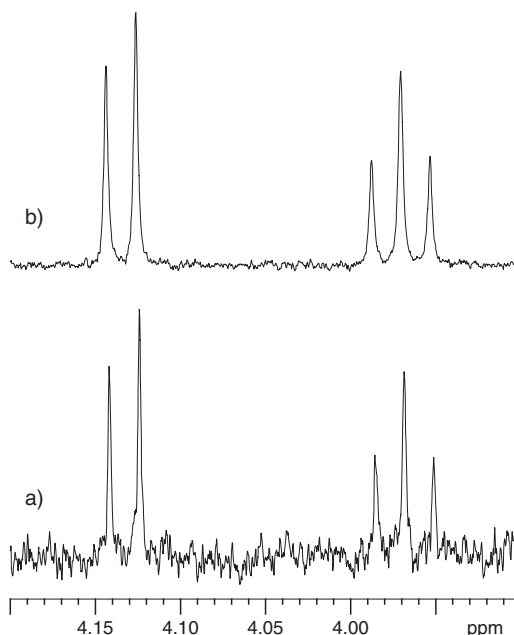


Figure 3.48. Partial ^1H spectra of 50 μg sucrose in D_2O recorded with probes of different dimensions, (a) 500 μL in 5 mm probe and (b) 5 μL in 1 mm probe. Spectrum (b) displays a fivefold gain in signal-to-noise relative to spectrum (a).

Microflow probes

An alternative approach to reducing sample volume requirements in probes is avoiding the use of sample tubes altogether and to use flow technology to place the sample within a fixed coil geometry. In the context of probe miniaturisation, this is achieved using capillary flow cells ('capillary NMR') in which the rf coil is wound directly around the capillary tube itself. Such probes have been developed, which have active volumes as little as 5 nL [33] although commercially available probes currently utilise active volumes of 2.5 μL that require working sample volumes of 5 μL and have a total *probe* volume of a mere 15 μL [34]. These probes employ rf coils that are surrounded by an inert perfluorinated fluid whose magnetic susceptibility matches that of the coil assembly, as needed to produce acceptable lineshapes. As there is no need to align the coil with the field axis for sample insertion, as is the case for tube-based designs, this allows the use of solenoid coils mounted perpendicular to the field, which give an intrinsic sensitivity advantage of approximately twofold over the traditional, vertically aligned saddle coil designs [35]. In accord with this, the data in Table 3.5 indicate the capillary probe to demonstrate a ~ 10 -fold mass sensitivity gain over a conventional 5 mm probe (or approximately 5-fold if Shigemi tubes are employed [36]). Furthermore, it is possible to tune the single coil to multiple frequencies simultaneously, making it possible to use the coil for both ^1H and ^{13}C observation without any loss of filling factor.

The flow cell design naturally requires a different approach to sample handling [37] with sample insertion performed via manual syringe injection, robotic injection or via direct hyphenation of the probe with capillary liquid chromatography (CapLC). The narrow capillaries ($<100\text{ }\mu\text{m}$ diameter) may be prone to blockage, and effective sample filtration and the routine use of in-line filters ($2\text{ }\mu\text{m}$) are essential. Arguably, the flow approach lacks the convenience of NMR tubes, which are effective storage vessels should one wish to undertake further analysis of a sample without further preparative steps. The practicalities of employing the flow probe in conjunction with CapLC has also been considered and contrasted with off-line sample separation coupled with tube-based analysis with a cryogenic probe [22].

Cryogenic probes [38, 39]

A fundamentally different approach to improving detection sensitivity is to reduce the background noise in spectra, and the most recent probe designs aim to do this by cooling both the rf detection coils and the preamplifier for optimum results. This is achieved through the use of a cold helium gas closed-cycle system, which cools the rf coils to typically 25 K and the preamplifier (which is housed within the body of the probe rather than being separate from this as with conventional probes) to around 70 K. This concept was proposed [26] and demonstrated experimentally [40, 41] some years before the first commercial systems became available (in 1999), a testament to the demanding technical challenges in the development of these systems, not least of which is the need to maintain the detection coil at around 25 K whilst the adjacent sample, a matter of some millimetres away, remains at ambient temperature. The result of cooling the rf coils leads to an approximately twofold gain in signal-to-noise whilst cooling of the preamplifier leads to a similar gain, resulting typically in a fourfold signal-to-noise gain relative to a conventional probe of similar dimensions³. The cooling of the rf coils is achieved through conduction from a cryo-cooled block on which the coils are mounted rather than by direct cooling of these by the flowing He gas so as to avoid disturbance of the coils.

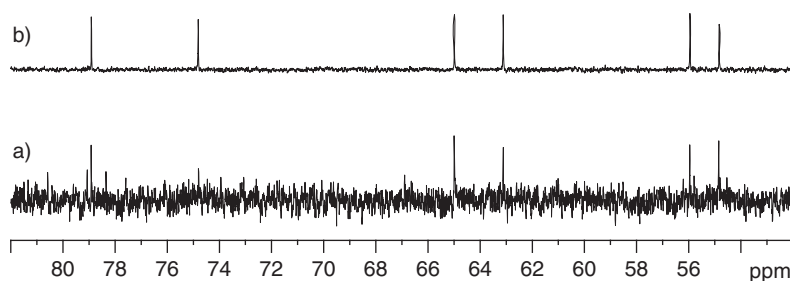
The gains arising from cryogenic cooling of probes may be described more formally by considering the noise contributions arising within the hardware. This may be summarised

$$S/N \propto \frac{1}{\sqrt{T_c R_c + T_a(R_c + R_s) + T_s R_s}} \quad (3.10)$$

where T_c and R_c represent the temperature and resistance of the coil, T_s is the sample temperature, R_s is the resistance generated in the coil by the sample itself (the 'sample resistance') and T_a is the effective noise temperature of the preamplifier. By cooling both the coils and the preamplifier, the first two terms of the denominator in Eq. (3.10) are reduced and so contribute to the higher sensitivity of cryogenically cooled probes. The third term, representing noise generated by the sample, becomes important for ionic solutions [42], as discussed below, and is of greater significance for cryogenic probes than conventional probes because of the smaller contributions of the first two terms. The comparison of

³The dewar assembly required for the cryoprobes actually results in a reduced filling factor that places limitations on the sensitivity improvement achievable through probe cooling; for example, a 5 mm cryogenic probe typically has the coil dimensions of a traditional 8 mm probe.

Figure 3.49. A comparison of the carbon-13 sensitivity performance of (a) a conventional room temperature probe and (b) a cryogenic probe operating at 500 MHz (^1H). Data were collected on the same sample under identical experimental conditions using a broadband (BB) observe probe tuned to carbon-13 and a cryogenic carbon-13 observe probe respectively.



cryogenic and conventional probes in Table 3.5 demonstrates sensitivity gains of (at least) 4, equating to a minimum 16-fold time-saving for a given sample mass or the ability to collect data on fourfold less material in a given data acquisition period. This advantage is demonstrated experimentally in Fig. 3.49 for a ^{13}C -optimised cryogenic probe for which there is observed a sixfold gain in signal-to-noise ratio relative to a room temperature probe operating under otherwise identical experimental conditions. Whilst the presence of some resonances is dubious in Fig. 3.49 trace (a), all are clearly revealed in trace (b). As the performance gain is not dependent on increased sample concentration, as is the case with microprobes, there is a simultaneous gain in both concentration and mass sensitivity with this technology. Further enhancements of mass sensitivity may thus be achieved by combining miniaturisation with cryogenic technology in the form of microcryogenic probes, as is illustrated by the figures for the 1.7 mm cryogenic probe.

It is also possible to manipulate the influence of sample noise to enhance sensitivity. Consider again the sample resistance, R_s , which may be represented in the form

$$R_s \propto \omega_0^2 l r^4 \sigma \quad (3.11)$$

where ω_0 is the observation frequency, l and r are the sample length and radius respectively and σ represents a term for the dielectric properties of the solution. This suggests that the noise contribution from the solution may be reduced by employing tubes of smaller diameter, the most common approach being a 3 mm tube used in a 5 mm probe. Whilst this approach is valid in principle for both conventional and cryogenic probes, the advantages are more pronounced for the cooled probes because the noise contribution from the sample relative to that of the instrument is more significant, as described above. Despite the associated reduction in filling factor, the measure of how much of the rf coil volume is filled with sample, a S/N gain of ~ 1.5 -fold has been demonstrated using this approach for a 3 mm vs a 5 mm tube containing identical sample masses when acquired in a 5 mm inverse cryogenic probe, [39] equating to a potential time-saving factor of ~ 2.2 . Personal experience suggests that comparable results to the 3 mm tubes are obtained for identical sample masses in both 2 and 1 mm diameter tubes, suggesting further possible gains in noise reduction are offset by the losses in filling factor. Greater gains may be anticipated, however, by the use of 3 mm matched ‘Shigemi’ tubes. For instances where sample concentration cannot be increased, this method is clearly inappropriate. It has been demonstrated that the contribution from sample noise may also be diminished through the use of NMR tubes with rectangular instead of circular cross-sections with the longer transverse axis of the tube aligned along the rf magnetic field [43]. Such tubes are now commercially available with typical dimensions of 6×3 mm and demonstrate a $\sim 20\%$ gain in S/N vs a 5 mm tube. Such gains are most pronounced for samples of high ionic strength, which compromise the performance of cryogenic probes because of the associated increase in the R_s term in Eq. (3.10). The most common examples of this are the buffered aqueous solutions widely employed in biomolecular studies. Whilst higher salt concentrations generally lead to decreased performance, the nature of the buffer itself can also have a significant effect, and the behaviour of many common buffers has been assessed in detail in this context [42]. Such adverse effects are generally less pronounced for organic solvents, although losses may still arise for the more polar solvents, especially methanol and dimethylsulfoxide. Finally, note also that Eq. (3.11) predicts that the use of cryogenic probe technology becomes less efficient at higher field strengths and for higher γ nuclides due to the ω_0 dependence of R_s . Thus, despite their high profile for biomolecular studies, the mid-range frequencies (400–600 MHz) routinely employed for organic chemistry combined with the prevalence of organic solvents and the importance of ^{13}C NMR might suggest this to be the home territory of cryogenic probe technology.

Despite the obvious benefits of these high-sensitivity probes, there are also, inevitably, some drawbacks in their use. These include the high initial purchase costs for the complete



Figure 3.50. A cryogenic probe installation. The cryogenic bay (A) contains the cold expansion head that generates cold helium gas that passes along the insulated transfer line (B) to the probe. The vacuum line (C) maintains the cryogenic probe vacuum and the base of the probe (D) houses the cold preamplifier stages. At the rear can be seen the high pressure lines (E) that carry helium gas to/from the remote compressor.

system, which can be over five times those of a conventional probe, the running and maintenance costs and the need to accommodate the additional instrumentation to operate the cryogenics. A complete system comprises the cold probe, the cryogenic platform or bay that directly supplies the probe with the cooled helium gas and acts as the master controller for the complete assembly, a remote helium gas compressor unit and some means of dissipating the considerable quantities of heat generated by the closed-cycle cooling system (Fig. 3.50). The probe contains the indirectly cooled rf coils and also houses the cooled preamplifiers that are fed with the same cold gas flow. The more recent probe designs cool the preamplifiers associated with both the inner and the outer coils providing enhanced detection sensitivity for multiple nuclei. The internal dewared probe assembly demands continuous pumping of the probe vacuum from the platform and/or the use of internal ion pumps to maintain the cryogenic efficiency. The cryogenic bay generates the cold helium gas through a compression/expansion cycle, producing the characteristic ‘chirping’ associated with the cryogenic probes. The heat generated by this refrigeration process is removed either through the use of a chilled water supply or by external air-cooled assemblies as used in air-conditioning systems. Selecting the most appropriate method can be a critical aspect of site planning these installations. The water-cooled systems may provide the convenience of local access, but the instrument is then beholden to the demands on the building-wide installation, whilst the external compressor systems require suitable routing either out of the building or into internal space with sufficient cooling capacity. The use of these probes also imposes different operational protocols, chiefly because of the time required to cool and warm the probes that are typically ~ 4 and ~ 2 h respectively. This, combined with the more complex nature of the probe and its installation, has led to most cryogenic probe assemblies being considered as permanent fixtures within an instrument and probe removal being reserved for maintenance procedures only.

In summary, it is apparent that there now exist a range of approaches to improving the sensitivity of NMR measurements using modern probe and tube technologies as an alternative to seeking higher field strengths. These often represent more economical and more practical routes to extending the range of samples that can be studied through probe miniaturisation and cryogenic cooling, through the appropriate use of microtubes and susceptibility-matched tubes, or indeed by a combination of these methods. Clearly, the adoption of appropriate probe technologies offers the chemist exciting new opportunities for the investigation of mass-limited samples.

3.4.3. Tuning the probe

The NMR probe is a rather specialised (and expensive) piece of instrumentation whose primary purpose is to hold the transmit and receive coils as close as possible to the sample to enable the detection of the weak NMR signals. For the coils to be able to transmit the rf pulses to the sample and to pick up the NMR signals efficiently, the electrical properties of the coil circuit should be optimised for each sample. The adjustments are made via variable capacitors that sit in the probe head a short distance from the coil(s) and comprise the tuning circuitry. There are two aspects to this optimisation procedure known as *tuning* and *matching*, although the whole process is more usually referred to as ‘tuning the probe’. The first of these, as the name implies, tunes the coil to the rf of the relevant nucleus and is analogous to the tuning of a radio receiver to the desired radio station. A poorly tuned probe will lead to a severe degradation in sensitivity, just as a radio broadcast becomes swamped with hissing noise. The second aspect aims to equalise (or match) the impedance (the total effective resistance to alternating current) of the coil/sample combination with that of the transmitter and receiver so that the maximum possible rf energy can pass from the

transmitter into the sample and subsequently from the sample into the receiver. As electrical properties differ between samples, the optimum tune and match conditions will also vary and so require checking for each new sample. These differences can be largely attributed to differences between solvents with the most significant changes occurring between low polarity organic solvents and ionic aqueous solutions.

Probe tuning is necessary for a number of reasons. Other than the fundamental requirement for maximising sensitivity, it ensures pulse widths can be kept short, which in turn reduces off-resonance effects and minimises the power required for broadband decoupling. A properly tuned probe is also required if previously calibrated pulse widths are to be reproducible, an essential feature for the successful execution of multipulse experiments.

Tuning and matching

The process of probe tuning involves applying rf to the probe, monitoring the response from it by some suitable means and making adjustments to the capacitors in the head of the probe (via long rods that pass through its base) to achieve the desired response. In the case of broadband probes, which may be tuneable over very wide frequency ranges, the capacitors may even need to be physically exchanged, either by removing them from the probe altogether or by means of a switching mechanism held within the probe. Various procedures exist for monitoring the response of the probe, but the most useful and the one supplied widely on modern instruments uses a frequency sweep back and forth over a narrow region (of typically a few megahertz) about the target frequency during which the probe response is compared to that of an internal $50\ \Omega$ reference load (all NMR spectrometers are built to this standard impedance). The display then provides a simultaneous measure of the tune and match errors (Fig. 3.51a), allowing one to make interactive adjustments to the tuning capacitors to achieve the desired result (Fig. 3.51b); the response reaches a minimum when the probe is matched to the $50\ \Omega$ load. With this form of display, it is possible to see the direction in which adjustments should be made to arrive at the correct tune and match condition. It is important to note that the tune and match controls are not mutually exclusive, and adjustments made to one are likely to alter the other, so a cyclic process of tune, match, tune and so on will be required to reach the optimum.

This process is clearly amenable to automation under computer control, and auto-tune configurations are now widely available through two distinct approaches. The first uses remote motorised units that connect to the probe tuning rods via flexible cables that drive this process and has the advantage of being compatible with standard probes. The second, more sophisticated, approach employs motors built into the base of the probe itself that perform the optimisation but has the limitation of being incompatible with older probe designs. The fact that the drive units operate within an intense magnetic field also poses rather severe technical challenges that have been overcome by the use of rare earth metals

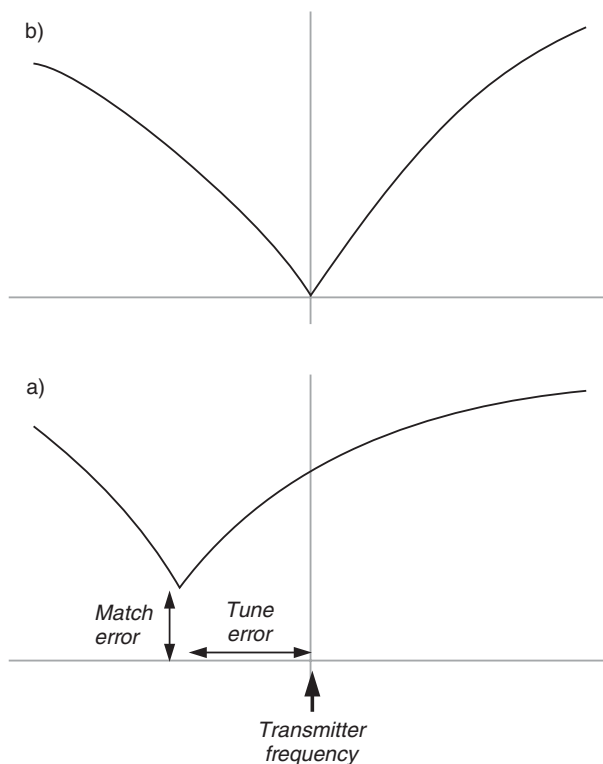


Figure 3.51. Tuning and matching a NMR probe head. The dark line represents the probe response seen for (a) a mis-tuned and (b) a correctly tuned probe head.

in the motorised units. Which ever approach is adopted the benefits of auto-tune probes mean that optimum tuning performance can be obtained under fully automated operation, a wide range of nuclei likewise become accessible without operator intervention (such as with broadband auto-tuneable probes), and for manually operated instruments, this provides the ease and convenience of not having to reach under magnets to tune, a sometimes awkward and potentially hazardous process.

The method for probe tuning on older spectrometers that are unable to produce the frequency sweep display is to place a *directional coupler* between the transmitter/receiver and the probe and to apply rf as a series of very rapid pulses. The directional coupler provides some form of display, usually a simple meter, which represents the total power being reflected back from the probe. The aim is to *minimise* this response by the tuning and matching process so that the maximum power is able to enter the sample. Unfortunately with this process, unlike the method described above, there is no display showing errors in tune and match separately, and there is no indication of the direction in which changes need be made, one simply has an indication of the overall response of the system. This method is clearly the inferior of the two, but may be the only option available.

When more than one nuclear frequency is of interest, the correct approach is to make adjustments at the *lowest* frequency first and work up to the highest. Tuning will also be influenced by sample and probe temperature and must be checked whenever changes are made. If large temperature changes are required, it is wise to quickly recheck the tuning every 10 or 20° so that one never becomes too far from the optimum; this is especially important if using the directional coupler method. Where the spectrometer is used in an open access environment, where interaction with the spectrometer is kept to a minimum or where the instrument runs automatically, probe tuning for each sample may not be not viable (unless an auto-tuneable probe is installed), in which case it is appropriate to tune the probe for the most frequently used solvent, and accept some degradation in performance for the others. Different solvents may require different pulse width calibrations under these conditions.

Finally in this section, we consider one particular situation in which it is beneficial to deliberately detune a probe. When performing studies in *protonated* water, the linewidth of the solvent resonance is broadened significantly in a well-tuned probe, because of the phenomenon of *radiation damping* [44]. This is where the FID of the solvent decays at an accelerated rate because the relatively high current generated in the coil by the intense NMR signal itself produces a secondary rf field that drives the water magnetisation back to the $+z$ axis at a faster rate than would be expected from natural relaxation processes alone. The rapid decay of the FID in turn results in a broadened water resonance. This only occurs for very intense resonances and has greatest effect when the sample couples efficiently with the coil, that is, when the probe is well tuned. Detuning the probe a little provides a sharper (and weaker) water resonance whose lineshape gives a better indication of the field homogeneity. Retuning the probe is essential for subsequent NMR observations employing solvent suppression schemes.

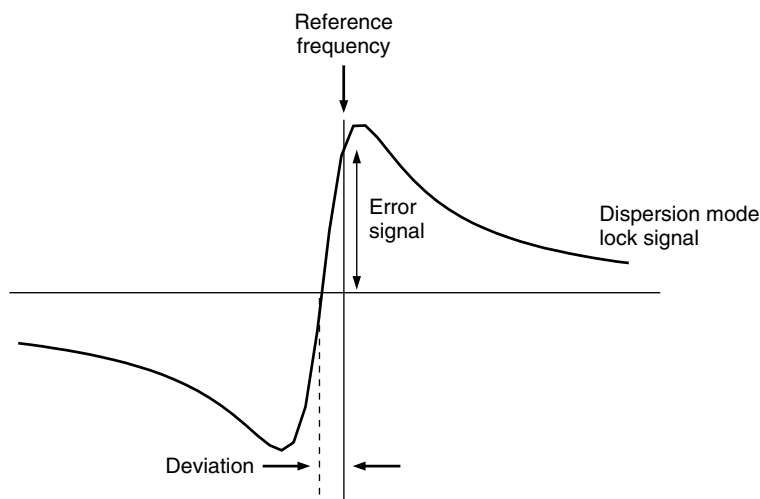
3.4.4. The field-frequency lock

Despite the impressive field stability provided by superconducting magnets, they still have a tendency to drift significantly over a period of hours, causing NMR resonances to drift in frequency leading to a loss of resolution. To overcome this problem, some measure of this drift is required so that corrections may be applied. On all modern spectrometers, the measurement is provided by monitoring the frequency of the deuterium resonance of the solvent. The deuterium signal is collected by a dedicated ^2H observe spectrometer within the instrument that operates in parallel with the principle channels, referred to as the *lock channel* or simply the *lock*.

The lock system

The lock channel regulates the field by monitoring the *dispersion* mode deuterium resonance rather than the absorption mode signal that is usually considered in NMR and aims to maintain the centre of this resonance at a constant frequency (Fig. 3.52). A drift in the magnetic field alters the resonance frequency and therefore produces an error signal that has both magnitude and sign (unlike the absorption mode resonance that has only magnitude). This then controls a feedback system that adjusts the field setting. The dispersion signal also has the advantage of having a rather steep profile, providing the greatest sensitivity to change. Monitoring the deuterium resonance also provides a measure of the magnetic field homogeneity within the sample since only a homogeneous field produces a sharp, intense resonance. In this manner, the lock signal may be used as a guide when optimising (shimming) the magnetic field (Section 3.4.5) where the absorption mode signal

Figure 3.52. The spectrometer lock system monitors the dispersion mode signal of the solvent deuterium resonance. A shift of the resonance frequency due to drift in the static field generates an error signal that indicates the magnitude and direction of the drift, enabling a feedback system to compensate this.



is presented to the operator as the lock display. Since the best field-frequency regulation is provided by the strongest and sharpest lock resonance, the more highly deuterated non-viscous solvents provide the best regulation. Water is especially poor since it tends to have a rather broad resonance because of exchange processes that are very dependent on solvent temperature and pH.

Optimising the lock

The first procedure for locking is to establish the resonance condition for the deuterium signal, which involves altering either the field or the frequency of the lock transmitter. Of these two options, the latter is preferred since it avoids the need for changing transmitter frequencies and is now standard on modern instruments. Beyond this, there are three fundamental probe-dependent parameters that need to be considered for optimal lock performance. The first of these is the lock transmitter *power* used to excite the deuterium resonance. This needs to be set to the highest usable level to maximise the ^2H signal-to-noise ratio but must not be set so high that it leads to lock *saturation*. This is the condition in which more energy is applied to the deuterium spins than can be dissipated through spin relaxation processes and is evidenced by the lock level drifting up and down erratically. The saturation level can be determined by making small changes to the lock power and observing the effect on the lock signal. If the power is increased, the lock signal should also increase, but if saturated it will then drift back down. Conversely, if the power is decreased, the lock level will drop but will then tend to creep up a little. The correct approach is to determine the point of saturation and then to be sure you are operating below this before proceeding. The second feature is the lock *gain*. This is the amplification that is applied to the detected lock signal and is generally less critical than the power although it should not be so high as to introduce excessive lock noise. The final feature is the lock *phase*. We have already seen that the field-frequency regulation relies on monitoring the pure dispersion mode lineshape, and this is present only when the resonance has been phased correctly. This is the case when the *observed* lock signal produces the maximum intensity as the lock phase is adjusted since only then does this have the required pure absorption lineshape.

Acquiring data unlocked

In cases where deuterium is not available for locking, for example, if one wishes to observe or decouple deuterium itself, then it may be necessary to acquire data 'unlocked' (although recent hardware developments allow the decoupling of deuterium during acquisition by appropriate blanking of the lock channel). Running unlocked is perfectly feasible over limited time periods, which depend on the drift rate of the magnet. For experiments that must run for very many hours for reasons of sensitivity, then a useful approach is to acquire a series of FIDs each being collected over a shorter time period, say 1 h. Drift within each data set will then be essentially negligible whereas any drift occurring over the whole duration of the experiment can be corrected by adding *frequency-domain spectra* for which any frequency drift has been compensated manually by internal spectrum referencing. The addition then provides the required enhancement of signal-to-noise without the deleterious effects of field drift.

3.4.5. Optimising the field homogeneity: shimming

NMR experiments require a uniform magnetic field over the whole of the NMR sample volume that sits within the detection coil. Deviation from this ideal introduces various lineshape distortions, compromising both sensitivity and resolution. Thus, each time a sample is introduced into the magnet it is necessary to ‘fine-tune’ the magnetic field and a few minutes spent achieving good resolution and lineshape is time well spent. For anyone actually using an NMR spectrometer, competence in the basic level of field optimisation is essential, but even if you only need to *interpret* NMR spectra, perhaps because someone else has acquired the data or if the whole process is performed through automation, then some understanding of the most common defects arising from remaining field inhomogeneities can be invaluable.

The shim system

Maintaining a stable magnetic field that is uniform to 1 part in 10^9 over the active volume within modern NMR probes (typically $0.1\text{--}1.5\text{ cm}^3$) is extremely demanding. This amazing feat is achieved through three levels of field optimisation. The first lies in the careful construction of the superconducting solenoid magnet itself, although the field homogeneity produced by these is rather crude when judged by NMR criteria. This basic field is then modified at two levels by sets of ‘shim’ coils. These coils carry electrical currents that generate small magnetic fields of their own, which are employed to cancel remaining field gradients within the sample. In fact, shims are small wedges of metal used in engineering to make parts fit together and were originally used in the construction of iron magnets to modify the position of the poles to adjust the field. Still in the present day where superconducting magnets dominate, this name permeates NMR, as does the term ‘shimming’, referring to the process of field homogeneity optimisation.⁴ The superconducting shim coils sit within the magnet cryostat and remove gross impurities in the magnet’s field. The currents are set when the magnet is first installed and do not usually require altering beyond this. The room temperature shims are set in a former that houses the NMR probe itself, the whole assembly being placed within the bore of the magnet such that the probe coil sits at the exact centre of the static field. These shims (of which there are typically around 20 to 40 on a modern instrument) remove any remaining field gradients by adjusting the currents through them, although in practice only a small fraction of the total number need be altered on a regular basis (see below).

The static field in vertical bore superconducting magnets also sits vertically and this defines, by convention, the z axis. Shims that affect the field along this axis are referred to as axial or Z shims, whereas those that act in the horizontal plane are known as radial or X/Y shims (Table 3.6). When acquiring high-resolution spectra, it is traditional practice to spin the sample (at about $10\text{--}20\text{ Hz}$) about the vertical axis. This has the effect of averaging field inhomogeneities in the $X\text{--}Y$ plane, so improving resolution. The averaging means that adjustments to shims containing an X or Y term must be made when the sample is static; hence, these shims are also commonly referred to as the ‘non-spinning shims’. Modern shim sets are capable of delivering non-spinning lineshapes that almost match those when spinning, and it is becoming increasingly common not to spin samples. For multidimensional studies, this is certainly the case, since sample spinning can introduce modulation effects to the acquired data, leading to unwanted artefacts particularly in the form of so-called t_1 noise (see Chapter 5).

Shimming

In order to achieve optimum field homogeneity, high quality samples are essential. The depth of a sample also has a considerable bearing on the amount of Z shimming required, which can be kept to a minimum by using solutions of similar depth each time. Most spectrometers possess software that is capable of carrying out the shimming process automatically, and clearly this is essential if an automatic sample changer is used. However, such systems are not infallible and can produce spectacularly bad results in some instances. Here, reproducible sample depths are vitally important for the auto-shimming procedures to be successful and to reach an optimum rapidly. It is also crucial for the whole of the sample to be at thermal equilibrium so that convection currents do not exist. For aqueous solutions away from ambient, this may demand $10\text{--}20\text{ min}$ equilibration in the probe.

To provide an indication of progress when shimming, one requires a suitable indicator of field homogeneity. Essentially, there are three schemes that are in widespread use, all of which have their various advantages and disadvantages; (1) the lock level, (2) the shape of the FID and (3) the shape of the NMR resonance. The ultimate measure of homogeneity is the NMR resonance itself, since defects apparent in the spectrum can often be related

⁴ Note that some (older) texts may refer to this process as “tuning”, which is now exclusively reserved for processes involving rf; for example, one may *shim* a magnet but will *tune* a probe.

Table 3.6. Shim gradients found on high-resolution spectrometers. Lower field instruments (<500 MHz) may utilise only 20 or so gradients (such as those in the left panel), whilst higher field spectrometers may employ in excess of 30. Shims up to third order are those most likely to need periodic optimisation as part of long-term spectrometer maintenance for which the most significant interacting shims are listed. Those shown in square brackets interact less strongly with the listed gradient whilst those that interact with Z^0 (the main field) may cause momentary disruption of the lock signal when adjusted

Shim gradient	Gradient order	Principle interacting shim gradients	Shim gradient	Gradient order
Z^0 (the main field)	0	–	XYZ^2	4
Z^1 (Z)	1	–	$(X^2 - Y^2)Z^2$	4
Z^2	2	Z	X^3Z	4
Z^3	3	Z, $[Z^2]$	Y^3Z	4
Z^4	4	$Z^2, Z^0, [Z, Z^3]$	XZ^4	5
Z^5	5	$Z^3, Z, [Z^2, Z^4]$	YZ^4	5
Z^6	6	$Z^4, Z^2, Z^0, [Z, Z^3, Z^5]$	XYZ^3	5
X	1	Y, $[Z]$	$(X^2 - Y^2)Z^3$	5
Y	1	X, $[Z]$	XZ^5	6
XZ	2	X, $[Z]$	YZ^5	6
YZ	2	Y, $[Z]$	XYZ^4	6
XY	2	X, Y	$(X^2 - Y^2)Z^4$	6
$X^2 - Y^2$	2	XY, $[X, Y]$	XYZ^5	7
XZ^2	3	XZ, $[X, Z]$	$(X^2 - Y^2)Z^5$	7
YZ^2	3	YZ, $[Y, Z]$		
XYZ	3	XY, $[X, Y, Z]$		
$(X^2 - Y^2)Z$	3	$X^2 - Y^2, [X, Y, Z]$		
X^3	3	X		
Y^3	3	Y		
XZ^3	4			
YZ^3	4			

directly to deficiencies in specific shim currents, as described below. Most often field homogeneity is monitored by the height of the deuterium lock resonance that one aims to maximise. Whilst conceptually this is a simple task, in reality it is complicated by the fact that most shims interact with others. In other words, having made changes to one it will then be necessary to re-optimize those with which it interacts. Fortunately, shims do not influence all others, but can be sub-divided into smaller groups that are dealt with sequentially during the shimming process. A detailed account of the shimming procedure has been described [45] and the fundamental physics behind field-gradient shims has also been presented [46], but here we shall be concerned more with addressing the lineshape defects that are commonly encountered in the daily operation of an NMR spectrometer.

When shimming, it is not always sufficient to take the simplest possible approach and maximise the lock level by adjusting each shim in turn, as this may lead to a ‘false maximum’, in which the lock level appears optimum yet lineshape distortions remain. Instead, shims must be adjusted interactively. As an example of the procedure that should be adopted, the process for adjusting the Z and Z^2 shims (as is most often required) should be:

1. Adjust the Z shim to maximise the lock level, and note the new level.
2. Alter Z^2 so that there is a noticeable change in the lock level, which may be up or down, and remember the direction in which Z^2 has been altered.
3. Readjust Z for maximum lock level.
4. Check whether the lock level is greater than the starting level. If it is, repeat the whole procedure, adjusting Z^2 in the same sense, until no further gain can be made. If the resulting level is lower, the procedure should be repeated but Z^2 altered in the opposite sense.

If the magnetic field happens to be close to the optimum for the sample when it is initially placed in the magnet, then simply maximising the lock level with each shim directly will achieve the optimum since you will be close to this already. Here again, a reproducible sample depth makes life very much easier.

Shimming is performed by concentrating on one interacting group at a time, always starting and finishing with the lowest order shim of the group. Principle interactions for selected shims are summarised in Table 3.6. Whenever it is necessary to make changes to a high order shim, it will be necessary to readjust all the low order shims within the same

interacting group, using a similar cyclic approach to that described for the adjustment of Z and Z^2 above. Generally, the order of optimisation to be followed will be:

1. Optimise Z and Z^2 interactively, as described above. If this is the first pass through Z and Z^2 , then adjust the lock phase for maximum lock level.
2. Optimise Z^3 : make a known change, then repeat step 1. If the result is better than previously, repeat this procedure, if not alter Z^3 in the opposite sense and repeat step 1.
3. Optimise Z^4 interactively with Z^3 , Z^2 and Z .
4. Stop the sample spinning (if applicable) and adjust Z to give the maximum response (this is likely to have changed a little as the position of the sample relative to the field will change). Adjust X and Y in turn to give the maximum response.
5. Optimise X and XZ interactively. Adjust Z to give the maximum response.
6. Optimise Y and YZ interactively. Adjust Z to give the maximum response.
7. Optimise XY interactively with X and Y .
8. Optimise X^2-Y^2 interactively with XY , X and Y .
9. Repeat step 1.

The higher the shim order, the greater the changes will be required, and when far from the optimum shim settings, large changes to the shim currents may have only a small effect on the lock level and the shim response will feel rather 'sluggish'. When close to the optimum, the response becomes very sensitive and small changes can have a dramatic effect. The above procedure should be sufficient for most circumstances and any field strength, unless the basic shims set has become grossly misset. If lineshape distortions remain, then it may be possible to identify the offending shim(s) from the nature of the distortion (see below), allowing the appropriate corrections to be applied.

Common lineshape defects

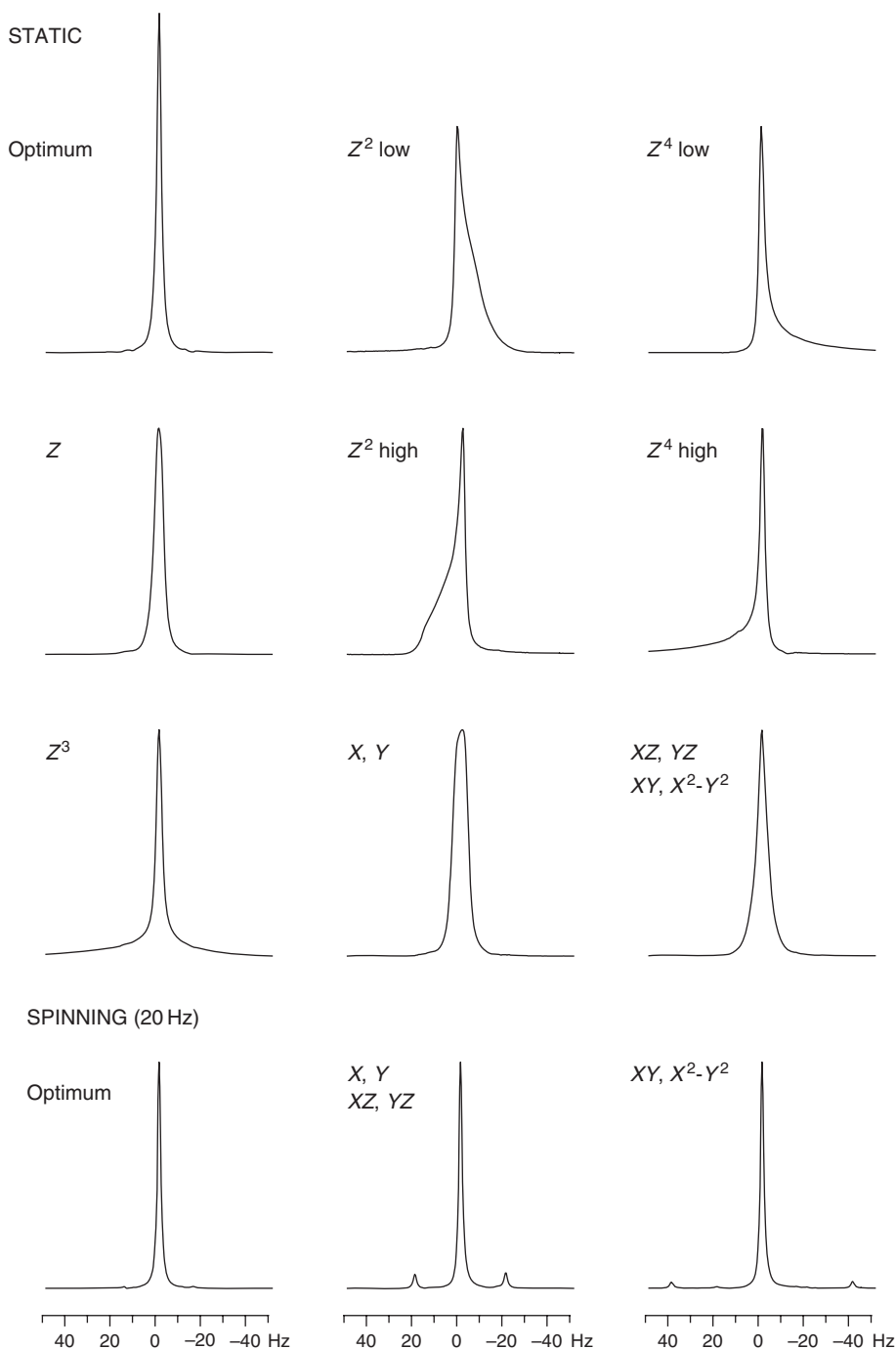
The NMR resonance lineshape gives the ultimate test of field homogeneity, and it is a useful skill to be able to recognise the common distortions that are caused by errors in shim settings (Fig. 3.53). Thus, the Z shims all influence the width of the NMR resonance, but in subtly different ways; impurities in the even order shims (Z^2 , Z^4 and Z^6) will produce unsymmetrical distortions to the lines whereas those in the odd orders (Z , Z^3 and Z^5) will result in symmetrical broadening of the resonance. In any case, the general rule is that the higher the order of the shim, the lower down the resonance the distortions will be seen. Errors in Z^3 usually give rise to a broadening of the base of a resonance and, since a broad resonance corresponds to a rapid decay of the FID, such errors are sometimes seen as a sharp decay in the early part of the FID. Another commonly observed distortion is that of a shoulder on one side of a peak, arising from poorly optimised Z and Z^2 shims (this is often associated with reaching a 'false maximum' simply by maximising the lock level with each shim and is usually overcome by making a significant adjustment to Z^2 , and following the procedure described above).

Errors in the low order X/Y shims give rise to the infamous 'spinning sidebands' (for a spinning sample). These are images of the main resonance displaced from it by multiples of the spinning frequency. Shims containing a single X or Y term produce 'first order sidebands' at the spinning frequency from the main line whereas XY and X^2-Y^2 give second order sidebands at double the spinning frequency. However, unless something has gone seriously amiss, you should not encounter more than the first order sidebands in every day NMR at most, and these should certainly be no greater than one or two percent of the main resonance. If there is any doubt as to the presence of sidebands, a simple test is to alter the spinning speed by say 5 Hz and re-acquire the data; only the sidebands will have moved. If the sample is not spinning, errors in the low order X/Y shims contribute to a general broadening of the resonance.

Shimming using the FID or spectrum

Although the lock level is used as the primary indicator of field homogeneity, it is not always the most accurate one. The lock level is dependent on only one parameter; the height of the deuterium resonance. This, whilst being rather sensitive to the width of the main part of the resonance, is less sensitive to changes in the broad base of the peak. The presence of such low level lumps can be readily observed in the spectrum (particularly in the case of protons), but for this to be of use when shimming, the spectrometer must be able to supply a 'real-time display' of a single-scan spectrum so that changes to the shim currents can be assessed rapidly. With modern host computers, the FT and phase correction of a spectrum can be performed very rapidly, allowing one to correct for lineshape distortions as one shims. Alternatively, the shape and the duration of the FID may be used as a more immediate indicator of homogeneity and can often be used simultaneously with the lock display. This approach works best when a singlet resonance dominates the spectrum (such

Figure 3.53. Lineshape defects that arise from inappropriate settings of various shims. These effects have been exaggerated for the purpose of illustration.



as for aqueous solutions) for which the shape of the FID should be a smooth exponential decay. Since with this method of shimming it is likely that changes to the shim currents will be made *during* the acquisition of the spectrum (which will certainly lead to a peculiar lineshape), it is essential that one assesses a later spectrum for which there have been no adjustments during acquisition to decide whether improvements have been made.

Gradient shimming

The most recent approach to field optimisation comes from the world of magnetic resonance imaging and makes use of field gradients to map B_0 inhomogeneity within a sample. This can then be cancelled by calculated changes to the shim settings [47, 48]. The results that can be attained by this approach are little short of astonishing when seen for the first time, especially for anyone who has had to endure the tedium of extensive manual shimming of a magnet, and this method now enjoys widespread popularity.

The discussions below assume some understanding of the action of PFGs, and the reader not familiar with these may wish to return to this section after they are introduced in Chapter 5. In any case, an appreciation of the capabilities of this method should be readily achieved from what follows. Here we shall consider the basis of the method with reference

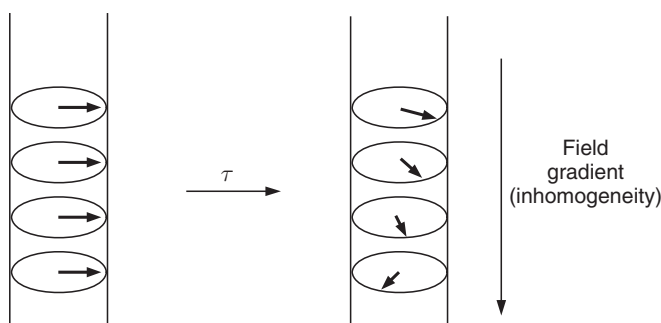


Figure 3.54. Errors in the local static field along the length of a sample are encoded as signal phases when magnetisation is allowed to precess in an inhomogeneous static field for a time τ . The resulting spatially dependent phase differences are used as the basis of gradient shimming.

to the optimisation of Z shims which requires z axis PFGs that are commonly found in modern probeheads (although the use of conventional shim assemblies to generate the appropriate gradients with so-called homospoil pulses has been demonstrated [49], which has the advantage of not requiring specialised gradient hardware). The underlying principle is that all spins throughout a sample contributing to a singlet resonance will possess the same precession (Larmor) frequency *only* if the static field is homogeneous throughout (of course this is what we aim for when shimming!). Any deviation from this condition will cause spins in physically different locations within the sample to precess at differing rates according to their *local* static field. If the excited spins are allowed to precess in the transverse plane for a fixed time period prior to detection, these differing rates simply correspond to different phases of their observed signals (Fig. 3.54). By detecting these signals in the presence of an applied field gradient, the *spatial distribution* of the spins becomes encoded as the *frequency distribution* in the spectrum allowing the inhomogeneity (encoded as phase differences) to be mapped along the length of the sample in the case of z axis gradients (or across the sample for x and y axis gradients).

A suitable scheme for recording this is the gradient echo of Fig. 3.55 in which spins are first dephased by a PFG and later rephased after a period of precession τ_1 to allow detection. The resulting spectrum is the one-dimensional spatial profile (or image) of the sample (Fig. 3.56). Recording a second echo with delay τ_2 and taking the difference yields the *phase map* in which only free precession during the period $(\tau_2 - \tau_1)$ is encoded. The phase distribution in this profile therefore directly maps the inhomogeneity along the sample. The necessary corrections to shim currents to remove these inhomogeneities are calculated from a series of reference phase maps recorded with known offsets in each of the Z shims. Once these reference maps have been recorded for a given probe, they can be used for the gradient shimming of all subsequent samples, with the whole process operating automatically. A more recent approach to optimisation [48] seeks to provide the best possible resonance lineshape rather than aiming for minimal B_0 field errors by simulating

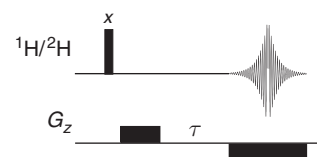


Figure 3.55. A gradient echo sequence suitable for z axis field gradient shimming. The pulsed field gradients provide the spatial encoding whilst the delay τ encodes the static field inhomogeneity as signal phase.

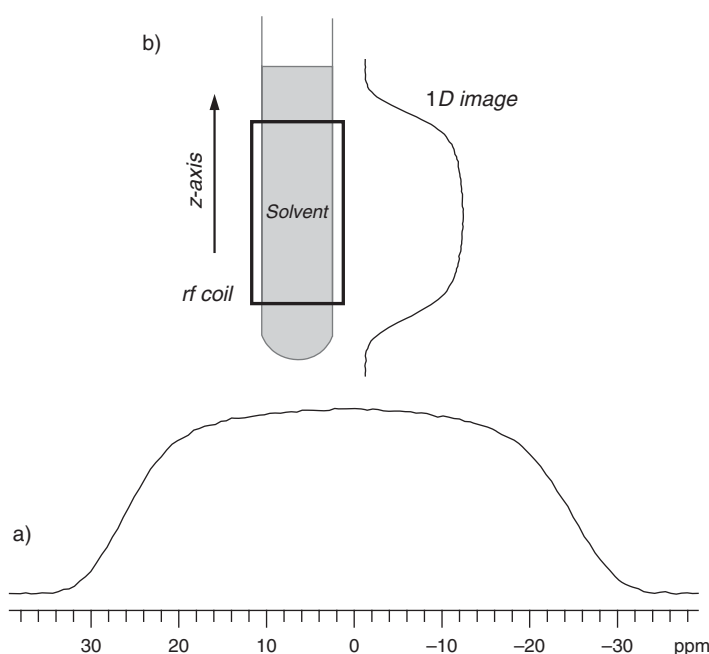
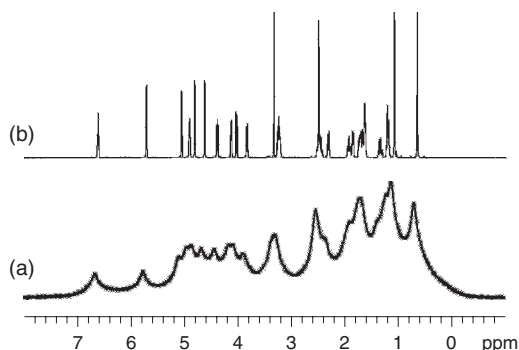


Figure 3.56. (a) The one-dimensional (1D) z axis deuterium image of dimethylsulfoxide (magnitude mode). (b) The frequency axis of this image encodes the spatial dimension along the length of the sample.

Figure 3.57. Automatic deuterium gradient shimming. Spectrum (a) was acquired with all z axis shims set to zero. After less than two minutes of gradient shimming of the $Z-Z^5$ shims (three iterations), spectrum (b) was obtained. The solvent was dimethylsulfoxide and one scan was acquired for each deuterium gradient echo collected via the probe lock coil. Automatic switching for deuterium observation was achieved with a home-built switching device.



peak shapes from the measured field maps and knowledge of the B_1 (rf) profile of the rf coil. This is better able to factor in field discontinuities at the ends of sample columns, for example, and is claimed to provide a more robust and reliable approach with higher quality results; experience suggest this is indeed case.

The primary experimental requirement for gradient shimming is a sample containing a dominant strong, singlet resonance. A good candidate for proton observation is 90% H_2O , but although this is ideal for biomolecular studies, it is clearly of little use for the majority of solvents used in organic spectroscopy. An alternative in this case is to observe the deuterium resonance of the solvent [50] (which in most cases is also a singlet) using the lock channel of the probe. The potential problem then is one of sensitivity and the need for appropriate hardware to allow deuterium observation on the lock coil without manually recabling the instrument each time a sample is shimmed. The necessary lock channel switching devices are commercially available, or, more recently, dedicated deuterium transmitters are built into lock systems to allow direct ^2H observation (and decoupling).

The remarkable power of gradient shimming is illustrated in Fig. 3.57. The lower spectrum was recorded with the $Z-Z^5$ shims all set to zero whilst the upper trace was the result of only three iterations of deuterium gradient shimming using the dimethylsulfoxide solvent resonance. The whole process took less than 2 min without operator intervention. Although a rather extreme example, the capabilities of this approach are clearly evident and explain the increasing use of this methodology, which now plays a valuable role in automated spectroscopy where irreproducible sample depths can lead to rather poor results with conventional simplex optimisation shim routines. The individual mapping of field errors within each and every sample overcomes these problems in a time-efficient manner.

3.4.6. Reference deconvolution

Despite efforts to achieve optimum shimming, either manually or through automated procedures, there are invariably instances when the field homogeneity in a sample does not meet expectations, leading to lineshape distortions in the resulting spectrum. Reference deconvolution is a post-processing strategy that aims to correct such distortions and is now a standard feature of some NMR processing software [51–54]. The process relies on the fact that shim errors will influence all resonances in the distorted spectrum in the same way. If the type and extent of lineshape error can be determined and a correction factor computed for a single resonance, this adjustment may be applied to all others to yield a spectrum free from the unwanted distortions. The principal limitation of the technique is in the selection of a suitable reference resonance with many common multiplet structures proving unsuitable since they give rise to null points in their FIDs; a singlet is thus most appropriate although multiplets have been employed [55]. The reference deconvolution procedure is summarised pictorially in Fig. 3.58. Having identified a suitable reference peak in the spectrum that is free from others, this is extracted (by zeroing all other data points) and its FID generated by inverse fourier transformation (iFT) of the reduced spectrum. An idealised lineshape is then chosen and the corresponding FID similarly computed. This shape will most likely have a Lorentzian profile, corresponding to an exponential decay of the FID, but others may also be selected including a Gaussian profile should resolution enhancement of the spectrum be desired. A comparison of the experimentally derived FID and the idealised FID is then made, a correction function computed and this applied to the original complete FID. This may then be transformed to yield the corrected spectrum.

The reference deconvolution procedure is not limited to addressing lineshape distortions in 1D spectra, having a number of potential applications in high-resolution NMR spectroscopy [53, 54] and has been shown to be effective in improving NOE difference spectra [52], for t_1 noise reduction in 2D spectra [56, 57] and in the production of high-resolution diffusion-ordered spectroscopy (DOSY) [58].

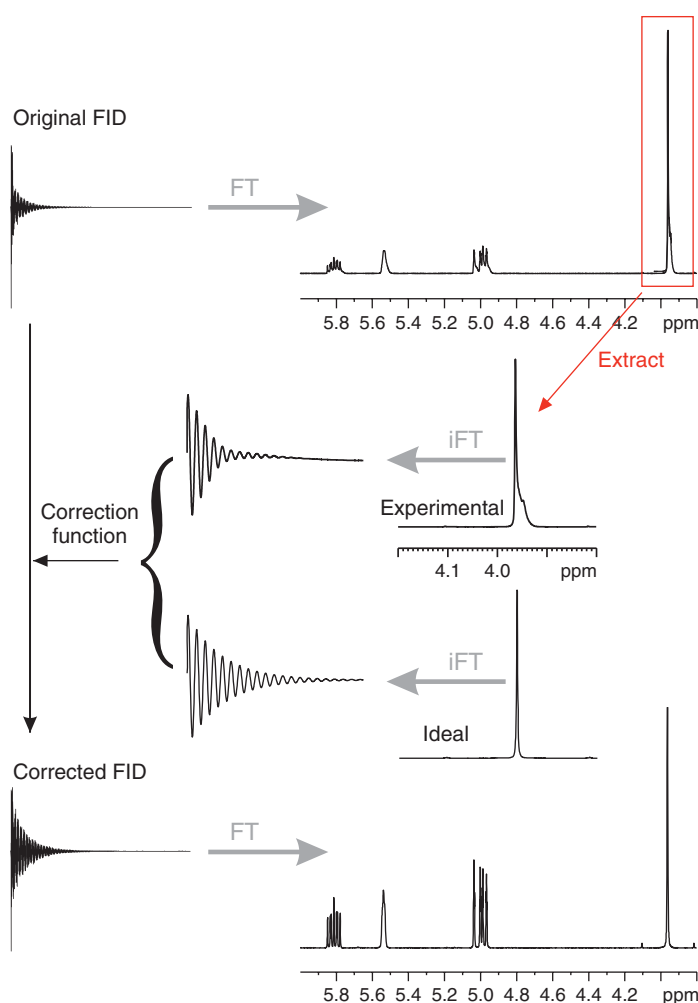


Figure 3.58. A schematic representation of the reference deconvolution procedure.

3.5. SPECTROMETER CALIBRATIONS

This section is primarily intended for those who need to set up experiments or those who have new hardware to install for which new calibrations are required. As with any analytical instrumentation, correct calibrations are required for optimal and reproducible instrument performance. All the experiments encountered in this book are critically dependent on the application of rf and gradient pulses of precise amplitude, shape and duration, and the calibrations described below are therefore fundamental to the correct execution of these sequences. Periodic checking of these calibrations, along with the performance tests described in the following section, also provides an indication of the overall health of the spectrometer.

3.5.1. Radiofrequency pulses

Modern multipulse NMR experiments are critically dependent on the application of rf pulses of known duration (the *pulse width*) that correspond to precise magnetisation tip angles, most frequently 90° and 180° . Pulse width calibrations are normally defined for the 90° pulse (PW_{90}), from which all other tip angles may be derived. Those with PW_{90} in the microsecond range generally excite over a rather wide bandwidth and are termed *non-selective* or *hard* pulses whilst those in the millisecond range are of lower power and are effective over a much smaller frequency window and are thus termed *selective* or *soft* pulses. A third class of pulse is the *frequency-swept* or *adiabatic* pulse, which can be effective over far wider frequency bandwidths than simple hard pulses and have become especially important with higher field spectrometers. The implementation of selective and adiabatic pulses and their calibration is considered separately in Chapter 10, whilst here we shall concentrate on the more widely used hard pulses and on weaker rf fields used for decoupling purposes.

The rf field strengths

The calibration of a pulse width is equivalent to determining the radiofrequency (B_1) field strength of the pulse, and, in fact, it is often more useful to think in terms of field strengths than pulse widths when setting up experiments. For example, excitation bandwidths and off-resonance effects are best considered with reference to field strengths, as are decoupling bandwidths. The relationship between pulse width and the rf field strength γB_1 is straightforward:

$$\gamma B_1 = \frac{1}{PW_{360}} \equiv \frac{1}{4PW_{90}} \text{ Hz} \quad (3.12)$$

Thus, a 90° pulse width of 10 μ s corresponds to a field strength of 25 kHz, a typical value for pulse excitation on a modern spectrometer.

Although spectrometer transmitters are frequently used at full power when applying single pulses, there are many instances when lower transmitter powers are required, for example, the application of decoupling sequences or of selective pulses. These lower powers are derived by attenuating the transmitter output, and the units used for defining the level of attenuation are decibel or dB. This is, in fact, a measure of the ratio between two power levels, P_1 and P_2 , as defined by:

$$\text{dB} = 10 \log_{10} \left(\frac{P_1}{P_2} \right) \quad (3.13)$$

When one speaks of attenuating the transmitter output by so many dBs, one must think in terms of a change in power relative to the original output. In fact, it is more convenient to think in terms of changes in output voltage rather than power, since the rf field strength and hence pulse widths (our values of interest) are proportional to voltage. Since power is proportional to the *square* of the voltage ($P = V^2/R$, where R is resistance), we may rewrite Eq. (3.13) as:

$$\text{dB} = 20 \log_{10} \left(\frac{V_1}{V_2} \right) \equiv 20 \log_{10} \left(\frac{PW_1}{PW_2} \right) \quad (3.14)$$

where PW_1 and PW_2 are the pulse widths for the same net tip angle at the two attenuations. Thus, if one wished to double a pulse width, an additional 20 log 2 dB (6 dB) attenuation is required. An alternative expression has the form:

$$+n\text{dB} \Rightarrow PW \times 10^{\frac{n}{20}} \quad (3.15)$$

or in other words, addition of n dB attenuation increases the pulse width by a factor of $10^{n/20}$. Some example attenuation values are presented in Table 3.7.

An illuminating experiment to perform, if your spectrometer is able to alter the output attenuation internally, is to determine the pulse width over a wide range of attenuations. A plot of pulse widths vs $\log_{10}(\text{attenuation})$ should yield a single straight line over the full range. Discontinuities in the plot may arise when different power amplifiers come into use or when large attenuators are switched in place of many smaller ones. More recent spectrometers make use of linear amplifiers together with calibrated power correction look-up tables, providing pulse output powers that are linear over the whole attenuation range. This means pulse calibrations at different power settings become superfluous since all pulse widths can be calculated with Eq. (3.14) from a single, accurate high power calibration, so it is worthwhile checking if your spectrometer is equipped for this. This also makes the application of more elaborate pulse profiles whose performance can be critically dependent on accurate pulse calibrations (such as the shaped pulses described in Chapter 10) far more straightforward since their calibrations may also be derived in this way.

Observe pulses: high sensitivity

We begin with the most basic NMR pulse calibration, that for the observed nucleus. When the spectrum of the analyte can be obtained in a single scan, or only a few scans, it is straightforward to calibrate the pulse width simply by following the behaviour of the

Table 3.7. Pulse width vs attenuation. The additional attenuation of transmitter output levels increases the pulse width by the factors shown. Thus, adding 6 dB will double the pulse width whereas removing 6 dB will halve it

Attenuation (dB)	1	3	6	10	12	18	20	24
Pulse width factor	1.1	1.4	2.0	3.2	4.0	7.9	10	15.8

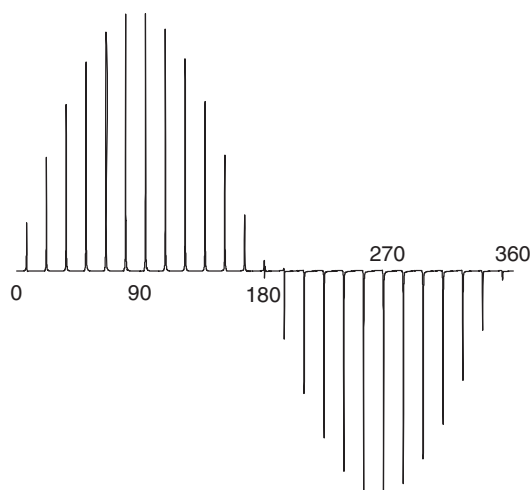


Figure 3.59. Pulse width calibration for the observe channel. A sequence of experiments is recorded with a progressively incremented excitation pulse. The maximum signal is produced by a 90° pulse and the first null with a 180° pulse. Either the 180° or the 360° condition can be used for the calibration (but be sure to know which of these you are observing).

magnetisation as the excitation pulse is increased, with nulls in the signal intensity occurring with a 180° or 360° pulse. To perform any pulse calibration, it is essential that the probe is first tuned for the sample so that the results are reproducible. The transmitter frequency should be placed on-resonance for the signal to be monitored, to eliminate potential inaccuracies arising from off-resonance effects, and a spectrum recorded with a pulse width less than 90° , say $2\text{--}3\ \mu\text{s}$. The resonance is used to define the phase correction for all subsequent experiments and is phased to produce the conventional positive absorption signal. The experiment is repeated with identical phase correction but with a progressively larger pulse width (Fig. 3.59). Passing through the 180° null then provides the PW_{180} calibration, whilst going beyond this yields an inverted resonance, so it becomes clear when you have gone too far. Detecting the null precisely can sometimes be tricky, as there is often some slight residual signal remaining, the exact appearance depending on the probe used, so in practice one aims for the minimum residual signal.

Between acquisitions there must be a delay sufficient for complete relaxation of the spins, and if signal averaging is used for each experiment, this must also be applied between each scan to obtain reliable calibrations. In proton spectroscopy, such delays are unlikely to be too much of a burden, but can be tediously long for slower relaxing spins. If you have some feel for what the pulse width is likely to be, perhaps from similar samples or a 'reference' calibration, then a better approach is to search for the 360° null. Since magnetisation remains close to the $+z$ axis, its recovery demands less time and the whole process can be performed more quickly. The process can be further simplified by monitoring the FID alone, which also has minimum amplitude with the 360° pulse. In any case, it is wise to check the null obtained is in fact the one you believe it to be, as all multiples of 180° give rise to nulls.

Observe pulses: single-scan nutation spectroscopy

A more recent approach to pulse calibration allows this to be performed in a single scan and provides a rapid and convenient method for ^1H pulse calibration in particular that is also well suited to use under automation [59]. The method has most significance for inverse and cryogenic probes where pulse calibrations may vary significantly between samples. The measurement derives from a single-scan nutation experiment in which an rf pulse is applied during each of the dwell periods of a FID acquisition between data point sampling in a manner similar to that used in the homonuclear decoupling experiments described in Section 4.2.2. No initial excitation pulse is required. Provided the rf pulses are applied on-resonance for a single peak, the associated magnetisation vector is driven by each of the pulses away from its equilibrium position along the longitudinal axis as the acquisition proceeds; the magnetisation is said to *nutate* about the applied rf field. The transverse magnetisation generated by this process describes an oscillatory behaviour along one axis during data acquisition (Fig. 3.60a). One may imagine this oscillatory behaviour as being equivalent to the *net* magnetisation generated by the evolution of a doublet that is on-resonance in the rotating frame (Fig. 3.60b; see also Fig. 2.13). Indeed, FT of the nutation data leads to the generation of an anti-phase doublet in the resulting nutation spectrum with a splitting $\Delta\nu$ Hz (Fig. 3.61). The oscillation frequency reflects the mean nutation frequency of the vector during data collection and equates to the frequency of evolution of each half of the hypothetical doublet $\Delta\nu/2$. This provides the mean γB_1 value of the applied rf and hence the pulse width calibration under the conditions used.

Figure 3.60. The behaviour of an on-resonance magnetisation vector during the nutation experiment. (a) As the rf field is applied during each of the FID dwell periods ($dw_1, dw_2, dw_3, \dots$) the vector is driven stepwise about the B_1 field (shown in shades of grey). The sampled transverse component (likewise greyed) oscillates along the y axis as the data acquisition proceeds and the total nutation angle progressively increments. (b) Equivalent behaviour arises from the evolution of an anti-phase doublet of splitting $\Delta\nu$.

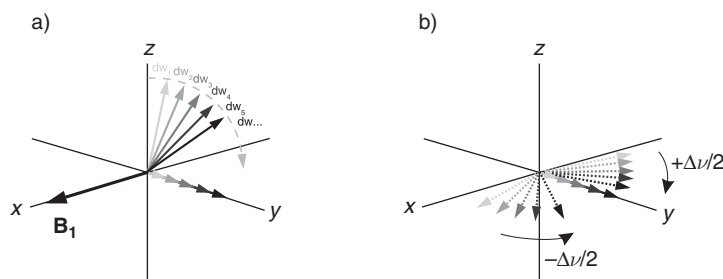
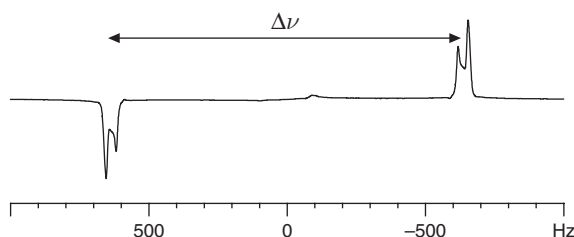


Figure 3.61. The single-scan nutation spectrum of an aqueous sample (12 dB attenuation, 10% duty cycle). The doublet splitting of 1268 Hz calibrates the high power 90° hard pulse at $9.86 \mu\text{s}$.



To avoid possible probe damage and sample heating, it is safe practice during the nutation experiment to reduce the rf amplitude from that used for hard pulses by typically 12 dB, corresponding to a fourfold increase in pulse width. It also becomes necessary to account for the fraction of time during which the rf pulse is applied within each dwell period of the FID, the so-called duty cycle, d . Values may range from 10–80% ($d = 0.1$ – 0.8), and the measured rf amplitude must be corrected for this. With the low power calibration complete the required hard pulse calibration may be calculated directly when linearised rf amplifiers are in use, as found on modern instruments, otherwise additional calibrations may be required to correct for the power differences. Assuming amplifier linearisation and the use of 12 dB attenuation in the nutation experiment, the high power pulse width may be computed as

$$t_{360} = \frac{d}{4(\Delta\nu/2)} \quad \text{or} \quad t_{90} = \frac{d}{8\Delta\nu} \quad (3.16)$$

The method is most appropriate when the spectrum is dominated by a single resonance, so has obvious application to samples in heavily protonated water but can be equally effective for organic solutions when a single dominant peak can be identified, such as a resolved methyl resonance for example. This must be placed on-resonance, and the smallest frequency separation between the anti-phase peaks in the nutation spectrum is then used in the appropriate calculation. The whole procedure is most conveniently executed through appropriate software routines, which may be supplied as standard by your instrument vendor.

Observe pulses: low sensitivity

When the sample is too weak to allow its observation within a few scans, one possible approach is to use the method of signal averaging and to search for the 360° null as described above [60], although this could become a rather laborious affair. An alternative approach [61] requires that only two spectra are collected with the pulse width of the second being exactly double that of the first ($PW_2 = 2PW_1$). Once again, a sufficient delay between scans is required to avoid saturation effects, and the spectrometer must be operating in an ‘absolute intensity’ scaling mode so that the relative signal intensities from both experiments can be compared. Heteronuclear spectra should also be acquired without enhancement by the NOE, and the use of the inverse-gated decoupling scheme (Section 4.2.3) allows such data to be collected with broadband decoupling during acquisition to aid sensitivity. Following some simple arithmetic [61], it can be shown that

$$\theta_1 = \cos^{-1} \left(\frac{I_2}{2I_1} \right) \quad (3.17)$$

where I_1 and I_2 correspond to the measured signal intensities in the first and second experiment, respectively. The duration of the pulse in the first experiment corresponds to the pulse angle θ_1 , from which the 90° pulse width can be derived. For optimum results, θ_1 should be greater than 30° and θ_2 , the tip angle in the second experiment, should be less than 180° , so again some rough feel for the likely answer is required for this approach to be efficient.

A more convenient approach in many instances is to perform the calibrations not on the sample of interest itself but on a suitable sample made up for the purposes of calibration that is strong enough to permit the observation of the $180/360^\circ$ null directly and quickly. The calibration can then be recorded and used in future experiments, as this should be quite reproducible if similar solvents are used and the probe correctly tuned. The largest discrepancies occur between aqueous and organic solvents, especially when the aqueous solutions are highly ionic, and separate calibrations may be required.

Indirect pulses

In contrast to above, the calibration of pulses for nuclei other than that being observed must be performed 'indirectly'. This is often referred to as the calibration of the 'decoupler' pulses although this is something of a misnomer nowadays since the pulses may have nothing to do with decoupling itself but may be an integral part of the pulse sequence. Most commonly, the indirect channel is used in two modes; the first is to apply short, hard pulses typically at maximum power, the second is to apply a pulsed decoupling sequence for longer periods of time, at significantly less power. It is therefore commonplace to record indirect calibrations at these two power levels, for which the following method is equally applicable, and having performed the calibration at high power, the low power attenuation can be estimated from Eq. (3.14) prior to its accurate determination. Alternatively, the use of accurately linearised amplifiers can avoid the need for low power calibrations altogether. More recent spectrometers make use of linear amplifiers together with calibrated power correction look-up tables, meaning the pulse output power is linear over the whole attenuation range. This means low power calibrations become superfluous since all pulse widths can be calculated from a single high power calibration with Eq. (3.14), so it is worthwhile checking if your spectrometer is equipped for this. This also makes the application of more elaborate pulse profiles, such as the shaped and adiabatic pulses described in Chapter 10, far more straightforward since their calibrations may also be computed for both observe and indirect pulses.

Indirect pulses on high abundance nuclides

Calibrations in which the indirect nuclide exists at high abundance are generally more straightforward because of the clear appearance of heteronuclear coupling. Thus, to relate the behaviour of the indirect spin, A, to that being observed, X, one exploits the mutual heteronuclear scalar coupling that must exist between them, J_{AX} , for the calibration to be possible. The simplest calibration sequence [62, 63] suitable for spin- $1/2$ nuclei is shown in Fig. 3.62. The A and X pulses should be on resonance, the X-pulse calibration must already be known, and the value of J_{AX} measured directly from the (coupled) X spectrum. The delay Δ is set to $1/2J_{AX}$ s for an AX group so that the X spin doublet vectors are anti-phase after this evolution period. The subsequent A pulse renders the x -magnetisation *unobservable* when $\theta = 90^\circ$ as this generates pure heteronuclear multiple-quantum coherence, which is unable to generate a detectable signal in the probe (see Chapter 5 for an explanation of this effect).

The approach is to begin with θ very small and to phase the spectrum so that the doublet lines are in anti-phase. As θ increases, the doublet intensity will decrease and become zero when θ is 90° , whilst beyond this the doublet reappears but with inverted phase (Fig. 3.63). If it is necessary to perform the calibration with an A_2X group, the delay Δ should be $1/4J$ and it is the outer lines of the triplet that behave as described above whilst the centre line remains unaffected. When calibrating lower powers for the purpose of broadband decoupling, it is usually more convenient to set the *duration* of the θ pulse according to the decoupler bandwidth required (Section 10.3) and to vary the output attenuation to achieve the null condition. As is often the way in experimental procedures, simplicity is best and the indirect pulse calibrations can most conveniently be performed on strong calibration samples using the sequence of Fig. 3.62. For ^1H - ^{13}C systems, CHCl_3 and (^{13}C -labelled) methanoic acid are convenient materials for organic and aqueous calibrations respectively.

A readily available alternative that is particularly suitable for (although not restricted to) proton calibration in ^1H -X systems is the distortionless enhancement by polarisation transfer (DEPT) sequence (Chapter 4). This improves observation sensitivity by making use of polarisation transfer from ^1H to X and is dependent on the faster relaxing protons for repetition rates in signal averaging. The 90° proton pulse is achieved when XH_2 or XH_3 groups pass through a null whereas XH groups have maximum intensity at this point (Fig. 3.64). One caveat here is that for the purposes of pulse calibration, all the proton pulses in the sequence

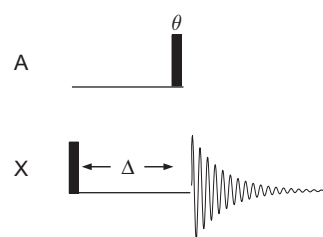


Figure 3.62. The sequence for the calibration of "indirect" transmitter pulses (those on nuclei other than that being observed). The period Δ is set to $1/2J_{AX}$.

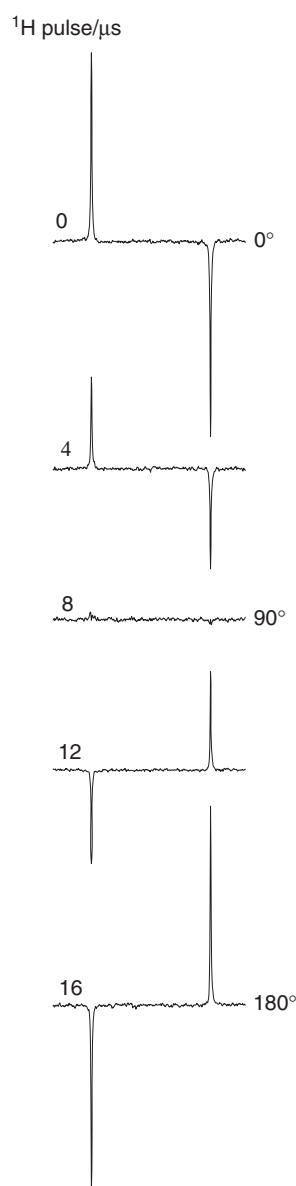


Figure 3.63. Indirect calibration of the proton pulse width with carbon observation using the sequence of Fig. 3.62. As the proton pulse width increases, the carbon signal diminishes until it disappears at the $^1\text{H}(90^\circ)$ condition. Going beyond this causes the signal to reappear but with inverted phase (the same phase correction is used for all spectra). The sample is ^{13}C -labelled methanoic acid in D_2O .

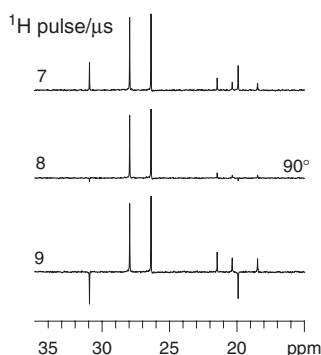


Figure 3.64. Calibration of indirect proton pulses using the DEPT sequence. When the proton editing pulse in the sequence is exactly 90° , only methine resonances are apparent (see Chapter 4 also). The sample is menthol in CDCl_3 .

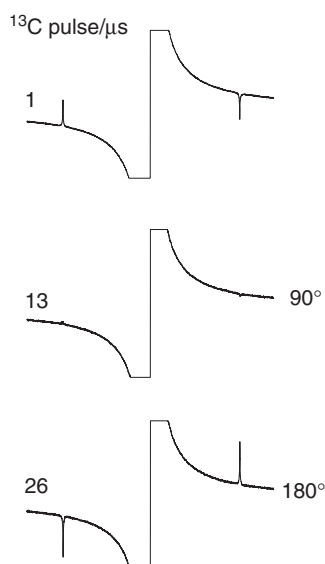


Figure 3.65. Calibration of indirect pulses (here ^{13}C) with proton observation using the sequence of Fig. 3.62. The parent $^1\text{H}(^{12}\text{C})$ resonance appears as a large dispersive signal, but the behaviour of the satellites is clear, again disappearing with a 90° pulse on the indirect channel. The sample is CHCl_3 .

need to be altered on each experiment, not just the final ' θ ' proton pulse (see Chapter 4 for an explanation of this sequence).

Indirect pulses on low abundance nuclides

When directly observing protons and indirectly calibrating nuclei of low abundance such as ^{13}C and ^{15}N (the so-called inverse configuration), the dominant resonance will be of no use and may interfere since it is the much less intense coupled satellites that must be employed for the calibration. Thus, the major ^{12}C line of CHCl_3 will be apparent as a large dispersive signal between the ^{13}C satellites of interests when using the sequence of Fig. 3.62, but this should not pose too great a problem so long as the sensitivity is sufficient for the satellites to be readily observed (Fig. 3.65). In cases when the major resonance hampers observation of the satellites, a PFG-based variant may be employed that removes this. The 2CALIS sequences [64] (calibration for indirect spins) are shown in Fig. 3.66 and may be most readily understood by considering $\theta = 90^\circ$, where the duration of θ is again the pulse calibration being sought and $\Delta = 1/2J_{\text{HX}}$. The initial $^1\text{H}-\text{X}$ spin-echo generates anti-phase ^1H doublet vectors that are then rendered longitudinal by the subsequent ^1H 90° pulse (this is the same initial behaviour as in the insensitive nuclei enhanced by polarisation transfer (INEPT) sequence described in Chapter 4). The strong purge gradient G_2 then destroys all magnetisation arising from non X-coupled protons, that is, the $^1\text{H}-^{12}\text{C}$ resonance in the case of carbon calibration. Following this, the simultaneous 90° pulses on both ^1H and X transforms the longitudinal, anti-phase ^1H magnetisation into unobservable multiple quantum coherence and so eliminates the satellites when $\theta = 90^\circ$, as for the simpler sequence of Fig. 3.62. Thus, as θ increases the satellite intensities decay toward this zero-crossing condition (Fig. 3.67). Improved suppression of the unwanted parent resonance may be achieved with additional X spin filtration by alternating the final X pulse between θ and 3θ (that is, 90° and 270° for the ideal calibration condition) combined with alternation of the receiver. The non-X-coupled proton magnetisation remains invariant to these pulses and so is cancelled by this receiver cycle. Optionally, an additional refocusing period may be employed, as in 2CALIS-1 (Fig. 3.66b), to yield in-phase ^1H doublets that may, again optionally, be X spin decoupled. This approach may prove advantageous when signal overlap makes the presence of anti-phase signals susceptible to accidental cancellation with neighbouring resonances. Note that for these sequences to generate any observable signals, 2θ must be close to 180° so that the heteronuclear spin-echo generates anti-phase magnetisation; if no X spin 180° pulse is applied (that is if $\theta = 0^\circ$), the heteronuclear coupling would be fully refocused. Thus, a reasonable estimate of θ is required before the calibration sequence begins as would typically be known from standard calibration samples. 2CALIS would then be employed for accurate calibration on the sample under study.

For the indirect calibration of natural abundance ^{15}N with ^1H observation, the dominant ^{14}N -bound proton resonance may be rather broad owing to the quadrupolar ^{14}N nucleus and may mask the small ^{15}N satellites. In such cases, the 2CALIS sequences may prove especially useful. Alternatively, the ^{14}N line can be preferentially suppressed without the need for PFGs, owing to its faster transverse relaxation rate, by the application of a spin-echo prior to the sequence of Fig. 3.62; see reference [65] for further details. A more convenient alternative is to use ^{15}N -labelled materials for calibration purposes. Calibration over long-range couplings is also possible, for example, when no one-bond coupling exist, for which the original CALIS-2 sequence [66] is best suited.

Homonuclear decoupling field strength

Homonuclear decoupling requires the decoupling (B_2) field to be on during the acquisition of the FID and, as described in Chapter 4, this demands the use of rapid gating of the decoupler during this period, so the methods presented above are therefore no longer applicable. The field strengths used in homonuclear decoupling are considerably less than those required for pulsing, and it is sometimes useful to have some measure of these when setting up decoupling experiments, to achieve the desired selectivity. A direct measure of the mean field strength produced during homonuclear decoupling can be arrived at through measurement of the Bloch–Siegert shift [67], which is the change in the resonance frequency of a line on the application of nearby irradiation. Provided the offset of the decoupler from the unperturbed resonance is greater than the decoupler field strength ($\nu_o - \nu_{\text{dec}} \gg \gamma B_2$), then

$$\gamma B_2 = ((\nu_{\text{BS}} - \nu_o) \cdot 2(\nu_o - \nu_{\text{dec}}))^{1/2} \text{Hz} \quad (3.18)$$

where ν_{BS} is the signal frequency in the presence of the irradiating field, ν_o is that in the absence of the irradiating field and ν_{dec} the frequency of the applied rf. An example of such a calibration is shown in Fig. 3.68.

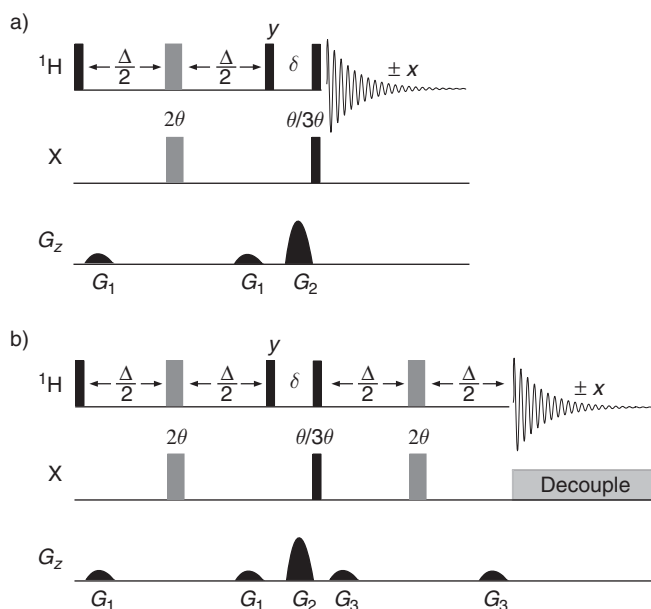


Figure 3.66. The 2CALIS sequences for the calibration of indirectly observed, low abundance nuclides. (a) 2CALIS-2 for anti-phase satellite display and (b) the 2CALIS-1 refocused version with X-spin decoupling. All pulse phases are x unless indicated and the optimum phase cycle is $y, y, -y, -y$ on the second proton 90° pulse with corresponding alternation of the receiver ($x, -x, x, -x$) as the final X pulse duration steps $\theta, 3\theta, 3\theta, \theta$.

Heteronuclear decoupling field strength

Complementary to the pulse methods described above for the measurement of the ‘indirect’ pulse widths is the measurement of the heteronuclear decoupling field strength. This method is applicable to the measurement of medium to low power outputs, such as those used in broadband decoupling, soft pulses and selective decoupling, and makes use of continuous, rather than pulsed, irradiation. Once again the method exploits the heteronuclear coupling between spins and observes the changes to this on the application of off-resonance heteronuclear decoupling [68]. Consider again the AX pair in which continuous A spin decoupling is applied so far from the A resonance that it has no effect; the X spectrum displays a doublet. As the decoupler frequency moves towards the A resonance, the observed coupling begins to collapse until eventually the X spectrum displays only a singlet when decoupling is on-resonance. Measurement of the reduced splitting in the intermediate stages provides a measure of the field strength according to [68]:

$$\gamma B_2 = \left[\left(\frac{J \cdot \nu_{\text{off}}}{J_r} \right)^2 + \frac{(J_r^2 - J^2)}{4} - \nu_{\text{off}}^2 \right]^{1/2} \text{ Hz} \quad (3.19)$$

where J is the AX coupling constant, J_r the reduced splitting in the presence of the decoupling field and ν_{off} the decoupler offset from the A resonance. When the decoupler field strength is much larger than the decoupler resonance offset, this simplifies to:

$$\gamma B_2 \approx \frac{J \cdot \nu_{\text{off}}}{J_r} \text{ Hz} \quad (3.20)$$

Thus, by measuring the resonance splitting in the absence and presence of the irradiation, the field strength is readily calculated. For ^1H – ^{13}C spectroscopy, the one-bond coupling is suitable for the medium to strong powers, whilst for very weak decoupler fields (<50 Hz) it may be necessary to utilise long-range ^1H – ^{13}C couplings to obtain significant changes in splitting. The calibration of proton field strength with this method is illustrated in Fig. 3.69.

3.5.2. Pulsed field gradients

Numerous modern NMR experiments utilise field-gradient pulses for signal selection or rejection (see Chapter 5), requiring these to be applied for well-defined durations at known gradient strengths. Experiments presented in the literature will (or should) state the gradient strengths required to achieve the desired results, so it is necessary to have some knowledge of the strengths provided by the instrument if wishing to implement these techniques. Practical procedures for determining gradient strengths and gradient recovery periods are described here, as it is these two parameters that must be defined by the operator when preparing a gradient-selected experiment. Discussions are restricted to static B_0 gradients rather than the far less widely used rf B_1 gradients [69, 70]. Despite the claimed advantages of B_1 gradients [71], these have yet to gain widespread acceptance.

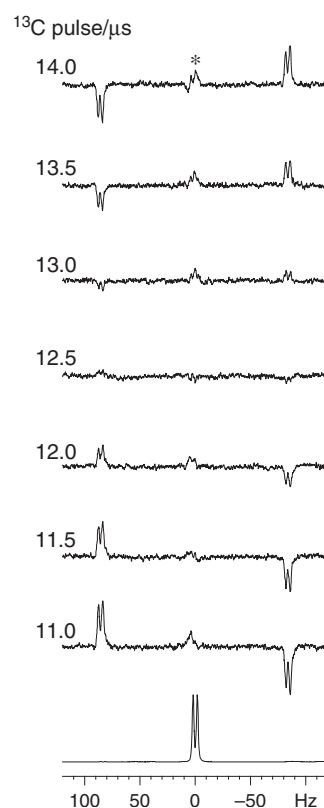


Figure 3.67. ^{13}C Indirect calibration with the 2CALIS-2 sequence performed on 50 mM sucrose. The natural abundance ^{13}C -satellites of the anomeric proton are shown and disappear when $\theta = 90^\circ$, here 12.5 μs . The asterisk indicates the residual ^{12}C parent signal (shown in the lower trace).

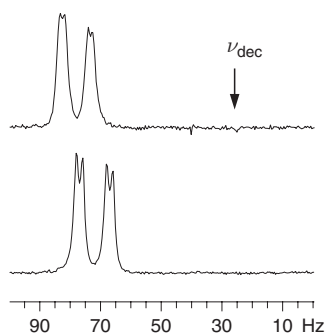


Figure 3.68. Calibration of the homonuclear decoupling field strength via the Bloch–Siegert shift. The decoupler offset from the unperturbed resonance was 47.5 Hz causing a shift of 5.5 Hz, indicating the mean rf field to be 23 Hz.

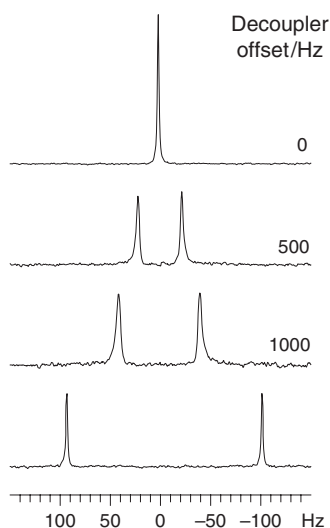


Figure 3.69. Calibration of the heteronuclear decoupler field strength via off-resonance proton decoupling. From the reduced splitting of the ^{13}C – ^1H doublet, the proton rf field strength is calculated to be 2.2 kHz, a typical value for broadband proton decoupling.

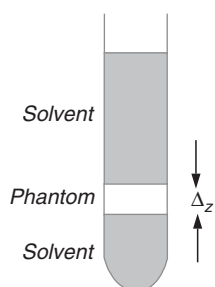


Figure 3.70. A phantom sample suitable for calibrating z axis gradient field strength. The teflon or rubber phantom has precise dimensions (Δz , typically 2–4 mm in height) and excludes solvent from a slice of the tube. It is positioned in the tube such that it sits at the centre of the rf coil when placed in the probe.

Gradient strengths

For the application of pulsed field gradients in high-resolution NMR, knowledge of the exact gradient strengths produced is not, in fact, crucial to the success of most experiments. This is in contrast to the application of rf pulses, where precise pulse widths are required. Provided the gradients are *sufficiently strong* for the experiment, the critical factors are pulse reproducibility and the ability to produce gradients pulses of precise amplitude *ratios*. The notable exception to this is in the measurement of self-diffusion coefficients by NMR spectroscopy (Chapter 9) for which very accurate gradient strengths calibrations are required. The procedures described below can be used to calibrate gradient strengths and to check the linearity of the gradient amplifier output.

Essentially there are two approaches to gradient calibration, one based on the measurement of molecular diffusion, the other using an image of a suitable calibration sample. The first requires precise experimental conditions, accurate temperature calibration and a reference solvent or solute with a known diffusion coefficient. Residual H_2O in D_2O is suitable for this, as described in Chapter 9 where diffusion NMR spectroscopy is considered in detail. With the relatively low gradient field strengths used in high-resolution NMR, the alternative imaging approach may be rather more straightforward and less dependent on solution conditions. The calibration of z -gradients is based on the measurement of a 1D image profile of a phantom of known spatial dimensions placed in a standard NMR tube containing H_2O (Fig. 3.70). The phantom is typically a disk 2–4 mm in length of rubber or teflon (a cut-down vortex suppressor for example), which is positioned in the tube such that it sits at the centre of the rf coil. The sample is not spun and need not be locked, in which case the lock field sweep must be stopped. The proton spectrum is first acquired in the absence of the field gradient and the transmitter placed on resonance. The spectrum is then acquired with a very large spectral width, say 50 kHz, in the presence of the field gradient. This can, in principle, be achieved by a simple pulse-acquire scheme with the gradient turned on throughout (Fig. 3.71a), although this approach has its limitations because the application of rf pulses in the presence of the gradient requires excitation over a very wide bandwidth. Thus, it is preferable to record the spectrum with a gradient spin-echo sequence (Fig. 3.71b), in which the second of the two gradients has the same strength but twice the duration of the first and is applied throughout the acquisition period during which the spin-echo refocuses. In either case, the acquisition time must be kept short (10 ms or less) and relatively low gradient strengths applied. The resulting data are processed with a *non-shifted* sine-bell window function (this matches the shape of the echo FID) and magnitude calculation. The spectrum then displays a dip in the profile corresponding to the region of the sample that contains the phantom, and hence no solution, (Fig. 3.72) from which the gradient strength may be calculated as:

$$G_z = \frac{2\pi\Delta\nu}{\gamma\Delta z} \equiv \frac{\Delta\nu}{4358 \times \Delta z} \text{ G cm}^{-1} \quad \text{or} \quad \frac{\Delta\nu}{4.358 \times 10^5 \times \Delta z} \text{ T m}^{-1} \quad (3.21)$$

where $\Delta\nu$ is the width of the dip in hertz and Δz is the height of the phantom in cm. The SI units for a field gradient are T m^{-1} , although it is common for values quoted in the literature to be given in G cm^{-1} ($1 \text{ G cm}^{-1} \equiv 0.01 \text{ T m}^{-1}$). For calibrations of x and y gradients, a similar method may be used, in which case the phantom is not required and the internal diameter of the NMR tube defines the sample width, with the areas outside the tube providing the empty region, and the width of the resulting ‘peak’ used in the above expression.

Repeating the above procedure with increasing gradient field strengths indicates the linearity of the amplifier, with larger gradients producing a proportionally wider dip in the profile. Figure 3.73 shows the result obtained for a 5 mm inverse z -gradient probe. Assuming linearity over the whole amplifier range, the maximum gradient strength for this system is 0.51 T m^{-1} (51 G cm^{-1}).

Gradient recovery times

As is discussed in Chapter 5, significant distortions in the magnetic field surrounding a sample will result following the application of a gradient pulse, and to acquire a high-resolution spectrum, the eddy currents responsible for these field distortions must be allowed to decay before data are collected. A simple scheme for the measurement of the gradient recovery period is shown in Fig. 3.74, in which the initial gradient pulse is followed by a variable recovery period, after which the data are acquired. Distortions of the spectrum are readily seen by observing a sharp proton singlet, such as that of chloroform. Initially, the recovery period, τ , is set to a large value (1 s) to establish the reference spectrum, and the value progressively decreased until distortions appear in the spectrum

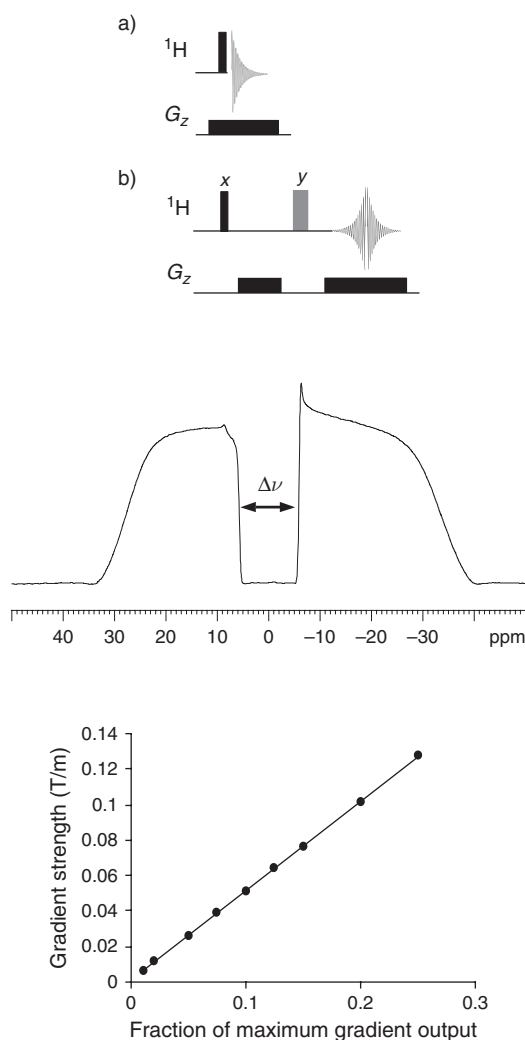


Figure 3.71. Sequences for collecting gradient profiles for the calibration of the z axis gradient field strength.

Sequence (a) collects the FID directly in the presence of the applied gradient whilst sequence (b) makes use of a gradient echo (see text). In (b), the second gradient has the same strength but twice the duration of the first.

Figure 3.72. A typical gradient profile collected with the sequence of Fig. 3.71b. Here the dip is 5896 Hz wide, corresponding to a gradient strength of 0.051 T m^{-1} (10% of maximum).

Figure 3.73. Calibration of the gradient amplifier linearity using the sequence of Fig. 3.71b. Assuming system linearity over the full output range and extrapolating the calibration, the maximum gradient strength provided by this system is 0.51 T m^{-1} (51 G cm^{-1}).

(Fig. 3.75). This provides some indication of the recovery period required following a gradient pulse, during which data should not be collected or further gradient or rf pulses applied. The success of high-resolution gradient-selected NMR is critically dependent on being able to use very short recovery periods, which are typically around $100 \mu\text{s}$ on modern instruments that have actively shielded probe heads. Whilst NMR experiments generally utilise *shaped* gradient pulses (as these help reduce eddy current generation), a more demanding test of hardware performance can be achieved by using *rectangular* gradients, as in Fig. 3.75. Performing this test with a half-sine-shaped gradient pulse showed complete recovery of the resonance within $10 \mu\text{s}$.

3.5.3. Sample temperature

Many spectrometers are equipped with facilities to monitor and regulate the temperature within a probe head. Usually the sensor takes the form of a thermocouple whose tip is placed close to the sample in the gas flow used to provide temperature regulation. However, the readings provided by these systems may not reflect the true temperature of the sample

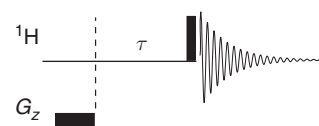


Figure 3.74. A simple scheme for investigating gradient recovery times.

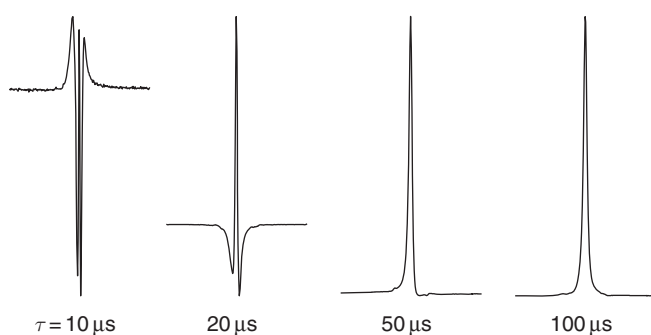


Figure 3.75. Gradient recovery after application of a 1 ms square gradient pulse of 0.25 T m^{-1} (50% of maximum output). The sample is chloroform, and data were acquired with an actively shielded 5 mm inverse z axis gradient probe head.

due to the finite heat capacity of the flowing gas [72] and must be subject to appropriate calibration for accurate temperature recording. One approach to such calibration is to measure a specific NMR parameter that has a known temperature dependence to provide a more direct reading of *sample* temperature. Whilst numerous possibilities have been proposed as reference materials [73], two have become accepted as the standard ^1H temperature calibration samples for solution spectroscopy with conventional probe heads. These are methanol for the range 175–310 K and 1,2-ethanediol (ethylene glycol) for 300–400 K. Such strong solutions are inappropriate for cryogenic probes however, and pure perdeuterated methanol offers a better alternative. For use as an *internal* temperature calibrant in ^{13}C measurements, tris(trimethylsilyl)methane is a robust material that may be used in a range of organic solvents, with the shift difference between the ^{13}C resonances of $\text{Si}(\text{CH}_3)_3$ and CH being indicative of sample temperature; see reference [74] for details of synthesis and calibration.

For the low temperature ^1H calibration, neat MeOH is used, with a trace of HCl to sharpen the resonances, for which the following equation holds [75, 76]:

$$T(\text{K}) = 403 - 29.53\Delta\delta - 23.87(\Delta\delta)^2 \quad (3.22)$$

where $\Delta\delta$ is the $\text{OH}-\text{CH}_3$ chemical shift difference (ppm). This expression may be approximated to three linear equations of the form:

$$175 - 220 \text{ K} : T(\text{K}) = 537.4 - 143.1\Delta\delta \quad (3.23)$$

$$220 - 270 \text{ K} : T(\text{K}) = 498.4 - 125.3\Delta\delta \quad (3.24)$$

$$270 - 310 \text{ K} : T(\text{K}) = 468.1 - 108.9\Delta\delta \quad (3.25)$$

which are presented as calibration charts in Fig. 3.76. For calibrations with neat ethane-1, 2-diol, the appropriate equation is [76]:

$$T(\text{K}) = 466.0 - 101.6\Delta\delta \quad (3.26)$$

where $\Delta\delta$ now represents the $\text{OH}-\text{CH}_2$ chemical shift difference (ppm) (Fig. 3.77). For both samples, the peak shift difference decreases as the sample temperature increases due to the reduced intermolecular hydrogen bonding, which in turn causes the hydroxyl resonance to move to lower frequency. Cryogenic probes are limited in their operating temperature range to within typically 280–330 K, but neat solutions produce too strong a signal for these probes, leading to radiation damping that both broadens and shifts the resonances. A more appropriate calibration sample is neat *perdeuterated* methanol for which the following holds [77] (Fig. 3.78):

$$T(\text{K}) = 419.1381 - 52.5130\Delta\delta - 16.7467(\Delta\delta)^2 \quad (3.27)$$

Before making temperature measurements, the sample should be allowed to equilibrate for at least 5–10 min at each new temperature, and it is also good practice to take a number of measurements to ensure they do not vary before proceeding with the calculation.

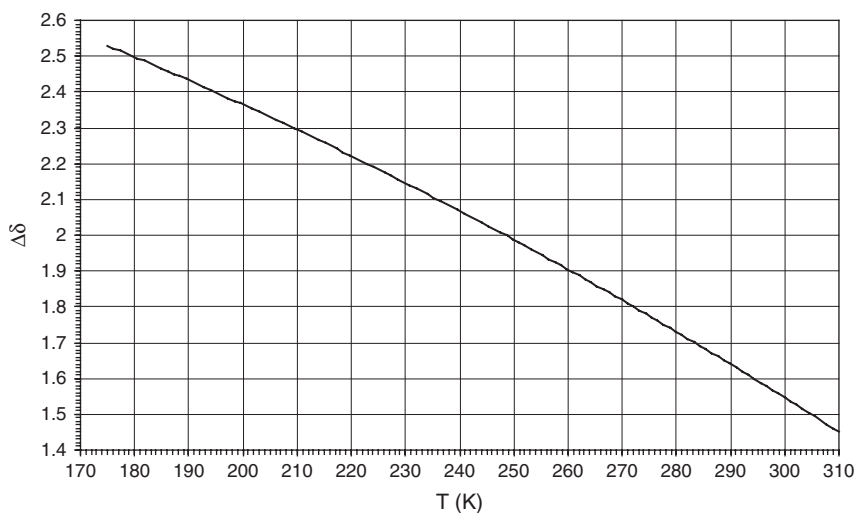


Figure 3.76. Temperature calibration chart for neat methanol. The shift difference $\Delta\delta$ (ppm) is measured between the CH_3 and OH resonances.

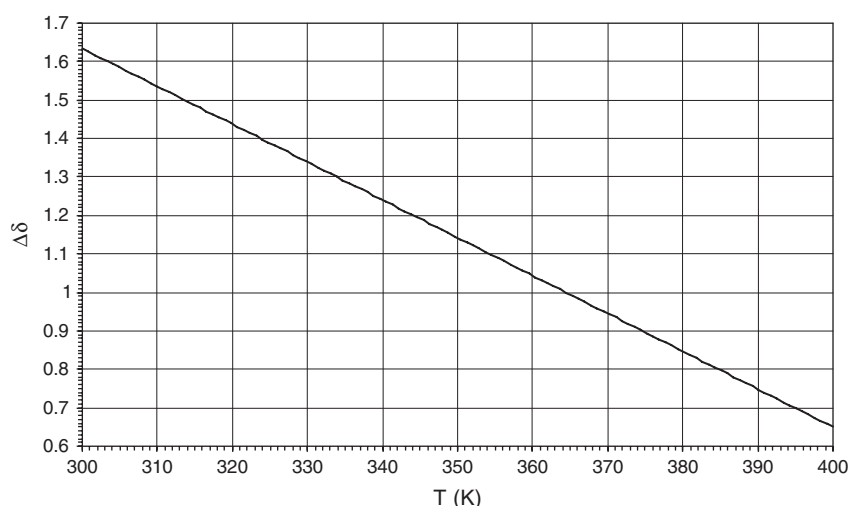


Figure 3.77. Temperature calibration chart for neat ethane-1,2-diol (ethylene glycol). The shift difference $\Delta\delta$ (ppm) is measured between the CH_2 and OH resonances.

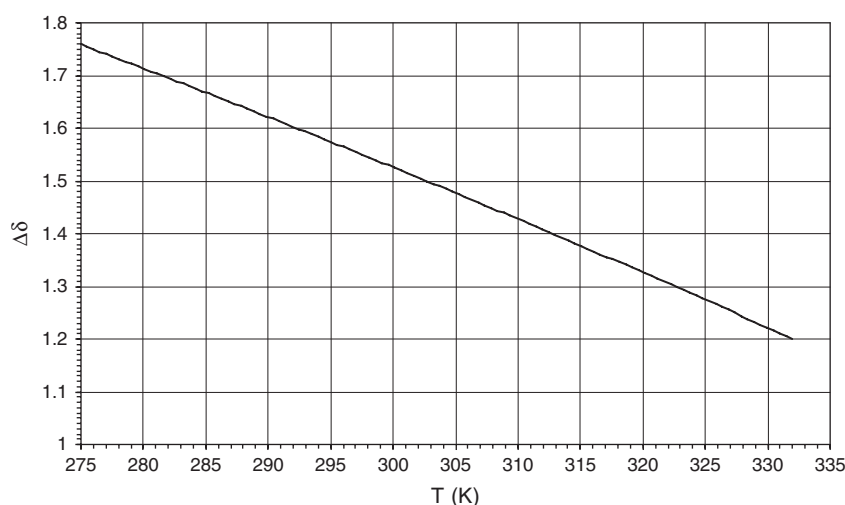


Figure 3.78. Temperature calibration chart suitable for cryogenic probes. The sample is neat d_4 -methanol, and the shift difference $\Delta\delta$ (ppm) is measured between the residual CHD_2 and OH resonances.

3.6. SPECTROMETER PERFORMANCE TESTS

There exist a large number of procedures designed to test various aspects of instrument performance, and it would certainly be inappropriate, not to mention rather tedious, to document them in a book of this sort. Instead I want to introduce briefly only those that are likely to be of use in a routine spectrometer 'health check', and as such, this section will be of most interest to anyone with responsibility for the upkeep of an instrument. These tests are generally accepted in the NMR community and by instrument manufacturers since they provide a quantitative measure of instrument performance and, in theory at least, also provides a comparison between the capabilities of different spectrometers. Naturally, this leads to aggressive claims by instrument vendors regarding their results, and some caution is required when comparing advertised figures. This demands attention to the details of procedures used, for example, whether a lineshape test was performed with or without sample rotation or how wide a region of the spectrum was used for estimating the noise level in a sensitivity test. When utilising these tests for in-house checks, consistency in your methodology is the important factor if the results are to be compared with previous measurements. Ideally, these tests should be performed at regular intervals, say every six months or once a year, and appropriate records kept. Additionally more frequent updating of the 'master' shim settings may also be required for optimal performance, although this tends to be very dependent on instrument stability (as well as that of the surrounding environment). One should also be aware that, perhaps not surprisingly, the installation test results achievable on a modern state-of-the-art instrument will certainly be substantially better than the initial test data of an instrument installed some years ago (see, e.g., the discussions on probes in Section 3.4.2 and see Fig. 3.46). The idea of what is an acceptable test result for an older instrument should therefore be scaled accordingly, and reference to previous records become essential if one wishes to gauge instrument performance.

3.6.1. Lineshape and resolution

The most important test, in may be argued, is that for the NMR resonance lineshape, since only when this is optimised can other quantities also be optimum. The test measures the properties of a suitable singlet resonance that, in a perfectly homogeneous field and in the absence of other distortions, produces a precise Lorentzian lineshape, or in other words, its FID possesses an exact exponential decay. Naturally, the only way to achieve this is through careful optimisation of the magnetic field via shimming, which can take many hours to achieve good test results, depending on the initial state of the system. Nowadays, both the lineshape and the resolution measurements are often taken from a single set of test data. The *lineshape* is defined by the width of the resonance at 0.55 and 0.11% of the peak height (these numbers having evolved from measurement of proton linewidths at the height of the ^{13}C satellites and at one-fifth thereof). The *resolution* is defined by the half-height linewidth, $\Delta\nu_{1/2}$ of the resonance. In fact, by definition, this cannot be a true measure of resolution (how can one measure resolving power with only a single line?), rather the ability of the instrument to separate neighbouring lines is implied by the narrowness of the singlet. For a genuine Lorentzian line, the widths at 0.55 and 0.11% should be 13.5 and 30 times that at half-height respectively, although a check for this is often never performed as attempts are often made (erroneously) to simply minimise all measurements. The tests may be performed on both spinning and static samples (be sure you know which if comparing results), the first being of relevance principally for highest resolution proton measurements whereas the second indicates suitability for non-spinning 1D and 2D experiments.

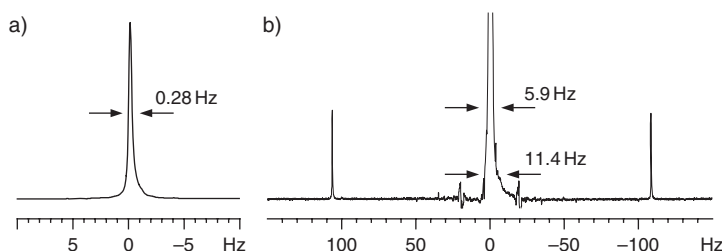
The proton lineshape test uses chloroform in deuterioacetone typically at concentrations of 3% at or below 400 MHz, and 1% at or above 500 MHz. Older instruments and/or probes of lower sensitivity or observations via outer 'decoupler coils' may require 10% at 200 MHz and 3% at 500 MHz to prevent noise interfering with measurements close to the baseline. A single scan is collected and the data recorded under conditions of high digital resolution (acquisition time of 16 s ensuring the FID has decayed to zero) and processed without window functions. Do not be tempted to make measurements at the height of the satellites themselves unless these are confirmed by measurement to be 0.55%. Since these arise from protons bound to ^{13}C , which relax faster than those of the parent line, they may be relatively enhanced if full equilibrium is not established after previous pulses. The test result for a 400 MHz instrument is shown in Fig. 3.79. The traditional test for proton resolution, which dates back to the continuous-wave (CW) era (*o*-dichlorobenzene in deuterioacetone), is now no longer employed and has become part of NMR history.

Lineshape and resolution tests on other nuclei follow a similar procedure to that above. Not all nuclei available with a given probe need be tested, and typically, only tests for 'inner' and 'outer' coil observations on multinuclear probes are required. This means the second test will often involve carbon-13 for which two samples are in widespread use; the ASTM (American Society for Testing and Materials) test sample (40% *p*-dioxane in deuterobenzene, also used for the sensitivity test) or 80% benzene in deuterioacetone. In either case, on-resonance continuous-wave (CW) decoupling of protons should be used as this provides improved results for a single resonance relative to broadband decoupling. Rather long (30–40 s) acquisition times will be required for a well-shimmed system.

3.6.2. Sensitivity

A great disadvantage of NMR spectroscopy relative to many other analytical techniques is the intrinsically low sensitivity from which it suffers. This, of course, is greatly outweighed by its numerous benefits, yet is still one of the more likely causes of experiment 'failure' and so deserves serious attention; instrumental approaches to enhancing NMR sensitivity are described in Section 3.4.2. The term 'sensitivity' strictly defines a minimum amount of material that is detectable under defined conditions and so represents the limit of

Figure 3.79. Proton resolution and lineshape tests for a static 3% CHCl_3 sample recorded on a 400 MHz spectrometer equipped with a dual $^1\text{H}(^{13}\text{C})$ inverse probe head. The ^{13}C satellites are clearly seen in (b) at $\pm 109\text{ Hz}$, whereas the artefacts at $\pm 20\text{ Hz}$ arise from floor vibrations.



detection for a measurement, but is used rather loosely throughout NMR and often interchangeably with ‘signal-to-noise ratio’. The instrument sensitivity test is indeed a signal-to-noise measurement in which the peak height of the analyte is compared with the noise level in the spectrum. For maximum peak height, optimum lineshape is essential, and generally, the lineshape and resolution test should be performed first and the shim settings re-optimised on the sensitivity sample (for similar reasons, sensitivity tests are traditionally performed with sample spinning). Likewise, the probe must be tuned for the test sample and the 90° pulse determined accurately.

The definition of noise intensity is of prime importance for this measurement since different approaches may lead to differing test results. This has particular significance nowadays as the older method of ‘manual’ noise estimation has been superseded by automated computational routines provided by instrument vendors. The signal-to-noise measurement compares peak intensity, P , to twice the root mean square (rms) noise level, N_{rms} , in the spectrum. The rms noise is nowadays computed over a defined bandwidth for a region of the spectrum devoid of resonances with a 200 Hz window now most commonly employed. The traditional use of a 2 ppm bandwidth has fallen out of favour with instrument vendors and, perhaps not surprisingly, the narrower bandwidth usually provides a greater signal-to-noise measurement (be sure you know which method is used if comparing results). In cases when a computational determination of N_{rms} cannot be made, this may be estimated from the peak-to-peak noise of the baseline, N_{pp} , whereby N_{rms} is one-fifth N_{pp} as measured directly on screen or from paper plots. The signal-to-noise is then given by

$$\frac{S}{N} = \frac{2.5P}{N_{\text{pp}}} \quad (3.28)$$

The peak-to-peak measurement must include all noise bands within the defined frequency window but is somewhat susceptible to what one might call ‘operator optimism’ in the exclusion of ‘spikes’ or ‘glitches’. Computational methods for determining noise have the advantage of removing such operator bias, and it is consistency of approach that is important for performance monitoring.

The proton sensitivity test uses 0.1% ethylbenzene in deuteriochloroform. As for all sensitivity tests, a single-scan spectrum is recorded following a 90° pulse on a fully equilibrated sample (relaxation delay of 60 s in this case). The spectrum is processed with matched filtration corresponding to a line broadening of 1 Hz for the methylene group on which the signal intensity is measured (this resonance is broadened slightly by unresolved long-range couplings to the aromatic protons). Results for a 400 MHz instrument are presented in Fig. 3.80 and show a signal-to-noise ratio of 575:1 or 622:1 with a 2 ppm and a 200 Hz noise bandwidth respectively.

The carbon sensitivity test comes in two guises, without and with proton decoupling. These give rise to quite different results, so it is again important to be aware of which approach has been used. Whilst the first provides an indication of absolute instrument sensitivity, the second represents a more realistic test of overall performance since it also takes into account the efficiency of proton decoupling and is thus more akin to how one performs genuine experiments. Both approaches acquire carbon spectra under atypical conditions of high digital resolution to correctly define peak shapes, demanding acquisition times of around 4 s. The proton-coupled version makes use of the ASTM sample again (40% *p*-dioxane in deuterobenzene) but this time measures peak height of the deuterobenzene triplet. A line broadening of 3.5 Hz is applied and the noise measured over the 80 to 120 ppm window. The proton-decoupled version uses 10% ethyl benzene in deuteriochloroform and employs broadband composite pulse decoupling usually via the

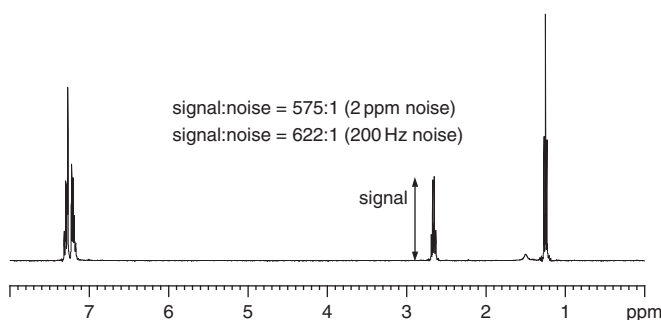


Figure 3.80. The proton sensitivity test for a 0.1% ethyl benzene sample recorded on a 400 MHz spectrometer equipped with a dual $^1\text{H}/^{13}\text{C}$ inverse probe head.

Table 3.8. Standard sensitivity test samples of some common nuclei

Nucleus	Sensitivity test sample	Notes
^1H	0.1% Ethylbenzene in CDCl_3	
^{13}C	10% Ethylbenzene in CDCl_3	Use broadband proton decoupling
^{13}C	40% dioxane in C_6D_6 (ASTM sample)	No decoupling, C_6D_6 used for measurement
^{31}P	0.0485 M triphenylphosphate in d_6 -acetone	No decoupling
^{19}F	0.05% trifluorotoluene in CDCl_3	No decoupling
^{15}N	90% formamide in d_6 -dimethylsulfoxide	Use inverse gated decoupling to suppress negative NOE
^{29}Si	85% hexamethyldisiloxane in d_6 -benzene	No decoupling

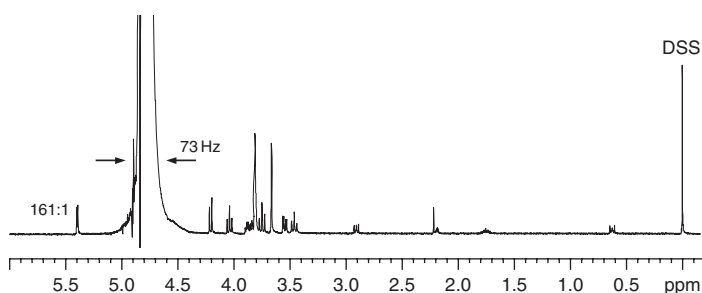
WALTZ-16 sequence (Chapter 10). A 0.3 Hz line broadening is used and the noise recorded over the same region, with the peak height determined for the tallest aromatic resonance. Tuning of the proton coil and appropriate calibration of the proton decoupling pulses are required in this case for optimum results. Test samples for other common nuclei are summarised in Table 3.8. Should you have frequent interests in other nuclei, a suitable standard should be decided upon and used for future measurements.

3.6.3. Solvent presaturation

Interest in biologically and medicinally important materials frequently demands NMR analysis to be undertaken on samples in 90% H_2O if solvent-exchangeable protons are also to be observed. The various tests presented so far have all made use of organic solvents; yet, the dielectric properties of these and of water are substantially different and a probe that performs well for, say, chloroform may not be optimum for an ionic aqueous solution, particularly with regard to sensitivity. Accurate tuning of the probe and pulse width calibration play an important role here, but protonated aqueous solutions also demand effective suppression of the solvent resonance if the analyte is to be observed (see Chapter 10 for discussions on suppression methods). The solvent suppression test makes use of solvent presaturation and can be used to measure a number of performance characteristics including the suppression capability, resolution and sensitivity. Good suppression performance places high demands on both static (B_0) and rf (B_1) field homogeneity. A narrow lineshape down to the baseline, and hence good B_0 homogeneity, is again a pre-requisite for good results and ensures all solvent nuclei resonate within a small frequency window. Good B_1 homogeneity means solvent nuclei in all regions of the sample experience similar rf power and are thus suppressed to the same degree. Much of the residual solvent signal that is observed following presaturation arises from peripheral regions of the sample that experience a reduced B_1 field.

The test sample is 2 mM sucrose with 0.5 mM sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) in 90 H_2O /10% D_2O , plus a trace of sodium azide to suppress bacterial growth. In the absence of suppression, only the solvent resonance is observed. The test involves on-resonance presaturation of the solvent for a 2 s period, followed by acquisition with a 90° pulse. The presaturation power is selected to attenuate the resonance significantly ($\gamma B_1 \approx 25$ Hz) and this setting used in future comparative tests. Naturally, higher powers will produce greater suppression, but these should not be so high as to reduce the neighbouring sucrose resonances. Typically 8 transients are collected following two “dummy” scans, which achieve a steady-state. The suppression performance is judged by measuring the linewidth of the residual solvent signal at 50 and 10% of the height of the DSS resonance. With probes of recent design that have appropriate screening of the rf coil leads, the 50% linewidth should be somewhat less than 100 Hz and reasonably symmetrical. Much older probes may show poorer performance and may be plagued by wide unsymmetrical humps (despite careful attention to

Figure 3.81. The proton solvent presaturation test. The data were acquired on a 400 MHz spectrometer equipped with a dual $^1\text{H}/^{13}\text{C}$ inverse probe, and the sample was 2 mM sucrose and 0.5 mM DSS in 90% H_2O /10% D_2O .



shim optimisation), which result from signal pick-up in unscreened coil leads [78]. Resolution is judged by the splitting of the anomeric proton doublet at around 5.4 ppm, which should be resolved at least down to 40% of the anomeric peak height. Sensitivity may also be measured from this resonance with the noise determined for the 5.5–7.0 ppm region using either of the methods described above (baseline correction may be required). Test results for a 400 MHz $^1\text{H}(^{13}\text{C})$ probe using a moderate presaturation power are shown in Fig. 3.81; further reductions in the water resonance could be achieved through the use of greater rf power.

REFERENCES

- [1] J.N. Shoolery, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1995, **28**, 37–52.
- [2] E. Bartholdi and R.R. Ernst, *J. Magn. Reson.*, 1973, **11**, 9–19.
- [3] M.B. Comisarow, *J. Magn. Reson.*, 1984, **58**, 209–218.
- [4] J.J. Led and H. Gesmar, *Chem. Rev.*, 1991, **91**, 1413–1426.
- [5] J.C. Hoch and A.S. Stern, *NMR Data Processing*, Wiley-Liss, New York, 1996.
- [6] W.F. Reynolds, M. Yu, R.G. Enriquez and I. Leon, *Magn. Reson. Chem.*, 1997, **35**, 505–519.
- [7] C.J. Turner and H.D.W. Hill, *J. Magn. Reson.*, 1986, **66**, 410–421.
- [8] D.I. Hoult and R.E. Richards, *Proc. Roy. Soc. (Lond.)*, 1975, **A344**, 311–340.
- [9] M.A. Delsuc and J.Y. Lallemand, *J. Magn. Reson.*, 1986, **69**, 504–507.
- [10] E. Kupce, J. Boyd and I. Campbell, *J. Magn. Reson. (A)*, 1994, **109**, 260–262.
- [11] M.E. Rosen, *J. Magn. Reson. (A)*, 1994, **107**, 119–125.
- [12] R.R. Ernst, *Adv. Mag. Reson.*, 1966, **2**, 1–135.
- [13] A.G. Ferrige and J.C. Lindon, *J. Magn. Reson.*, 1978, **31**, 337–340.
- [14] G.A. Pearson, *J. Magn. Reson.*, 1987, **74**, 541–545.
- [15] D.D. Traficante and G.A. Nemeth, *J. Magn. Reson.*, 1987, **71**, 237–245.
- [16] C.R. Pacheco and D.D. Traficante, *J. Magn. Reson. (A)*, 1996, **120**, 116–120.
- [17] J.N.S. Evans, *Biomolecular NMR Spectroscopy*, Oxford University Press, Oxford, 1995.
- [18] J. Cavanagh, W.J. Fairbrother, A.G. Palmer, N.J. Skelton and M. Rance, *Protein NMR Spectroscopy: Principles and Practice*, 2nd ed., Academic Press (Elsevier), San Diego, 2006.
- [19] R.K. Harris, E.D. Becker, S.M. Cabral de Menezes, R. Goodfellow and P. Granger, *Pure Appl. Chem.*, 2001, **73**, 1795–1818.
- [20] R.K. Harris and E.D. Becker, *J. Magn. Reson.*, 2002, **156**, 323–326.
- [21] R.W. Dykstra, *J. Magn. Reson. (A)*, 1995, **112**, 255–257.
- [22] R.J. Lewis, M.A. Bernstein, S.J. Duncan and C.J. Sleigh, *Magn. Reson. Chem.*, 2005, **43**, 783–789.
- [23] V. Exarchou, M. Krucke, T.A. van Beek, J. Vervoort, I.P. Gerothanassis and K. Albert, *Magn. Reson. Chem.*, 2005, **43**, 681–687.
- [24] K. Albert, *On-line LC-NMR and Related Techniques*, Wiley, Chichester, 2002.
- [25] M.D. Grynbaum, D. Kreidler, J. Rehbein, A. Pura, P. Schuler, W. Schaal, H. Czesla, A. Webb, V. Schurig and K. Albert, *Anal. Chem.*, 2007, **79**, 2708–2713.
- [26] D.I. Hoult and R.E. Richards, *J. Magn. Reson.*, 1976, **24**, 71–85.
- [27] J.N. Shoolery, In: *Topics in Carbon-13 NMR Spectroscopy*, Vol. 3 (Ed.: G.C. Levy), Wiley Interscience, New York, 1979, pp. 28–38.
- [28] R.C. Crouch and G.E. Martin, *Magn. Reson. Chem.*, 1992, **30**, S66–S70.
- [29] G.E. Martin and C.E. Hadden, *Magn. Reson. Chem.*, 1999, **37**, 721–729.
- [30] G.E. Martin, R.C. Crouch and A.P. Zens, *Magn. Reson. Chem.*, 1998, **36**, 551–557.
- [31] G. Schlotterbeck, A. Ross, R. Hochstrasser, H. Senn, T. Kuhn, D. Marek and O. Schett, *Anal. Chem.*, 2002, **74**, 4464–4471.
- [32] P.A. Keifer, L. Baltusis, D.M. Rice, A.A. Tymiak and J.N. Shoolery, *J. Magn. Reson. (A)*, 1996, **119**, 65–75.
- [33] D.L. Olson, T.L. Peck, A.G. Webb, R.L. Magin and J.V. Sweedler, *Science*, 1995, **270**, 1967–1970.
- [34] D.L. Olson, J.A. Norcross, M. O'Neil-Johnson, P.F. Molitor, D.J. Detlefsen, A.G. Wilson and T.L. Peck, *Anal. Chem.*, 2004, **76**, 2966–2974.
- [35] A.G. Webb, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1997, **31**, 1–42.
- [36] M. Gronquist, J. Meinwald, T. Eisner and F.C. Schroeder, *J. Am. Chem. Soc.*, 2005, **127**, 10810–10811.
- [37] F.C. Schroeder and M. Gronquist, *Angew. Chem. Int. Ed.*, 2006, **45**, 7122–7131.
- [38] H. Kovacs, D. Moskau and M. Spraul, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2005, **46**, 131–155.
- [39] G.E. Martin, *Ann. Rep. NMR. Spectrosc.*, 2005, **56**, 1–96.
- [40] P. Styles, N.F. Soffe and C.A. Scott, *J. Magn. Reson.*, 1989, **84**, 376–378.
- [41] P. Styles, N.F. Soffe, C.A. Scott, D.A. Cragg, F. Row, D.J. White and P.C.J. White, *J. Magn. Reson.*, 1984, **60**, 397–404.
- [42] A.E. Kelly, H.D. Ou, R. Withers and V. Dotsch, *J. Am. Chem. Soc.*, 2002, **124**, 12013–12019.
- [43] T.M. de Swiet, *J. Magn. Reson.*, 2005, **174**, 331–334.
- [44] N. Bloembergen and R.V. Pound, *Phys. Rev.*, 1954, **95**, 8–12.
- [45] W.W. Conover, In: *Topics in Carbon-13 NMR Spectroscopy*, Vol.4 (Ed.: G.C. Levy), Wiley, New York, 1983, pp. 37–51.
- [46] G.N. Chmurny and D.I. Hoult, *Concept. Magn. Reson.*, 1990, **2**, 131–159.
- [47] P.C.M. Vanzijl, S. Sukumar, M.O. Johnson, P. Webb and R.E. Hurd, *J. Magn. Reson. (A)*, 1994, **111**, 203–207.

- [48] M. Weiger, T. Speck and M. Fey, *J. Magn. Reson.*, 2006, **182**, 38–48.
- [49] H. Barjat, P.B. Chilvers, B.K. Fetler, T.J. Horne and G.A. Morris, *J. Magn. Reson.*, 1997, **125**, 197–201.
- [50] S. Sukumar, M.O. Johnson, R.E. Hurd and P.C.M. van Zijl, *J. Magn. Reson.*, 1997, **125**, 159–162.
- [51] G.A. Morris, *J. Magn. Reson.*, 1988, **80**, 547–552.
- [52] G.A. Morris and D. Cowburn, *Magn. Reson. Chem.*, 1989, **27**, 1085–1089.
- [53] G.A. Morris, H. Barjat and T.J. Horne, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1997, **31**, 197–257.
- [54] K.R. Metz, M.M. Lam and A.G. Webb, *Concept. Magn. Reson.*, 2000, **12**, 21–42.
- [55] H. Barjat, G.A. Morris, A.G. Swanson, S. Smart and S.C.R. Williams, *J. Magn. Reson. (A)*, 1995, **116**, 206–214.
- [56] T.J. Horne and G.A. Morris, *J. Magn. Reson. (A)*, 1996, **123**, 246–252.
- [57] A. Gibbs, G.A. Morris, A.G. Swanson and D. Cowburn, *J. Magn. Reson. (A)*, 1993, **101**, 351–356.
- [58] H. Barjat, G.A. Morris, S. Smart, A.G. Swanson and S.C.R. Williams, *J. Magn. Reson. (B)*, 1995, **108**, 170–172.
- [59] P.S.C. Wu and G. Otting, *J. Magn. Reson.*, 2005, **176**, 115–119.
- [60] J.R. Wesener and H. Günther, *J. Magn. Reson.*, 1985, **62**, 158–162.
- [61] E. Haupt, *J. Magn. Reson.*, 1982, **49**, 358–364.
- [62] D.M. Thomas, M.R. Bendall, D.T. Pegg, D.M. Doddrell and J. Field, *J. Magn. Reson.*, 1981, **42**, 298–306.
- [63] A. Bax, *J. Magn. Reson.*, 1983, **52**, 76–80.
- [64] A.J. Benie and O.W. Sorensen, *J. Magn. Reson.*, 2006, **182**, 348–352.
- [65] E. Kupce and B. Wrackmeyer, *J. Magn. Reson.*, 1991, **94**, 170–173.
- [66] A.J. Benie and O.W. Sorensen, *J. Magn. Reson.*, 2006, **180**, 317–320.
- [67] F. Bloch and A. Siegert, *Phys. Rev.*, 1940, **57**, 522–527.
- [68] S.D. Simova, *J. Magn. Reson.*, 1985, **63**, 583–586.
- [69] W.S. Price, *Annu. Rep. NMR. Spectrosc.*, 1996, **32**, 51–142.
- [70] D.G. Cory, F.H. Laukien and W.E. Maas, *J. Magn. Reson. (A)*, 1993, **105**, 223–229.
- [71] W.E. Maas, F. Laukien and D.G. Cory, *J. Magn. Reson. (A)*, 1993, **103**, 115–117.
- [72] N.M. Loening and J. Keeler, *J. Magn. Reson.*, 2002, **159**, 55–61.
- [73] M.L. Martin, J.-J. Delpuech and G.J. Martin, *Martin, Practical NMR Spectroscopy*, Heydon, London, 1980.
- [74] W.H. Sikorski, A.W. Sanders and H.J. Reich, *Magn. Reson. Chem.*, 1998, **36**, S118–S124.
- [75] A.L. van Geet, *Anal. Chem.*, 1970, **42**, 679–680.
- [76] D.S. Raidford, C.L. Fisk and E.D. Becker, *Anal. Chem.*, 1979, **51**, 2050–2051.
- [77] M. Findeisen, T. Brand and S. Berger, *Magn. Reson. Chem.*, 2007, **45**, 175–178.
- [78] R.W. Dykstra, *J. Magn. Reson.*, 1987, **72**, 162–167.

Chapter 4

One-dimensional techniques

The approach to any structural or mechanistic problem will invariably start with the acquisition of 1D spectra. Since these provide the foundations for further work, it is important that these be executed correctly and full use be made of the data they provide before more extensive and potentially time-consuming experiments are undertaken. This chapter describes the most widely used 1D techniques in the chemistry laboratory, beginning with the simple single-pulse experiment and progressing to consider the various multipulse methods that enhance the information content of our spectra. The key characteristics of these are summarised briefly in Table 4.1. This chapter does not cover the wide selection of techniques that are strictly 1D analogues of 2D experiments, as these are more appropriately described in association with the parent experiment and are found throughout the following chapters.

4.1. THE SINGLE-PULSE EXPERIMENT

Chapter 3 described procedures for the optimum collection of an FID, and how these were dictated by the relaxation behaviour of the nuclei and by the digital resolution required in the resulting spectrum, which in turn defines the data acquisition time. When setting up an experiment, one also needs to consider the optimum pulse excitation angle that should be used and how rapidly pulses can be applied to the sample for signal averaging (Fig. 4.1). These parameters also depend on the spin relaxation times. There are two extreme cases to be considered for the single-pulse experiment, in particular whether one is striving for optimum sensitivity for a given period of data accumulation or whether one requires accurate quantitative data from the sample. The experimental conditions for meeting these criteria can be quite different, so each shall be considered separately.

4.1.1. Optimising sensitivity

If one were to apply a 90° pulse to a spin system at thermal equilibrium, it is clear that the maximum possible signal intensity would result since all magnetisation is placed in the transverse plane. This may therefore appear to be the optimum pulse tip angle for maximising sensitivity. However, one is usually interested in performing signal averaging, so before applying subsequent pulses it becomes necessary to wait many times T_1 for the system to relax and recover the full signal once again; a period of $5T_1$ leads to 99.3% recovery of longitudinal magnetisation (see Fig. 2.20), complete for all practical purposes. Such slow repetition is in fact not the most efficient way of signal averaging and it turns out

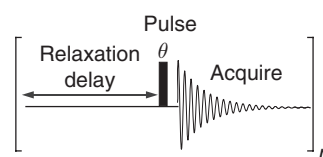
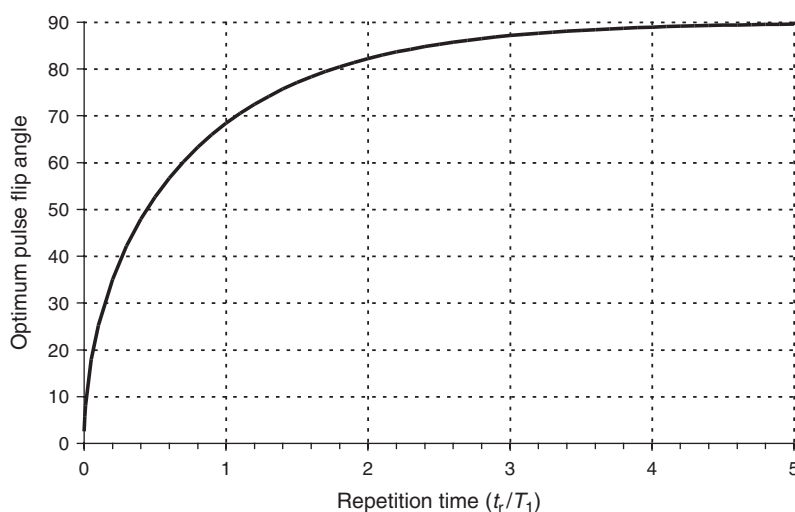


Figure 4.1. The essential elements of the single-pulse NMR experiment: the relaxation (recovery) delay, the pulse excitation angle and the data acquisition time.

Table 4.1. The principal applications of the multipulse techniques described in this chapter

Technique	Principal applications
<i>J</i> -modulated spin-echo or APT	Editing of heteronucleide spectra (notably carbon-13) according to resonance multiplicity.
INEPT	Enhancement of a low- γ nuclide through polarisation transfer from a high- γ nuclide, e.g. ^1H , ^{31}P , ^{19}F . Editing of spectra according to resonance multiplicity.
DEPT	Enhancement of a low- γ nuclide through polarisation transfer, with editing according to resonance multiplicity. Experimentally more robust for routine carbon-13 spectra than INEPT. No responses observed for quaternary carbons.
DEPTQ	As for DEPT but with retention of quaternary carbons.
PENDANT	Enhancement of a low- γ nuclide through polarisation transfer from a high- γ nuclide, e.g. ^1H , ^{31}P , ^{19}F . Editing of spectra according to resonance multiplicity with retention of quaternary carbons.
RIDE or ACOUSTIC	Observation of low-frequency quadrupolar nuclei with very broad resonances. Sequence suppresses 'acoustic ringing' responses from probehead.
ERETIC	Quantification of solute concentration relative to an external rf reference

Figure 4.2. The Ernst angle for optimum sensitivity when signal averaging. The pulse tip angle is dictated by the pulse repetition time, t_r , relative to the longitudinal relaxation time, T_1 .



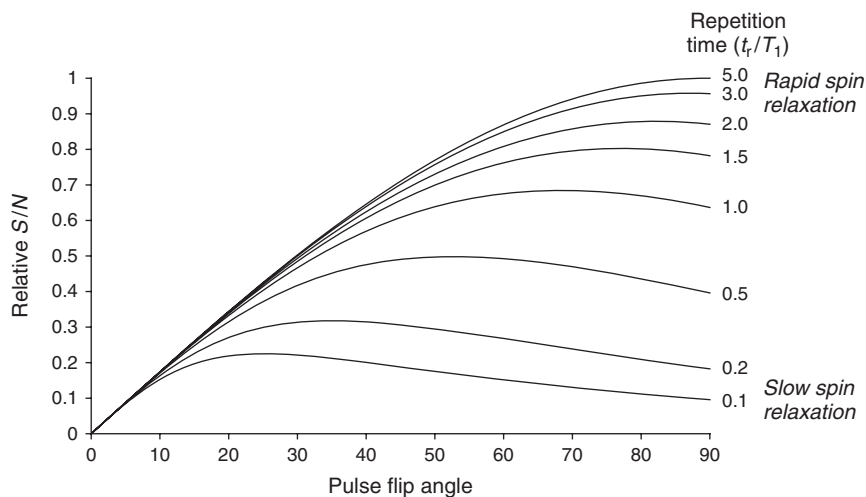
to be better to do away with the recovery delay altogether and to adjust pulse conditions to maximise the *steady-state* z -magnetisation produced.

Under conditions where there is complete decay of transverse magnetisation between scans (i.e. the FID decays to zero) the optimum tip angle for a pulse repetition time t_r , known as the *Ernst angle*, α_e , is given by [1, 2]:

$$\cos \alpha_e = e^{-t_r/T_1} \quad (4.1)$$

and is illustrated in Fig. 4.2. As the repetition time decreases relative to the spin relaxation rate, that is when faster pulsing is used, smaller tip angles produce the optimum signal-to-noise ratio. Proton spectra are typically acquired with sufficient digital resolution to reveal multiplet fine structure, and acquisition times tend to be of the order of $3T_2^*$, which is sufficient to enable the almost complete decay of transverse magnetisation between scans ($3T_2^*$ corresponds to 95% decay). Furthermore, since for small to medium-sized molecules $T_2 = T_1$ and for proton observation in a well-shimmed magnet $T_2^* \approx T_2$, we have $t_r \approx 3T_1$, and thus, from Fig. 4.2, the pulse angle for *maximising sensitivity* will typically be $>80^\circ$. However, there will exist a range of T_1 values for the protons in a molecule and it will not be possible to use optimum conditions for all. In such cases, a large pulse angle is likely to lead to significant intensity differences for protons with widely differing relaxation times, with the slower relaxing spins displaying reduced signal intensity [3]. This is exemplified in the signal-to-noise plots shown in Fig. 4.3. When proton integrals are required to indicate the number of nuclei giving rise to each resonance, as for routine proton acquisitions, it is wise to optimise the pulse tip angle for the longer T_1 values and thus use shorter pulses (see the following section for details of *accurate* quantitative measurements). Assuming the longest T_1 to be around 4 s and an acquisition time of 3 s, the optimum tip angle will be around 60° , although the plots of Fig. 4.3 clearly possess rather broad maxima, meaning the precise setting of the pulse tip angle is not critical. Pulsing too rapidly, that is, using very short

Figure 4.3. The dependence of signal-to-noise ratio on pulse tip angle for different pulse repetition rates. The maximum of each curve corresponds to the Ernst angle. When a molecule contains nuclei with very different relaxation times (T_1 s), pulsing with a large flip angle produces a large difference in their signal intensities and makes even semi-quantitative measurements unreliable. Optimising the tip angle for the slowest relaxing spins reduces these intensity differences. The plots have been generated from the equations given in [3].



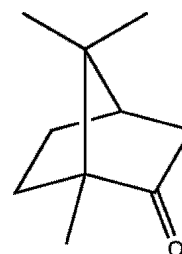
repetition times relative to T_1 , leads to a substantial decrease in signal-to-noise until in the extreme case magnetisation has no time to recover between pulses and so no signal can be observed. This condition is known as *saturation* and causes resonances to be lost completely, which can be a nuisance or a bonus depending on your point of view. If you are interested in observing the signal then clearly this must be avoided, whereas if the signal is unwanted, for example a large solvent resonance, then this is to be encouraged (Chapter 10).

Conditions for the acquisition of heteronuclear spectra are usually rather different to those for proton observations. Often one does not require the spectra to be well digitised when there is little or no fine structure to be observed; therefore acquisition times are kept comparable to T_2^* , which is often less than T_2 . Under such conditions, transverse magnetisation does not decay completely between scans and such rapid pulsing gives rise to steady-state spin-echoes [2, 4, 5] where transverse magnetisation remaining from one pulse is refocused by subsequent pulses. These echoes can give rise to phase and intensity distortions in resulting spectra, which are a function of resonance offset. The use of rapid pulsing, where repetition times are considerably shorter than longitudinal relaxation times (as in the case of routine carbon-13 observations), further contributes to distortions of the relative intensities because of partial saturation effects. This is the principal reason for quaternary carbon centres (which relax rather slowly owing to the lack of attached protons) appearing with often characteristically weak intensities. In extreme cases these can become fully saturated and effectively lost altogether if pulsing is too rapid and/or if the pulse tip angle is too large. Thus, routine carbon spectra are typically acquired with a somewhat reduced pulse angle ($<45^\circ$) and with a relaxation delay of a second or so. Similar considerations apply to other heteronuclei. For routine observations there is always some compromise needed between optimum sensitivity and the undesirable intensity anomalies that arise for nuclei with widely differing relaxation times. If problematic, one approach towards reducing these anomalies is the addition of paramagnetic relaxation agents such as chromium(III) acetylacetonate, $\text{Cr}(\text{acac})_3$ with the aim of reducing and, in part, equalising longitudinal relaxation times to allow faster signal averaging (Fig. 4.4; around 10–100 mM relaxation agent suffices and the solution should take on a slight pink–purple hue).

Finally, we consider the situation where one is compelled by the pulse sequence to use 90° pulses in which case the above arguments no longer apply. This is in fact the case for most multipulse and multidimensional sequences. Under such conditions a compromise is required between acquiring data rapidly and the unwelcome effects of saturation, and it has been shown [6] that for *steady-state* magnetisation, the sensitivity is maximised by setting the repetition rate equal to $1.3T_1$. This leads to a signal-to-noise ratio that is approximately 1.4 times greater than that obtained using a recycle time of $5T_1$ for a given period of data collection (Fig. 4.5). Thus, for most sequences, a repetition time of $1.3T_1$ is the target.

4.1.2. Quantitative measurements and integration

The extensive use of integration in proton NMR arises from the well-used doctrine that the area of an NMR resonance is proportional to the relative number of nuclei giving rise to it. In fact this is strictly true only under well-defined experimental conditions, and in routine proton spectra integrals may only be accurate to within 10–20% or so. Whilst this level of accuracy is usually sufficient for the estimation of the relative proton count within a molecule, it is clearly inadequate for quantitative measurements where accuracy to within



4.1

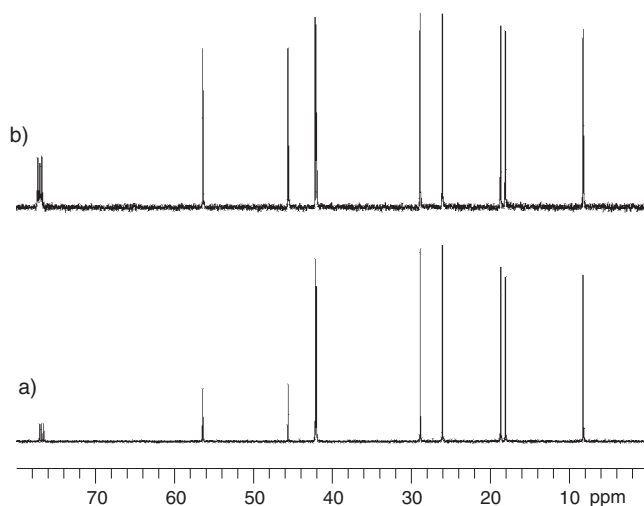
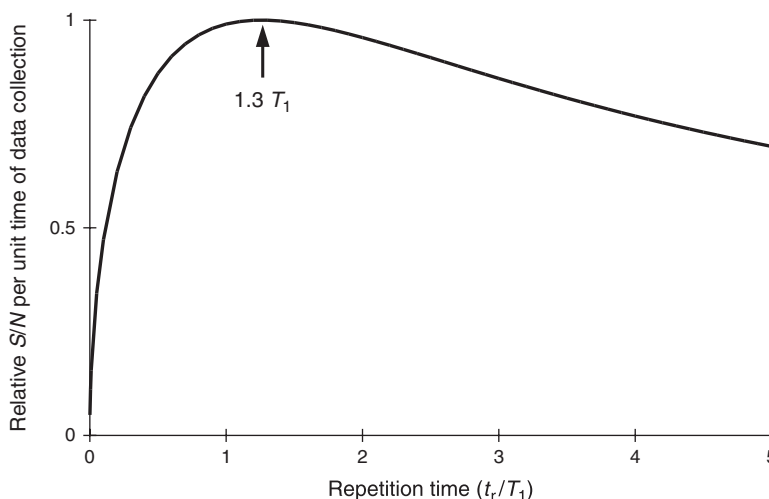


Figure 4.4. Carbon-13 spectra of camphor **4.1** acquired (a) without and (b) with the addition of $\text{Cr}(\text{acac})_3$ under otherwise identical standard observation conditions (1 s relaxation delay, 30° excitation pulse, 0.5 s acquisition time). In (a) the two quaternary resonances display reduced signal intensities as a result of partial saturation. In (b) this difference is largely eliminated by the addition of the relaxation agent, whilst the reduced signal intensity of the protonated resonances arises from the suppression of ^1H – ^{13}C NOE enhancements and the line-broadening caused by the relaxation agent.

Figure 4.5. Optimum pulse repetition time when using 90° pulses. The curve has been calculated from the equation in [3].



a few per cent is required. The fact that integrals are typically reported to many decimal places by NMR software can draw one into believing such figures are significant, thus inflating one's expectations of what can be derived from routine spectra. Conversely, it is widely taught in introductory NMR lectures that it is not possible to integrate carbon-13 spectra. Whilst this is indeed inappropriate for routine acquisitions, not just for carbon-13 but also for many heteronuclei, it is nevertheless quite possible to obtain meaningful integrals when the appropriate protocols are employed, as described below. In all cases it is important to be conscious of the fact that NMR measurements are always *relative* measures of signal intensities. There exists no NMR equivalent to the extinction coefficients of ultraviolet spectroscopy, and so, *absolute* sample quantities cannot be determined directly.

Data collection

There are three features of specific importance to quantitative measurements, aside from the obvious need for adequate signal-to-noise in the spectrum. These are the avoidance of differential saturation effects, the need to characterise the NMR resonance lineshape properly and the need to avoid differential NOE enhancements. As has been mentioned in the previous section, acquiring data whilst pulsing rapidly relative to spin relaxation times leads to perturbation of the relative signal intensities in the spectrum so to avoid this it is *essential* to wait for the spins to fully relax between pulses, demanding recycle times of least $5T_1$ of the *slowest relaxing nuclei*. This allows the use of 90° observation pulses, providing the maximum possible signal per transient. Clearly, one requires some knowledge of the T_1 values for the sample of interest, which may be estimated by the inversion recovery methods described in Chapter 2 or estimates made from prior knowledge of similar compounds. Whilst recycle times of the order of $5T_1$ are usually bearable for proton work, they can be tediously long in the study of heteronuclei, which may demand many minutes between scans. Here relaxation reagents can be employed to reduce these periods to something more tolerable.

The second fundamental requirement is for the data to be sufficiently well digitised for the lineshape to be defined properly. To minimise intensity errors it is necessary to have *at least four* acquired data points covering the resonance linewidth, although many more than this are preferable; so it is beneficial to use the minimum spectral width compatible with the sample and to adjust the acquisition times accordingly. The spectral width should not be too narrow to ensure that the receiver filters do not interfere with resonance intensities at the edges of the spectrum.

A further source of intensity distortions in heteronuclear spectra recorded with broadband proton decoupling arises from the NOE produced by proton saturation (Chapter 8). Clearly, differential enhancements will prevent the collection of meaningful intensity data; so it is necessary to take measures to suppress the NOE, yet it is still desirable to collect proton-decoupled spectra for optimum signal-to-noise and minimal resonance overlap. The solution to this apparent dichotomy is to employ the inverse-gated decoupling scheme described in the following section. The lack of the NOE and the need to pulse at a slow rate means quantitative measurements can take substantially longer than the routine observation of the same sample. The addition of a relaxation agent will again speed things along by reducing recycle delays and will also aid suppression of the NOE as it will eliminate the dipolar relaxation responsible for this enhancement (see Fig. 4.4).

Data processing

Besides the necessary precautions to ensure the acquired data genuinely reflects the relative ratios within the sample, appropriate processing can further enhance results. The spectra of heteronuclei in particular benefit from the application of an exponential function that broadens the lines. In addition to improving the signal-to-noise ratio, this helps to ensure the data are sufficiently well digitised. For proton spectroscopy, achieving adequate sensitivity is not such a demanding problem, although the use of a matched exponential window will again help to ensure sufficient digitisation. The use of zero-filling will further assist with the definition of the lineshape and is highly recommended, although this *must not* be used as a substitute for correct digitisation of the acquired FID. Careful phasing of the spectrum is also essential. Deviations from pure absorption-mode lineshapes will reduce integrated intensities with contributions from the negative-going components; in the extreme case of a purely dispersive lineshape, the integrated intensity is zero. Another potential source of error arises from distortions of the spectrum baseline, which have their origins in the spectrometer receiver stages. These errors mean the regions of the spectrum that should have zero intensity, that is, those that are free from signals, have a non-zero value, and make a positive or negative contribution to measurements. NMR software packages incorporate suitable correction routines for this.

The final consideration when integrating is where the integral should start and finish. For a Lorentzian line, the tails extend a considerable distance from the centre and the integral should, ideally, cover 20 times the linewidth each side of the peak if it is to include 99% of it. For proton observation this is likely to be 10–20 Hz each side. In practice it may not be possible to extend the integral over such distances before various other signals are met. These may arise from experimental imperfections (such as spinning sidebands), satellites from coupling to other nuclei or from other resonances in the sample. Satellites can be particularly troublesome in some cases as they may constitute a large fraction of a total signal, owing to high natural abundance of the second nuclide, and one must decide on whether to include them or exclude them *for all measurements*. In some instances satellites can be used to one's advantage in quantitative measurements. One example is in the estimation of enantiomeric or diastereomeric excesses by proton NMR where the minor isomer is present at only a few per cent of the major. In such cases, the *ee* or *de* measurement demands the comparison of a very large integral versus a very small one, a situation prone to error. Comparison of similar size integrals can be made if one considers only the carbon-13 satellites of the major species (each present at 0.55%) and scales the calculation accordingly.

4.1.3. Quantification with ERETIC

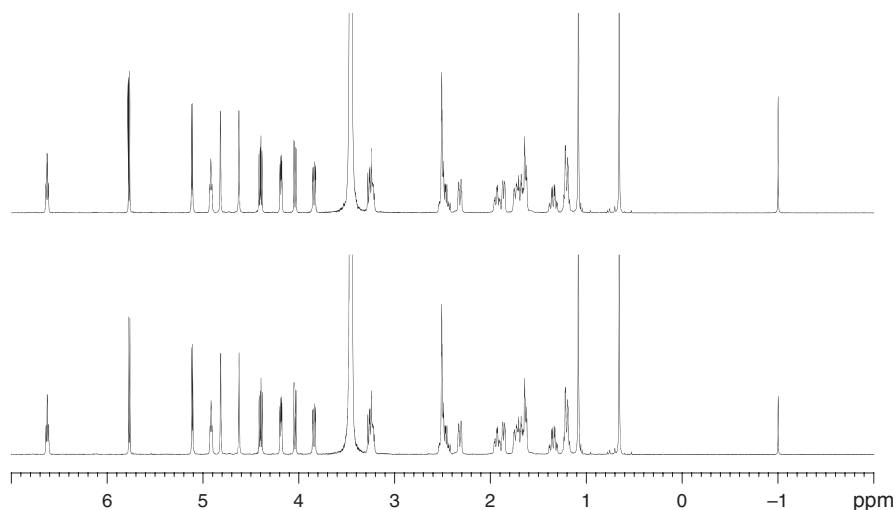
When seeking to determine the concentration of a solute by NMR spectroscopy, it is always necessary to measure it against that of a known calibrant. Typically, this would demand the addition of a suitable reference compound to the analyte solution against which the integrated intensity of a solute resonance may be compared following the protocols described above for quantitative measurements. However, the use of an internal chemical reference may impose constraints that may not be readily met, making this approach undesirable. The reference material needs to be soluble in the analyte solution, it must be inert to the solute, its resonance(s) must not overlap that of the solute and its relaxation properties should be similar to that of the solute. In addition to these practical considerations, one simply may not wish to add a reference 'contaminant' to a precious sample. An alternative approach to this use of an internal *chemical* reference is to introduce a *synthetic* rf reference signal as in the ERETIC method (Electronic REference To access In Vivo Concentrations) [7, 8].

The basic approach is to 'leak' a small amount of an rf signal into the NMR detector during the acquisition of the solute FID such that an additional resonance line appears in the final spectrum that arises from the synthesised rf signal. Since this is generated from the NMR spectrometer itself it is under the control of the operator, meaning the reference line can be placed in any area of the spectrum free from analyte peaks and its amplitude defined. The application of the method then involves two stages. The first is to *calibrate* the intensity of the synthetic signal itself against a chemical reference solution of known concentration. This will define the effective concentration of the ERETIC signal for the second stage of the process, the *quantification* of subsequent samples of unknown concentration. As an illustration, a spectrum with the addition of the ERETIC reference signal at -1.0 ppm is shown in Fig. 4.6.

Experimental implementation

The precise details for implementing the ERETIC method will be dependent upon the make and vintage of your instrument and here it is possible only to describe the general approach to this; your instrument manufacturer should be able to provide further guidance.

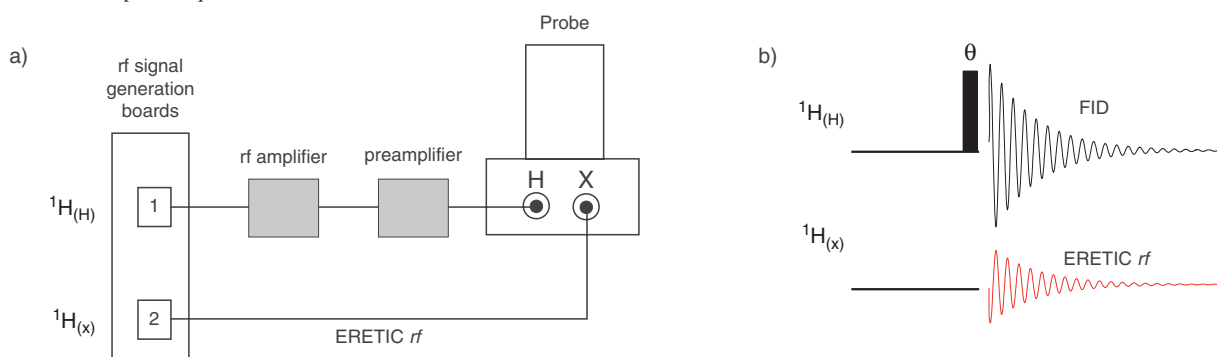
Figure 4.6. The use of the synthetic ERETIC reference signal for concentration measurement in ^1H NMR. The ERETIC reference signal appears at -1.0 ppm. The ERETIC intensity in the upper spectrum is precisely twice that in the lower due to a 6 dB increase in the ERETIC signal intensity.



The original approach to introduce the synthetic rf signal is to feed this in via a second rf coil of the probe in use; for the typical case of observing ^1H this would then employ the carbon or broadband (X) channel of the probe. This does not need to be tuned to ^1H since we only require a small degree of signal leakage between the coils (Fig. 4.7a). The rf signal does not need to be amplified since the detected signal intensity need only be comparable to the NMR response and as such it is typically taken directly from the second rf signal generation board to the probe, the first being used for the normal pulse-acquire process. To generate a Lorentzian lineshape for the reference signal in the final NMR spectrum, the synthetic signal must exhibit an exponential intensity decay during the data acquisition period (as would the true solute FID), which may be possible with the pulse-shaping tools of the spectrometer; that is, the ERETIC pulse can be applied as a single exponentially decaying decoupler pulse throughout the FID acquisition (Fig. 4.7b). The rate of signal decay will dictate the observed linewidth of the ERETIC reference signal and as such it is under the experimentalist's control. The final parameter that will need optimisation (through trial and error) is the phase of the synthetic rf signal fed to the probe, which should be adjusted, preferably under software control, so that the ERETIC reference line has an identical absorption phase to the solute signals after FT and phase correction of the NMR data (see Fig. 4.6).

For the method to be reproducible across different samples a number of factors need to remain constant throughout the measurements. The final intensity of the ERETIC signal is dictated by the coupling between the two rf coils, which is influenced by probe tuning and by the dielectric properties of the solvent. Thus, the optimum approach is to use the same solvent throughout, including that of the known reference compound used to calibrate the ERETIC signal itself, and to keep the probe tuning constant. If a broadband channel has been used to supply the ERETIC signal, this must be tuned to the same nucleus for all measurements and generally higher frequencies have been observed to give better results, meaning ^{31}P would be favoured if no other constraints apply. The ERETIC method has been demonstrated to have accuracy and precision that is at least as good as that provided by use of an internal reference [8, 9] and has more recently also been applied to 2D spectroscopy [10].

Figure 4.7. A schematic implementation of the ERETIC method: (a) the rf signal routing and (b) the ERETIC pulse sequence.



4.2. SPIN DECOUPLING METHODS

The use of spin–spin decoupling is no doubt familiar to most regular users of solution NMR spectroscopy, and it has constituted an important tool for the chemist since the early applications of NMR to problems of chemical structure [11]. It is now so widely used in the observation of heteronuclear spectra (when did you last see a fully proton-coupled carbon spectrum?) that one can almost become oblivious to the fact that it is an integral part of numerous pulse experiments. Nowadays, scalar spin–spin decoupling is most often applied with one of two goals in mind: either the *selective* decoupling of a single resonance in an attempt to identify its coupling partner(s) or non-selective (*broadband*) decoupling of one nuclide to enhance and simplify the spectrum of another. Decoupling is usually classified as being *homonuclear*, in which the decoupled and observed nuclides are the same, or *heteronuclear*, in which they differ. That which experiences the decoupling rf is conventionally distinguished by placing it in curly brackets so, for example, $^{13}\text{C}\{^1\text{H}\}$ would indicate carbon observation in the presence of proton decoupling, whilst $^1\text{H}\{^{13}\text{C},^{31}\text{P}\}$ would represent proton observation in the presence of simultaneous carbon and phosphorus decoupling (or more generally carbon and phosphorus pulsing). One may also find spin decoupling being referred to as one example of a ‘double-resonance’ experiment, a term originating from CW experiments in which the observe and decouple rf were applied simultaneously. Likewise, the H, C, P example above may be termed a ‘triple-resonance’ experiment as it requires three rf channels.

4.2.1. The basis of spin decoupling

A simplified [12] yet convenient description of how spin decoupling operates considers two spin- $\frac{1}{2}$ nuclei, A and X, that share a mutual scalar coupling of J Hz. The resonant frequency of the X spins will depend on whether their coupled partners are oriented parallel (α orientation) or anti-parallel (β orientation) to the applied static field. For a spin ensemble we can assume there exists an equal number of A nuclei in the α and β states, owing to the very small energy difference between the two orientations, and thus the X spectrum displays the familiar doublet pattern. Application of an rf field, designated the B_2 field (recall the transmitter field is termed B_1), at the frequency of the A spins causes these to undergo continuous, rapid transitions between the α and β orientations by continually inverting these spins. If this reorientation is fast relative to the coupling constant, the X spin doublet coalesces into a singlet since the lifetimes of the α and β orientations are no longer sufficient for the coupling to be distinguished. Thus, if the A spins are irradiated during data acquisition with a sufficiently strong field such that $\gamma B_2 > J$ Hz, the X resonance displays no coupling to A and the spins are said to be decoupled.

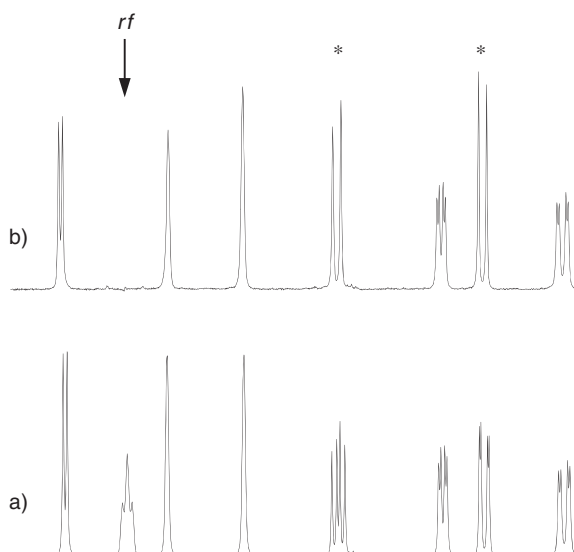
Whilst the removal of scalar spin–spin coupling is the usual goal of such experiments, a number of additional effects can arise from the application of the additional rf field, which may be beneficial or detrimental depending on the circumstances. Incomplete decoupling can introduce residual line broadening or, even worse, leave rather esoteric partially decoupled multiplets, whilst the non-uniform irradiation of a resonance can introduce population transfer effects that cause intensity distortions *within* multiplets (Section 4.4). Population disturbances caused by the rf may also produce intensity changes that arise from the NOE (Chapter 8), which operates quite independently from J -coupling. Finally, changes in the resonant positions of signals close in frequency to the applied rf may also be observed, so-called Bloch–Siegert shifts. In many cases it is possible to have some control over these factors, according to the experimental protocol used, as described in later sections.

4.2.2. Homonuclear decoupling

Homonuclear decoupling involves the selective application of a coherent decoupling field to a target resonance with the aim of identifying scalar spin-coupled partners, and is most often applied in proton spectroscopy (Fig. 4.8). Although the use of this method for identifying coupled ^1H – ^1H spins has been largely superseded by 2D correlation methods (Chapter 5), selective decoupling can still be a very useful and convenient tool in the NMR armoury. It is very simple to set up, providing rapid answers to relatively simple questions, and can be particularly useful in identifying spins that share very small couplings that do not always reveal themselves in 2D correlation experiments. In very crowded spectra where only a specific interaction is to be investigated, the affected resonances may not be obvious and the use of *difference spectroscopy* can aid interpretation [13, 14]. Here, a control spectrum recorded in the absence of decoupling¹ is subtracted from that collected in the

¹ For any form of difference spectroscopy it is better practice to collect the control FID with the decoupler frequency applied far away from all resonances rather than being turned off completely.

Figure 4.8. Homonuclear decoupling allows the rapid identification of coupled partners by removing couplings to the irradiated spin. (a) Control spectrum and (b) decoupled spectrum.



presence of on-resonance decoupling to reveal any changes that arise. In such cases a 2D correlation experiment may, however, be more appropriate.

One of the limitations to the use of selective decoupling lies in the need to irradiate only a single resonance to identify the coupling partners of the desired target. Any saturation spillover onto neighbouring resonances introduces a degree of ambiguity into the interpretation. Where the target multiplet is free from other resonances this poses little problem and the decoupler power can be set sufficiently high to ensure complete decoupling ($\gamma B_2 > J\text{Hz}$). When other resonances are close in frequency to the target, it may be necessary to reduce the decoupler power and thus reduce the frequency spread to avoid disturbing neighbouring resonances. The penalty for reducing the decoupler power may be incomplete decoupling of the target spins, although changes within the fine structure of other resonances should be sufficient to identify coupled partners.

Bloch–Siebert shifts

The application of an rf field during the acquisition of the FID may also move signals that resonate close to the decoupler frequency. This effect is known as the *Bloch–Siebert shift* [15, 16] and, more formally, it occurs when γB_2 becomes comparable to the shift difference in hertz between the decoupling frequency and the resonance. It arises because the decoupling field acts on the neighbouring spins such that they experience a modified effective field that is inversely dependent upon their resonance offsets from the decoupling frequency but proportional to $(\gamma B_2)^2$, which causes resonances to move *away* from this decoupling frequency (Fig. 4.9). The effect is principally limited to homonuclear decoupling experiments, where resonances may be very close to the decoupling frequency, but is of no concern in most pulse NMR experiments since the rf pulse is turned off prior to data collection. A notable exception is when solvent presaturation is applied during the evolution time (t_1) of proton homonuclear 2D experiments. This can lead to shifts of f_1 frequencies of resonances close to the solvent, but since this is not present in f_2 , it

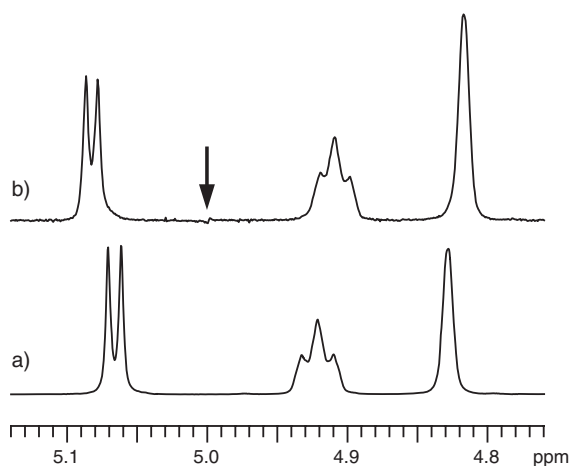


Figure 4.9. The Bloch–Siebert shift causes resonances near to an applied rf to move away from its point of application. (a) is the conventional spectrum and (b) is that required with a decoupling field applied at the position of the arrow.

introduces asymmetry in the shifts of crosspeaks associated with these resonances. In homonuclear decoupling experiments it is rarely a major problem since the requirement for selectivity limits the B_2 field and thus keeps the shifts small. However, such small shifts may still introduce subtraction artefacts into decoupling difference spectra since the reference will not contain Bloch–Siegert shifts, so one should be cautious not to interpret these as evidence of coupling. Caution is also required should one need to measure accurate chemical shifts from decoupled spectra. In Section 3.5.1 the Bloch–Siegert shift is used quantitatively as a means of calibrating decoupler powers.

Experimental implementation

The application of homonuclear irradiation whilst acquiring the FID poses some challenging instrumental problems. Whilst needing to detect the responses of the excited spins, one must not have the receiver open when the decoupler is on since this will simply swamp the NMR signal. The solution lies in the discrete sampling of the FID, and the application of homonuclear irradiation *only* when data points are *not* being sampled, that is, during the FID dwell time [17]. Spectrometers have purpose-built homonuclear decoupling modes to handle the necessary gating internally. The time in which the decoupler is gated on is thus only a small fraction of the total acquisition time, this so-called *duty cycle* typically being 20% or less. The low duty cycle means the effective mean decoupler power is somewhat less than the instantaneous B_2 field; hence rf powers are usually greater than those required for presaturation of a resonance where such receiver conflict does not arise. If the gating off of the decoupler is not perfect during data collection, a number of spectrum artefacts may be introduced [18, 19]. Most notable are a reduction in signal-to-noise ratio, owing to ‘leakage’ of the decoupler rf into the receiver, and a significant ‘spike’ occurring at the decoupler frequency, although this is really only a question of aesthetics. The leakage problem varies greatly, it seems, from one instrument to another and if a serious problem may be cured by instrumental modification [19]. The second may be eliminated simply by setting the *transmitter* frequency to match that of the decoupler [20] so that the usual phase-cycling routines employed in 1D acquisitions remove the unsightly ‘zero-frequency’ spike (Fig. 4.10).

4.2.3. Heteronuclear decoupling

$X\{^1H\}$ decoupling

The application of broadband proton decoupling during the acquisition of carbon-13 spectra is now universally applied. The removal of all ^1H – ^{13}C couplings concentrates all the carbon resonance intensity into a single line, providing a significant increase in signal intensity and simplification of the spectrum. As an added bonus, the continuous saturation of the proton spins provides further enhancement of the signal (by as much as 200% in the case of carbon-13) due to the NOE (Chapter 8). For these reasons, the use of broadband proton decoupling is standard practice for the routine observation of most commonly encountered nuclides.

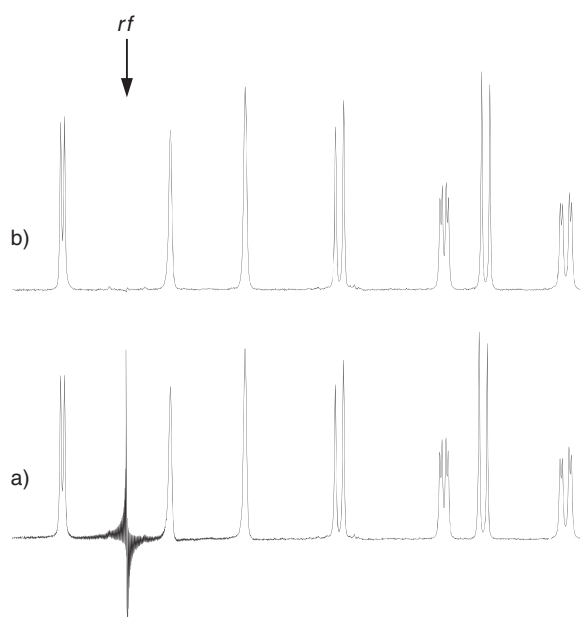


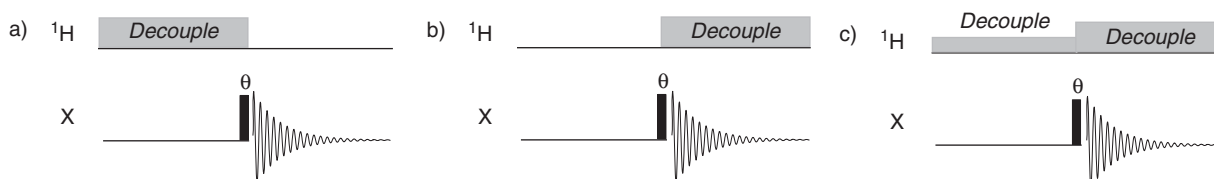
Figure 4.10. The aesthetically unappealing decoupler frequency ‘spike’ sometimes observed in homonuclear decoupling experiments as in (a) can be readily removed by setting the transmitter and decoupler frequencies to be the same, as in (b).

The principles behind heteronuclear decoupling are no different from the homonuclear case, the major difference being the very much larger frequency separation between the decoupling and observed frequencies, implying Bloch–Siegert shifts and receiver interference are no longer a problem. The use of broadband heteronuclear decoupling is itself associated with a number of other practical problems. Firstly, there is the need to decouple uniformly over the whole of the proton spectrum and this requires the applied rf to be effective over a far greater frequency window. Decoupling bandwidths are typically of the order of many kilohertz and are greater at higher field strengths. A 10 ppm proton spectrum covers 4 kHz at 400 MHz but 6 kHz at 600 MHz, for example. Generally, a wider decoupling bandwidth can be achieved by increasing the decoupler power, although the continuous application of high-power rf is more likely to destroy the probe and sample than achieve the desired results. High powers also give rise to the problem of rf sample heating, which is most pronounced in ionic samples, and can be disastrous for heat-sensitive compounds. The effect is insidious as the heating occurs within the sample itself; so the usual method of sample temperature monitoring (a thermocouple placed within the airflow surrounding the sample) is largely insensitive to its presence. The usual indicator is a drift of the lock level when the pulse sequence is started. To overcome the problem of decoupling over wide bandwidths without the need for excessive powers, specially designed modulated decoupling schemes are employed (which include the so-called *composite pulse decoupling* sequences and more recently *adiabatic pulses*, described in Chapter 10). The careful design of probes can also assist by reducing the sample heating, which is caused by the *electric* component of the rf. As with pulse excitation, the required frequency spread can be kept to a minimum by placing the decoupler frequency in the *centre* of the region to be decoupled. If your heteronuclear spectra display unexpectedly broad or even split resonances, this may be due to the decoupler frequency being incorrect, and/or the decoupler channel being poorly tuned or wrongly calibrated (Chapter 3). If these perturbations occur only at the edges of the spectrum, the effective bandwidth for the decoupling scheme may be inadequate.

Sample heating can also be reduced by gating off the rf when not essential to the experiment (Fig. 4.11). Gating the decoupler off during the recovery delay (*inverse-gated decoupling*, Fig. 4.11b) also removes the NOE and provides a decoupled spectrum *without* NOE enhancements. This is because any NOE that builds up during the acquisition time affects only longitudinal magnetisation and so does not influence the detected (transverse) signals, and is then allowed to decay during the recovery delay, so again has no influence. This method is particularly significant in the observation of nuclei with negative magnetogyric ratios, since the NOE causes a *decrease* in signal intensity for such nuclei (Chapter 8). It is also employed for accurate quantitative measurements, as discussed in the previous section. Conversely, running the decoupler during the relaxation delay but gating it off during the acquisition period (*gated decoupling*, Fig. 4.11a) results in a spectrum that retains spin coupling and is enhanced by the NOE. A further method for reducing sample heating provides a decoupled spectrum with enhancement by the NOE and is known as *power-gated decoupling* (Fig. 4.11c). Here, a high decoupler power is applied during acquisition to achieve complete decoupling, but a reduced power is applied between scans to maintain a degree of saturation and hence develop the NOE. This approach is particularly applicable to studies at high field where larger bandwidths demand relatively high decoupler powers. A comparison of these various decoupling schemes is illustrated in Fig. 4.12 for the terpene α -pinene, **4.2**.

**4.2**

Figure 4.11. Possible schemes for applying proton decoupling when observing a heteronucleus. (a) Gated decoupling (coupled spectrum with NOE), (b) inverse-gated decoupling (decoupled spectrum without NOE) and (c) power-gated decoupling where the rf is applied at two different powers (decoupled spectrum with NOE).



Just like homonuclear decoupling described above, *selective* heteronuclear decoupling is also possible. The decoupling of a single proton resonance could in principle identify coupled partners through the observation of collapsed multiplet structure in the heterospin spectrum. This however requires the decoupling rf to be effective over the *satellites* of the parent proton resonance, which may be far apart if $^1J_{\text{XH}}$ is large. One-bond proton–carbon couplings exceed 100 Hz and may be hard to remove whilst retaining sufficient selectivity, so here a 2D heteronuclear correlation spectrum is likely to be a more efficient approach. The selective removal of long-range (2 or 3-bond) proton–carbon couplings is more readily achieved since these are typically less than 10 Hz. An example of this is illustrated by the identification of configurational isomers of **4.3** through the measurement of three-bond

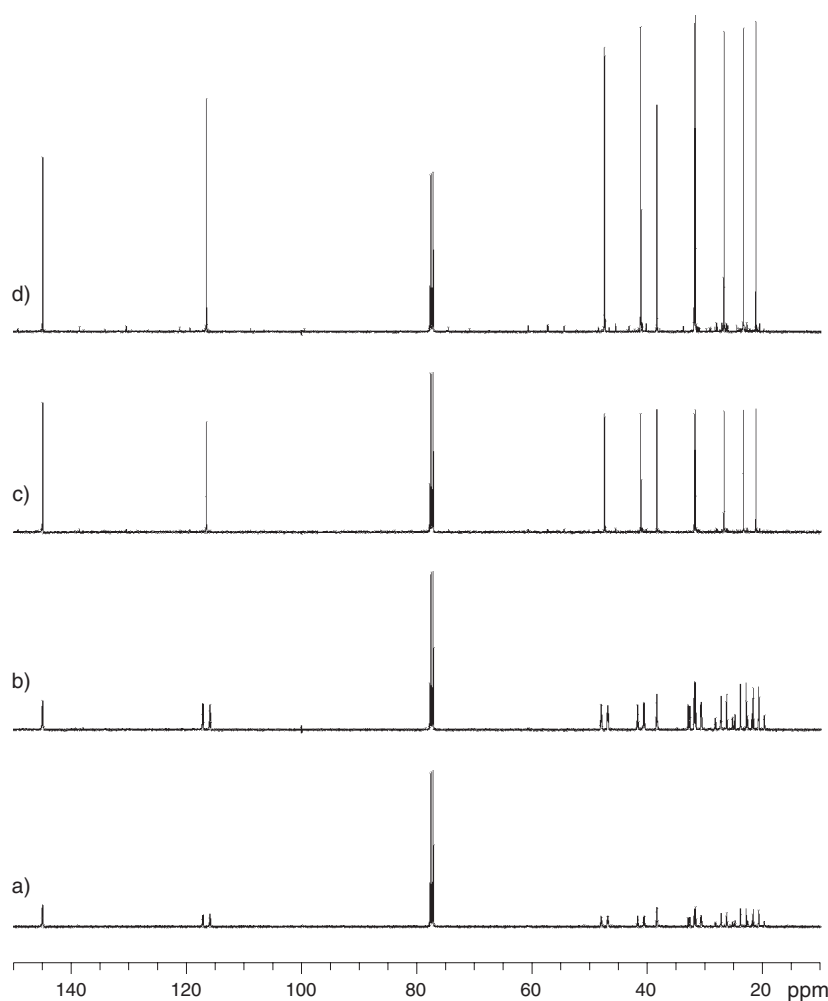
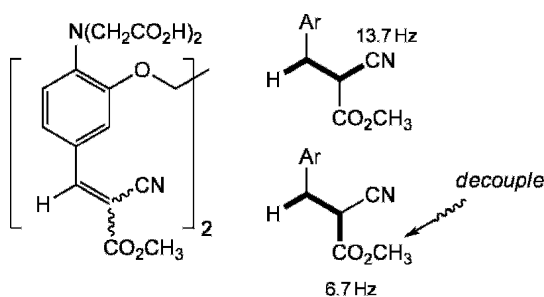


Figure 4.12. The carbon-13 spectrum of α -pinene acquired (a) without proton irradiation at any stage (coupled spectrum without NOE), (b) with gated decoupling (coupled spectrum with NOE), (c) with inverse-gated decoupling (decoupled spectrum without NOE) and (d) power-gated decoupling (decoupled spectrum with NOE). All other experimental conditions were identical and the same absolute scaling was used for each plot.



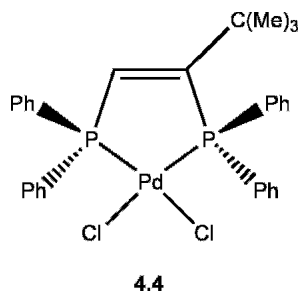
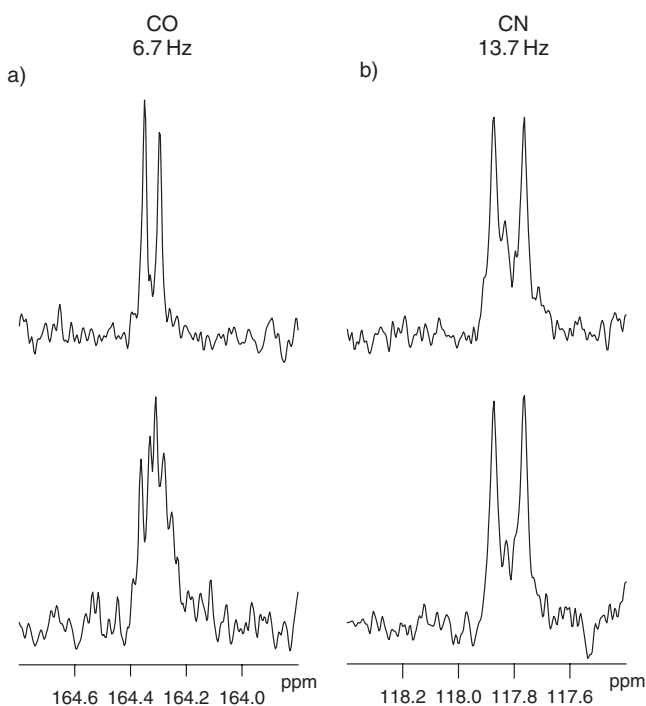
4.3

proton–carbon couplings (Fig. 4.13), which share a Karplus-type dependence on dihedral bond angles. The magnitude of the proton couplings to the carbon atoms of the nitrile and the ester across the alkene would therefore indicate their relative stereochemistry, but whilst the nitrile coupling presented a clear doublet in the proton-coupled ^{13}C spectrum, the carbonyl coupling was masked by additional three-bond couplings to the ester methyl protons. Selective decoupling of these methyl protons eliminated this interference resulting in a carbonyl doublet, sufficient to identify the nitrile as being trans to the alkene proton.

$^1\text{H}\{\text{X}\}$ decoupling

Traditionally, FT spectrometers were built with the ability to decouple only protons whilst observing the heteronucleus, as described above. Modern instruments now have the capability of providing X-nucleus pulsing and decoupling whilst observing protons, $^1\text{H}\{\text{X}\}$ (the ‘inverse’ configuration). The bandwidths required for the broadband decoupling of, for example, carbon-13, far exceed those needed for proton decoupling, and the composite pulse or adiabatic decoupling methods described in Chapter 10 become essential for success. Thus, carbon-13 decoupling over 150 ppm (a typical range for proton-bearing carbons) at 400 MHz- ^1H requires a bandwidth of 15 kHz and the rf heating problems

Figure 4.13. The application of selective proton decoupling in the measurement of heteronuclear long-range proton–carbon coupling constants. Lower traces are from the fully proton-coupled carbon-13 spectrum and the upper traces from that in which the methyl ester protons of **4.3** were selectively decoupled to reveal the three-bond coupling of the carbonyl carbon across the alkene.



mentioned above are generally more problematic with heteronuclear decoupling, especially at high fields. Broadband X-nucleus decoupling is most frequently applied as part of a multidimensional pulse sequence to simplify crosspeak structures, although it can be a useful tool in interpreting 1D proton spectra when a heteronucleus has high natural abundance and thus makes a significant contribution to coupling fine structure, for example ^{19}F or ^{31}P . Fig. 4.14 demonstrates the simplification of the ^1H spectrum of the palladium phosphine **4.4** on application of broadband ^{31}P decoupling, and this procedure often aids the interpretation of multiplet structures or the extraction of homonuclear proton couplings. Likewise, if the X-nucleus chemical shifts are known, *selective* X-decoupling can be used to identify the coupled partners in the proton spectrum, and in simple cases this may serve as a ready alternative to a 2D heteronuclear correlation experiment.²

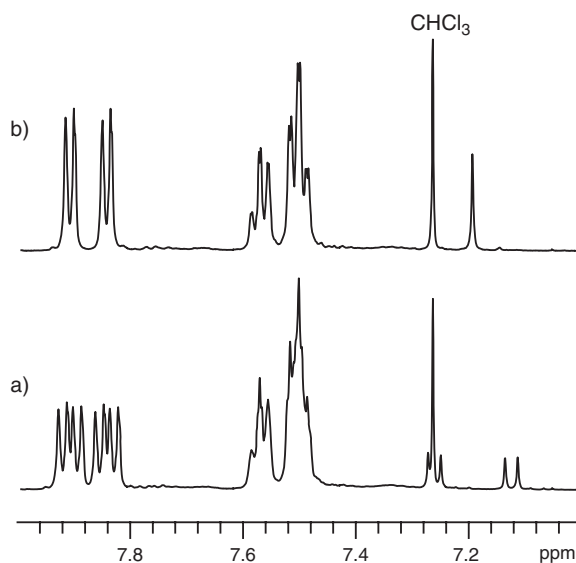


Figure 4.14. Simplification of the conventional proton spectrum (a) of the palladium phosphine **4.4** in CDCl_3 by the application of broadband phosphorus decoupling (b). All long-range ^1H – ^{31}P couplings are removed, as is most apparent for the alkene proton (7.2 ppm) and the *ortho* protons of the phenyl rings (above 7.8 ppm).

² In fact, this approach was used to identify the resonant frequencies of heteronuclei prior to the advent of the pulse-FT methods [11] which made their direct observation possible. Thus, the proton spectrum was recorded whilst applying a second rf field to the heteronucleus. Successively stepping the decoupler frequency and repeating the measurement would ultimately indicate the X resonance position when the X– ^1H coupling was seen to disappear from the proton spectrum.

4.3. SPECTRUM EDITING WITH SPIN-ECHOES

The principal reason behind the application of broadband proton decoupling of heteronuclei is the removal of the coupling structure to concentrate signal intensity, thereby improving the signal-to-noise ratio and reducing resonance overlap (Fig. 4.12 above). Additional gains such as signal enhancements from the NOE and the clarification of any remaining homonuclear couplings may also arise. Set against these obvious benefits is the loss of multiplicity information present in the proton-coupled spectrum (Fig. 4.12a), meaning there is no way, *a priori*, of distinguishing, for example, a methine from a methylene carbon resonance. It is therefore desirable to be able to record fully proton-decoupled spectra yet still retain the valuable multiplicity data. Some of the earliest multipulse sequences were designed to achieve this aim. These were based on simple spin-echoes and provided spectra in which the multiplicities were encoded as signal intensities and signs. Despite competition from the methods based on polarisation transfer described shortly, these techniques still find widespread use in organic chemistry laboratories. As they are also rather easy to understand, they provide a suitable introduction to the idea of spectrum multiplicity editing. The sections that follow utilise the rotating-frame vector model to explain pictorially the operation of the experiments and familiarity with the introduction in Chapter 2 is assumed.

4.3.1. The *J*-modulated spin-echo

One of the simplest approaches to editing is the *J*-modulated spin-echo sequence [21] (Fig. 4.15a, also referred to as SEFT, Spin-Echo Fourier Transform, [22]), which can be readily appreciated with reference to the vector model. The key to understanding this sequence is the realisation that the evolution of carbon magnetisation vectors under the influence of $^1J_{CH}$ coupling only occurs when the proton decoupler is gated off whilst at all other times only carbon chemical shifts are effective. Further simplification comes from ignoring carbon chemical shifts altogether since shift evolution during the first Δ period is precisely refocused during the second by the $180^\circ(C)$ refocusing pulse (see Section 2.2). Thus, to understand this sequence one only need consider the influence of heteronuclear coupling during the first Δ period.

Consider events with $\Delta = 1/J$ s, for which the relevant vector evolution for different multiplicities is illustrated in Fig. 4.16. Since chemical shifts play no part, quaternary carbons remain stationary along $+y$ during Δ (long-range C–H coupling that may exist will be far smaller than the one-bond coupling and may be considered negligible). Doublet vectors for a methine pair evolve at $\pm J/2$ Hz, so each will rotate through one-half cycle in $1/J$ s and hence meet once more along $-y$. As these now have a 180° phase difference with respect to the quaternary signals, they will ultimately appear inverted in the final spectrum. Applying these arguments to the other multiplicities shows that methylene vectors evolving at $\pm J$ Hz will align with $+y$ whilst methyl vectors evolving at $\pm J/2$ and $\pm 3J/2$ Hz will terminate along $-y$. More generally, by defining an angle θ such that $\theta = 180 J \Delta$ degrees, the signal intensities of carbon multiplicities, I , vary according to:

$$C: \quad I = 1$$

$$CH: \quad I \propto \cos \theta$$

$$CH_2: \quad I \propto \cos^2 \theta$$

$$CH_3: \quad I \propto \cos^3 \theta$$

as illustrated in Fig. 4.17. The spectrum for $\Delta = 1/J$ s ($\theta = 180^\circ$) will therefore display quaternaries and methylenes positive with methines and methyls negative if phased as for

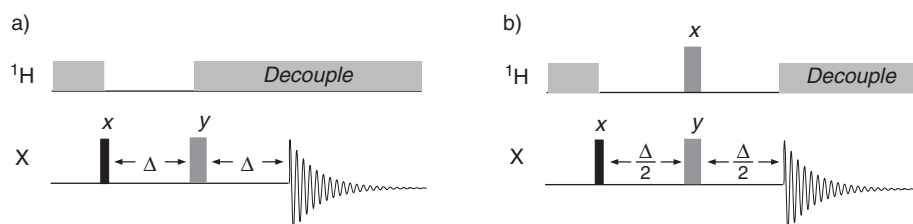


Figure 4.15. *J*-modulated spin-echo sequences. (a) The decoupler-gated variant and (b) the pulsed variant.

Figure 4.16. The evolution of carbon magnetisation vectors under the influence of proton–carbon couplings.

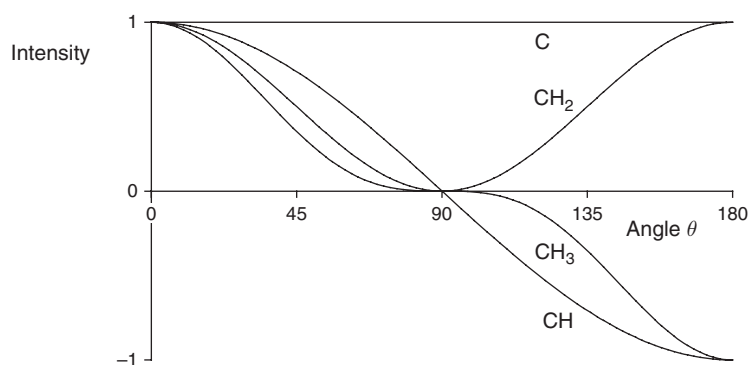
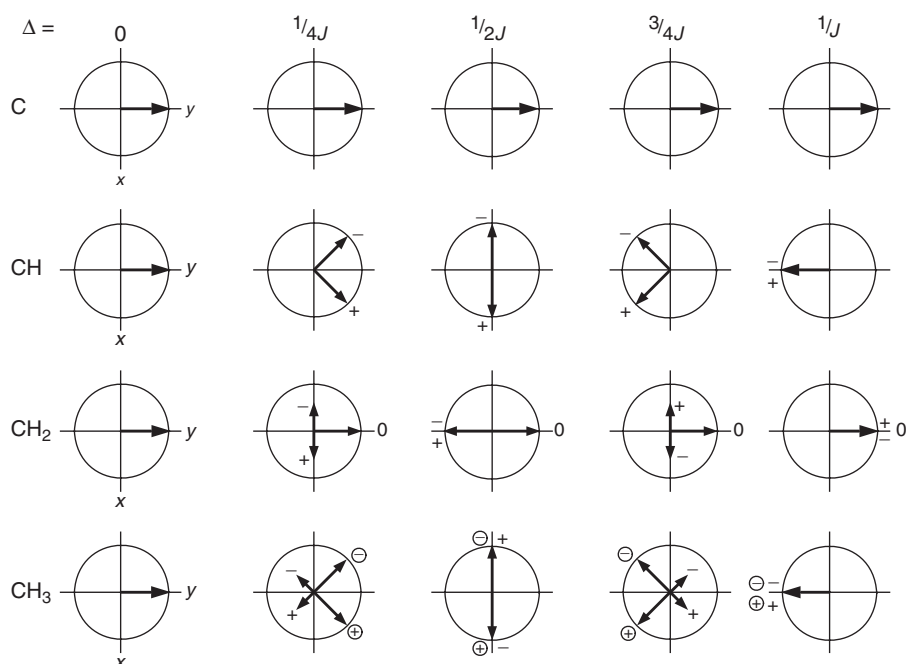


Figure 4.17. The variation of carbon signal intensities in the J -modulated spin-echo as a function of the evolution time Δ ($\theta = 180J\Delta^\circ$).

the one-pulse carbon spectrum (Fig. 4.18b), although edited spectra are often presented with methine resonances positive and methylene negative, so local convention should be checked. The carbon multiplicities therefore become encoded as signal intensities and at least some of the multiplicity information lost in the single-pulse carbon experiment is recovered, whilst still benefiting from decoupled resonances. The application of proton decoupling for all but a short time period Δ also ensures spectra are acquired with enhancement from the NOE. Setting $\Delta = 1/2J$ s corresponds to a null for all protonated carbons (Fig. 4.17), thus producing a quaternary-only spectrum (Fig. 4.18c). The accuracy of editing is clearly critically dependent on the correct setting of Δ , which is in turn

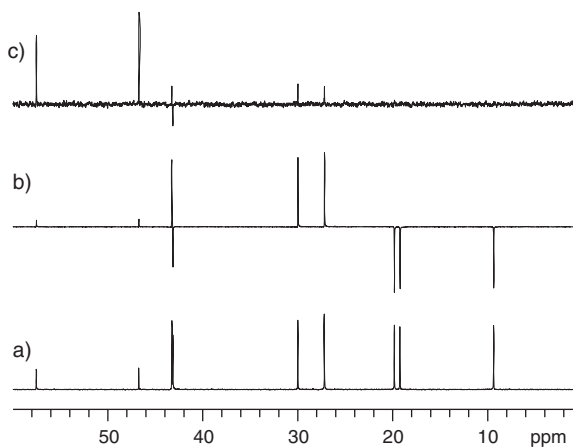


Figure 4.18. Carbon spectrum of camphor **4.1** edited with the J -modulated spin-echo sequence. (a) Conventional carbon spectrum (carbonyl not shown), and edited spectra with (b) $\Delta = 1/J$ ($\theta = 180^\circ$) and (c) $\Delta = 1/2J$ ($\theta = 90^\circ$) with J assumed to be 130 Hz. Some breakthrough of protonated carbons is observed in (c) due to variations in coupling constants within the molecule.

Table 4.2. Typically ranges for one-bond proton–carbon coupling constants

Carbon environment	Typical $^1J_{\text{CH}}$ range/Hz
Aliphatic, CH_n –	125–135
Aliphatic, CH_nX (X = N, O, S)	135–155
Alkene	155–170
Alkyne	240–250
Aromatic	155–165

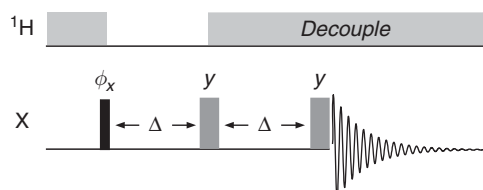
dependent upon J , and as there is likely to be a wide variety of J values within a sample one is forced to make some compromise setting for Δ . One-bond proton–carbon couplings range between 125 and 250 Hz, although they are more commonly between 130 and 170 Hz (Table 4.2), so a typical value for Δ would be ≈ 7 ms ($^1J_{\text{CH}} \approx 140$ Hz) when aromatics and carbon centres bearing electronegative heteroatoms are anticipated. If couplings fall far from the chosen value the corresponding resonance can display unexpected and potentially confusing behaviour. Alkyne carbons are particularly prone to this, owing to exceptionally large $^1J_{\text{CH}}$ values.

The spin-echo experiment is particularly simple to set up as it does not require proton pulses or their calibration, a desirable property when the experiment was first introduced but of little consequence nowadays. The same results can, in fact, be obtained by the use of proton 180° pulses rather than by gating of the decoupler [23] (Fig. 4.15b). In this case the Δ period is broken in two periods of $1/2J$ separated by the simultaneous application of proton and carbon 180° pulses. These serve to refocus carbon chemical shifts but at the same time allow couplings to continue to evolve during the second $\Delta/2$ period (Section 2.2). Hence, the total evolution period in which coupling is active is $1/J$, as in the decoupler-gating experiment above, and identical modulation patterns are produced. It is this shorter pulsed form of the heteronuclear spin-echo that is widely used in numerous pulse sequences to refocus shift evolution yet leave couplings to evolve.

4.3.2. APT

The principal disadvantage of the J -modulated spin-echo described above is the use of a 90° carbon excitation pulse that, as discussed in Section 4.1, is not optimum for signal averaging and may lead to signal saturation, notably of quaternary centres. The preferred approach using an excitation pulse width somewhat less than 90° requires a slight modification of the J -modulated experiment, giving rise to the Attached Proton Test (APT) sequence [24] (Figure 4.19). Use of a small tip-angle excitation pulse leaves a component of magnetisation along the $+z$ axis, which is inverted by the $180^\circ(\text{C})$ pulse that follows. In systems with slowly relaxing spins, this inverted component may cancel magnetisation arising from the relaxation of the transverse components, leaving little or no net signal to observe on subsequent cycles. It is therefore necessary to add a further $180^\circ(\text{C})$, which returns the problematic $-z$ component to $+z$ prior to acquisition, thereby eliminating possible cancellation. Transverse components also experience a 180° rotation prior to detection, but are otherwise unaffected beyond this phase inversion. The APT experiment is commonly used with excitation angles of 45° or less and is thus better suited to signal averaging than the basic echo sequence above, but otherwise gives similar editing results.

The poor editing accuracy of spin-echoes in the presence of a wide range of J values and the inability to fully characterise all carbon multiplicities are the major limitations of these techniques. More complex variations on the pulsed J -modulated spin-echo are to be found that do allow a complete decomposition of the carbon spectra into C, CH, CH_2 and CH_3 sub-spectra [25] and that also show greater tolerances to variations in $^1J_{\text{CH}}$ [26]. Likewise, J -compensated APT sequences have been developed for greater tolerance to a spread of J values [27], and for the direct generation of complete sub-spectra [28]. Invariably, the simplest sequences still find widest use.

**Figure 4.19.** The Attached Proton Test (APT) sequence.

4.4. SENSITIVITY ENHANCEMENT AND SPECTRUM EDITING

One of the principal concerns for the chemist using NMR spectroscopy is the relatively poor sensitivity of the technique when compared to other analytical methods, which stems from the small energy differences and hence small population differences between spin states. Developments that provide increased signal intensity relative to background noise are a constant goal of research within the NMR community, be it in the design of the NMR instrumentation itself or in novel pulse techniques and data processing methods. The continued development of higher field magnets clearly contributes to gains in sensitivity, and is paralleled by other instrumental advances such as the probe developments described in Section 3.4.2. Although that section considered the issue of sensitivity with reference to probe design we now return to this topic in relation to pulse techniques and will again review some of the principal factors that govern this.

Aside from instrumental developments, optimum sensitivity is provided by observing the nuclear species giving rise to the strongest signal, as judged by the intrinsic sensitivity of the nucleus and its natural abundance. The intrinsic sensitivity depends upon the magnetogyric ratio, γ , of the spin in three ways, with a greater γ contributing to:

- a high resonant frequency, which in turn implies a large transition energy difference and hence a greater Boltzmann population difference,
- a high magnetic moment and therefore a stronger signal, and
- a high *rate* of precession that induces a greater signal in the detection coil (just as cycling faster would cause a bicycle dynamo lamp to glow brighter, should you happen to possess one).

Thus, in general, the strength of an NMR signal is proportional to γ^3 for a single nuclide, but as noise itself increases with the square root of the observation frequency [29] the signal-to-noise ratio scales as $\gamma^{5/2}$. Notice also that as two of the above terms depend upon resonant frequency, the signal-to-noise ratio for a single nuclide would be expected to scale with the static field according to $B_0^{3/2}$ (all other things being equal). When more than one nuclide is involved in a sequence, a general expression for the signal-to-noise ratio of a 1D experiment may be derived:

$$\frac{S}{N} \propto NAT^{-1} B_0^{3/2} \gamma_{\text{exc}} \gamma_{\text{obs}}^{3/2} T_2^* (NS)^{1/2} \quad (4.2)$$

where N is the number of molecules in the observed sample volume, A is a term that represents the abundance of the NMR active spins involved in the experiment, T is temperature, B_0 is the static magnetic field, γ_{exc} and γ_{obs} represent the magnetogyric ratios of the initially excited and the observed spins, respectively, T_2^* is the effective transverse relaxation time and NS is the total number of accumulated scans. The high magnetogyric ratio of the proton, in addition to its 100% abundance (and ubiquity), explains why proton observation is favoured in high-resolution NMR. Indeed, many of the newly developed multipulse heteronuclear experiments utilise direct proton observation to achieve greater sensitivity, whilst the heteronuclear spin is observed indirectly, as described in Chapter 6. This section deals with those 1D pulse methods in widespread use that assist in the *direct* observation of relatively low- γ nuclei, here termed X-nuclei, for example, ^{13}C , ^{15}N , ^{29}Si and so on. These methods are characterised by initial excitation of a high- γ spin, typically ^1H , ^{19}F or ^{31}P , followed by *polarisation transfer* onto the low- γ nucleus to which it is scalar spin-spin coupled. This process introduces γ_{high} as the γ_{exc} term in Eq. (4.2) in place of γ_{low} , and so provides sensitivity gains by a factor of $\gamma_{\text{high}}/\gamma_{\text{low}}$.

It has been alluded to in the previous section, and will be considered in full in Chapter 8, that significant enhancements of low- γ nuclei may be obtained on saturation of, for example, protons, owing to the NOE. This effect, however, can be ill-suited to nuclei that possess a negative γ , since the NOE then causes a *reduction* in signal intensity and may, in the worst case, lead to a complete loss of signal. Polarisation transfer methods do not suffer such disadvantages and, in many cases, are able to provide considerably greater enhancements than the NOE alone. In addition, these experiments provide another means for the multiplicity editing of spectra and enable the differentiation of groups that possess differing numbers of attached nuclei. The ability to edit carbon spectra and hence to distinguish quaternary, methine, methylene and methyl groups offers an alternative to the spin-echo methods of the previous section and these polarisation transfer techniques, the DEPT experiment in particular, have become routine experiments in the organic chemist's repertoire. Methods that relate to the multiplicity editing of proton spectra are principally dealt with in Chapter 6. More recently developed and experimentally more complex technologies that potentially provide very significant sensitivity gains far beyond those attainable with

the methods described below are also emerging. These rely on the generation of very large population differences, one notable example being *dynamic nuclear polarisation* (DNP). As this currently remains emerging technology it is introduced briefly in Chapter 10 (Section 10.8.2) but in due course may itself make important contributions to the types of polarisation transfer methods described below.

4.4.1. Polarisation transfer

Polarisation transfer methods enhance signal intensity by transferring the greater population difference (or *polarisation*) of a high- γ spin onto its spin-coupled low- γ partner(s). Through this they replace one of the three γ_{low} signal intensity dependencies described above with γ_{high} , leading to a signal enhancement by a factor of $(\gamma_{\text{high}}/\gamma_{\text{low}})$. The principles behind all polarisation transfer methods can be understood by considering a spin- $1/2$ scalar-coupled pair, which for illustrative purposes is taken to be a ^1H - ^{13}C pair *both with 100% abundance*, whose energy level diagram is shown in Fig. 4.20. At thermal equilibrium, the populations for each of the four spin transitions are governed by the Boltzmann law as represented in Fig. 4.21a. From this the population *differences* across the transitions are $2\Delta\text{H}$ and $2\Delta\text{C}$ for the proton and carbon spins, respectively, and since these scale linearly with γ , the ratio $2\Delta\text{H}/2\Delta\text{C}$ equates to $\gamma_{\text{H}}/\gamma_{\text{C}}$ or ≈ 4 . The resonance intensities for proton are thus four times greater than those of carbon when judged by these populations (Fig. 4.21b). Now consider the result of selectively inverting *one* line of the H spin doublet, say H^1 (with a weak selective pulse for example), which equates to inverting the population difference across the corresponding transition (Fig. 4.20c). As H^2 remains untouched, the population difference across the H^2 transition is no different to that before the inversion. The salient point is that both the C spin population differences have now been altered by this process. Thus, the C^1 population difference becomes $-2\Delta\text{H} + 2\Delta\text{C}$ whilst that of C^2 is $2\Delta\text{H} + 2\Delta\text{C}$ and the population differences previously associated with the protons have been transferred onto the carbons. Since $2\Delta\text{H}$ is four times greater than $2\Delta\text{C}$, the C-transitions will display relative intensities of $-3:5$ so that sampling the carbon magnetisation at this point would produce a spectrum with one half of the doublet inverted and with signal intensities greater than in the single-pulse carbon spectrum (Fig. 4.20d). For a ^1H - ^{15}N pair, signal intensities following polarisation transfer from protons are $+11$ and -9 , ($\gamma_{\text{H}}/\gamma_{\text{N}} \approx 10$) and the signal enhancement is even more impressive. Since one half of the H spin populations have been inverted but the line intensities are otherwise unaffected, there has been no *net* transfer of magnetisation from proton to carbon. The *integrated intensity* of the whole carbon doublet is the same as in the absence of polarisation transfer, so we say there has been a *differential* transfer of polarisation.

The experiment described above is termed *selective population transfer* (SPT), or more precisely in this case with proton spin inversion, *selective population inversion*, (SPI). It is important to note, however, that the complete inversion of spin populations is not a requirement for the SPT effect to manifest itself. Any *unequal perturbation* of the lines

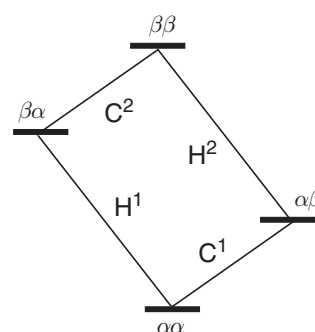


Figure 4.20. The schematic energy level diagram for a two-spin ^1H - ^{13}C system. The two transitions for each nucleus correspond to the two lines in each doublet.

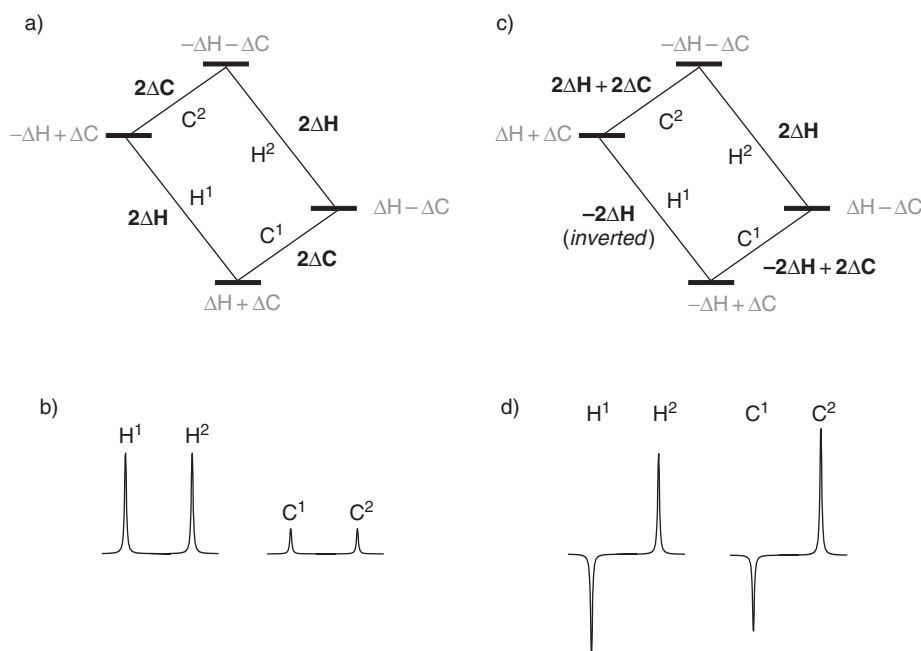
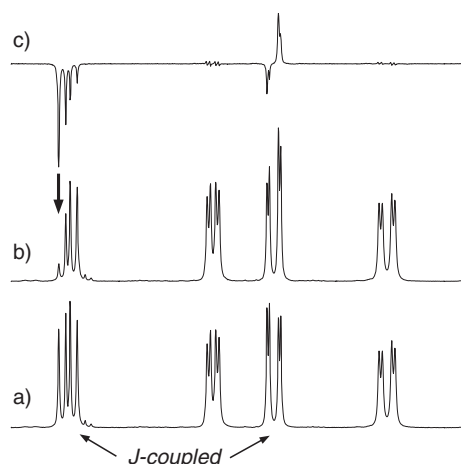


Figure 4.21. Polarisation transfer in a two-spin system. (a) Populations (in grey) and population differences (in bold) for each transition at equilibrium and (b) the corresponding spectra obtained following pulse excitation illustrating the four-fold population difference (see text). (c) The situation after selective inversion of one-half of the proton doublet and (d) the corresponding spectra showing the enhanced intensity of the carbon resonances.

Figure 4.22. Selective population transfer (SPT) distorts proton multiplet intensities when the resonance of a coupled partner is unevenly saturated (b). These perturbations are more apparent in the difference spectrum (c), when the unperturbed spectrum (a) is subtracted.



within a multiplet will suffice, so, for example, saturation of one proton line would also have altered the intensities of the carbon resonance. In heteronuclear polarisation (population) transfer experiments, it is the heterospin-coupled satellites of the parent proton resonance that must be subject to the perturbation to induce SPT. The effect is not restricted to heteronuclear systems and can appear in proton spectra when homonuclear-coupled multiplets are subject to unsymmetrical saturation. Figure 4.22 illustrates the effect of selectively but unevenly saturating a double doublet and shows the resulting intensity distortions in the multiplet structure of its coupled partner, which are most apparent in a difference spectrum. Despite these distortions, the integrated intensity of the proton multiplet is unaffected by the presence of the SPT because of the equal positive and negative contributions (see Fig. 4.21d). Distortions of this sort have particular relevance to the NOE difference experiment described in Chapter 8.

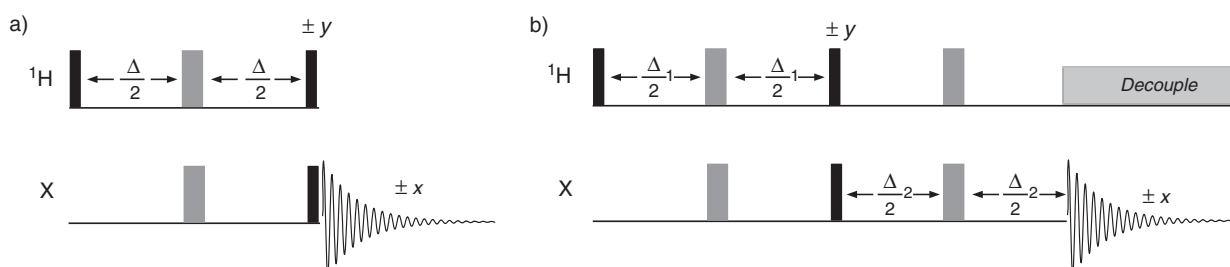
The greatest limitation of the SPI experiment is its lack of generality. Although it achieves the desired polarisation transfer, it is only able to produce this for one resonance in a spectrum at a time. To accomplish this for all one would have to repeatedly step through the spectrum, inverting satellites one-by-one and performing a separate experiment at each step. Clearly, a more efficient approach would be to invert one half of each proton doublet simultaneously for all resonances in a single experiment and this is precisely what the INEPT sequence achieves.

4.4.2. INEPT

The INEPT experiment [30] (Insensitive Nuclei Enhanced by Polarisation Transfer) was one of the forerunners of many of the pulse NMR experiments developed over subsequent years and still constitutes a feature of some of the most widely used multidimensional experiments in modern pulse NMR. Its purpose is to enable *non-selective* polarisation transfer between spins, and its operation may be readily understood with reference to the vector model. Most often it is the proton that is used as the source nucleus and this discussion will relate to XH spin systems throughout, although it should be remembered that any high- γ spin- $\frac{1}{2}$ nucleus constitutes a suitable source.

The INEPT sequence (Fig. 4.23a) provides a method for inverting one-half of each XH doublet in a manner that is independent of its chemical shift, requiring the use of non-selective pulses only. The sequence begins with excitation of all protons, which then evolve under the effects of chemical shift and heteronuclear coupling to the X spin. After a period $\Delta/2$, the proton vectors experience a 180° pulse that serves to refocus chemical shift evolution (and field inhomogeneity) during the second $\Delta/2$ period. The simultaneous

Figure 4.23. (a) The INEPT sequence and (b) the refocused INEPT sequence.



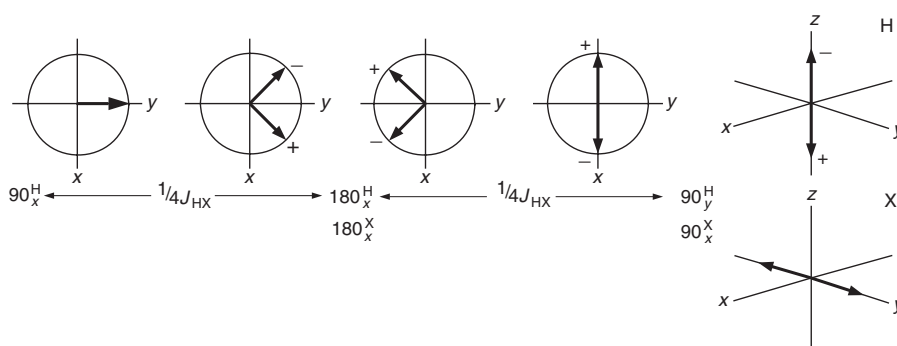


Figure 4.24. The evolution of proton vectors during the INEPT sequence. Following initial evolution under J_{XH} the 180° (H) pulse flips the vectors about the x axis and the 180° (C) pulse inverts their sense of precession. After a total evolution period of $1/2J_{XH}$ the vectors are anti-phase and are subsequently aligned along $\pm z$ by the 90°_y (H) pulse. This produces the desired inversion of one half of each H–X doublet for all resonances.

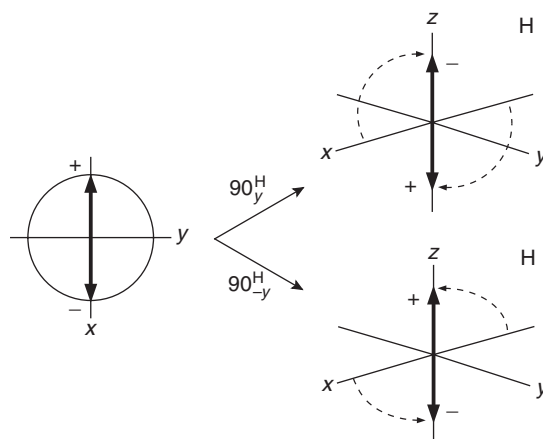


Figure 4.25. Inverting the phase of the last proton pulse in INEPT inverts the other half of the proton doublet (compare Fig. 4.24).

application of a 180° (X) pulse ensures that the heteronuclear coupling continues to evolve by inverting the proton vector's sense of precession. This is once again a spin-echo sequence in which only coupling evolution requires consideration. For an XH pair, a total Δ period of $1/2J$ ($\Delta/2 = 1/4J$) leaves the two proton vectors opposed or *anti-phase* along $\pm x$, so that the subsequent 90°_y (H) pulse aligns these along the $\pm z$ axis (Fig. 4.24). This therefore corresponds to the desired inversion of one-half of the proton doublet, as for SPI, but for all spin pairs simultaneously. The 90° (X) pulse samples the newly created population differences to produce the enhanced spectrum. In practice, these last two pulses are applied simultaneously, although in this pictorial representation it is convenient to consider that the proton pulse occurs first.

The asymmetrical peak intensities produced by the SPI effect, -3 and $+5$ for a CH pair, arise from the contribution of the natural X spin magnetisation, which may be removed by the use of a simple phase cycle. Thus, repeating the experiment with the phase of the last proton pulse at $-y$ causes the anti-phase lines to adopt the inverted disposition relative to the $+y$ experiment, that is, what was $-z$ now becomes $+z$ and *vice versa* (Fig. 4.25). The resulting X spin doublets are likewise inverted, although the natural x -magnetisation, being oblivious to the proton pulse, is unchanged. Subtraction of the two experiments by inverting the receiver phase causes the polarisation transfer contribution to add but cancels the natural magnetisation (Fig. 4.26). This two-step phase cycle is the basic cycle required for INEPT. As only the polarisation transfer component is retained by this process, a feature of pure polarisation transfer experiments is the lack of responses from nuclei without significant proton coupling.

Refocused INEPT

One problem with the basic INEPT sequence described above is that it precludes the application of proton spin decoupling during the acquisition of the X spin FID. Since this removes the J -splitting it causes the anti-phase lines to become coincident and cancel to leave no observable signal. The addition of a further period, Δ_2 , after the polarisation transfer allows the X spin magnetisation to refocus under the influence of the XH coupling, giving the *refocused-INEPT* sequence [31] (Fig. 4.23b), the results of which are illustrated in Fig. 4.27. Once again, the refocusing period is applied in the form of a spin-echo to remove chemical shift dependencies, and for the XH system to fully refocus the appropriate period is again $1/2J$. Since it is now the X spin magnetisation that refocuses, it is necessary to consider coupling to all attached protons. Choosing $\Delta_2 = 1/2J$ is not appropriate for nuclei

Figure 4.26. Experimental carbon-13 INEPT spectra of methanoic acid recorded with the phase of the last proton pulse set to (a) γ and (b) $-\gamma$. Subtracting the two data sets (c) cancels natural magnetisation that has not been generated by polarisation transfer and equalises the intensity of the two lines.

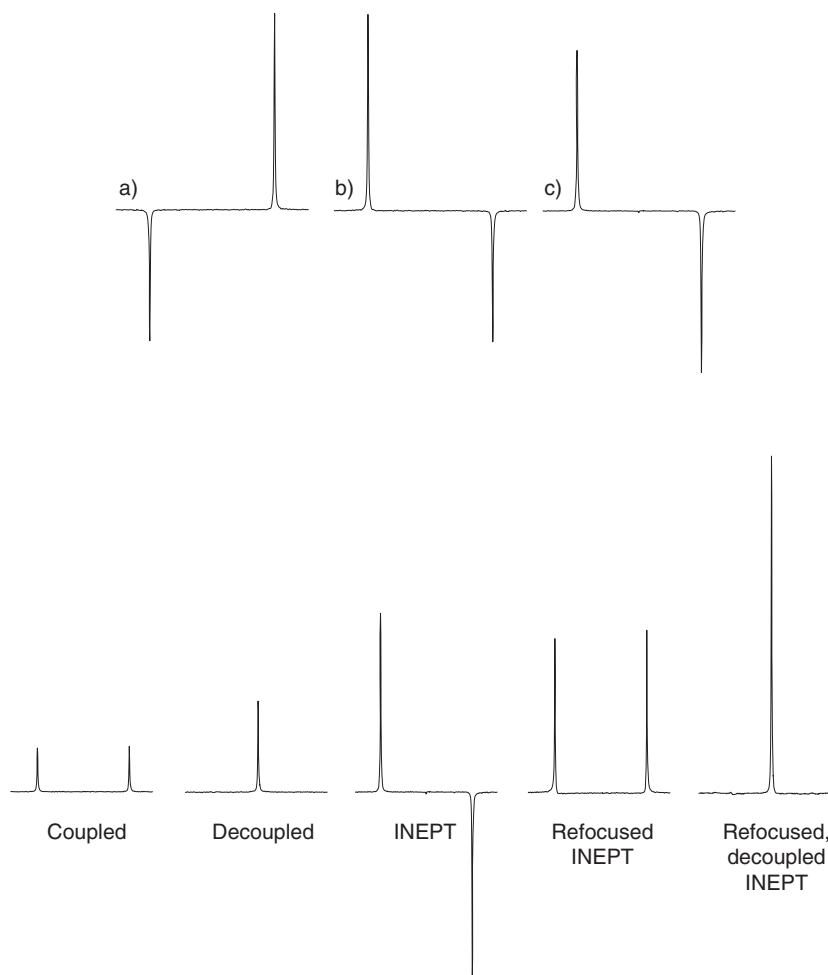


Figure 4.27. Experimental carbon-13 spectra of methanoic acid acquired without and with INEPT illustrating the intensity gains arising from polarisation transfer.

coupled to more than one proton and does not lead to the correct refocusing of vectors; rather they remain anti-phase and produce no signal on decoupling (this provides a clue as to how one might use INEPT for spectrum editing). It has been shown [32] that for optimum sensitivity a refocusing period for an XH_n group requires:

$$\Delta_2 = \frac{1}{\pi J} \sin^{-1} \left(\frac{1}{\sqrt{n}} \right) \quad (4.3)$$

where the angular term must be calculated in radians. For carbon spectroscopy this corresponds to $1/2J$, $1/4J$ and $\approx 1/5J$ for CH , CH_2 and CH_3 groups, respectively. If all multiplicities are to be observed simultaneously, a compromise setting of $\approx 1/3.3J$ is appropriate, which for an assumed 140 Hz coupling gives $\Delta_2 = 2.2$ ms. A spectrum recorded under these conditions but without proton decoupling will display significant phase errors since the refocusing period will not be optimal for any multiplicity; such errors are removed with proton decoupling however.

In cases where the XH coupling fine structure is of interest and proton decoupling is not applied, the relative line intensities within multiplets are distorted relative to the coupled spectrum without polarisation transfer, and this is one potential disadvantage of the above INEPT sequences. Even with suppression of the contribution from natural X spin magnetisation, intensity anomalies remain for XH_n groups with $n > 1$ (Fig. 4.28). A potentially confusing feature is the disappearance of the central line of XH_2 multiplets. One sequence proposed to generate the usual intensities within multiplets is INEPT^+ , comprising the refocused-INEPT with an additional proton 'purge' pulse to remove the magnetisation

Figure 4.28. Relative multiplet line intensities in coupled INEPT spectra. (a) Conventional multiplet intensities and those from INEPT (b) without and (c) with suppression of natural magnetisation.

	(a)	(b)	(c)
CH	1 1	-3 +5	-1 1
CH ₂	1 2 1	-7 2 +9	-1 0 1
CH ₃	1 3 3 1	-11 -9 15 13	-1 -1 1 1

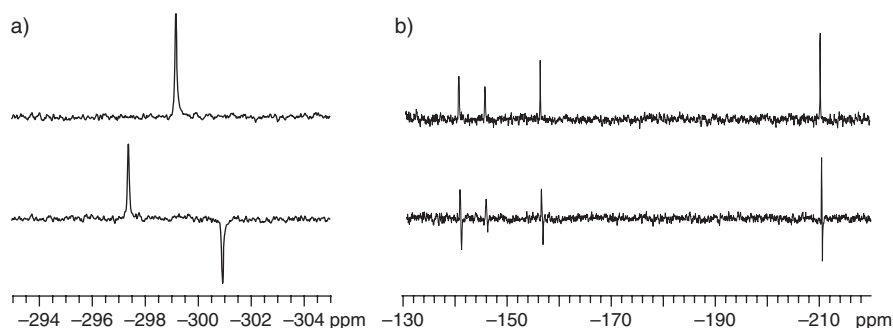
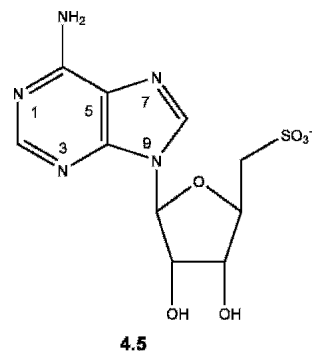


Figure 4.29. Natural abundance ^{15}N INEPT spectra of adenosine-5'-monosulphate **4.5**. Lower traces show results with the INEPT sequence and upper traces with the refocused-INEPT sequence. Delays were calculated assuming (a) $J = 90$ Hz and (b) $J = 10$ Hz. For (a) the lower trace displays the 1:0:–1 pattern of the NH_2 group whilst in (b) all resonances display anti-phase two-bond ^1H – ^{15}N couplings. Spectra are referenced to nitromethane.

terms responsible for the intensity anomalies [33]. In practice, the DEPT-based sequences described below are preferred as these show greater tolerance to experimental mis-settings.

Finally note that these INEPT discussions assume that the evolution of magnetisation is dominated by the coupling between the X and H spins, with all other couplings being negligibly small. In situations where homonuclear proton coupling becomes significant ($J_{\text{HH}} \approx \geq \frac{1}{3} J_{\text{XH}}$), it is necessary to modify the Δ_1 period for optimum sensitivity, and analytical expressions for this have been derived [34]. Such considerations are most likely to be significant with polarisation transfer from protons via long-range couplings, that is, when the protons are not directly bound to the heteronucleus. Examples may include coupling to tertiary nitrogens, quaternary carbons or to phosphorus. In some instances, the situation may be considerably simplified if the homonuclear couplings are removed by the application of *selective* proton decoupling during the proton Δ_1 evolution periods [35]. Figure 4.29 illustrates the acquisition of ^{15}N spectra of adenosine-5'-sulphate **4.5** by polarisation transfer through one-bond and long-range (two-bond) couplings. All nitrogen centres in the purine unit were observed in two experiments in which delays were optimised for $J = 90$ Hz and 10 Hz, which are the assumed values for one- and two-bond couplings, respectively.



Sensitivity gains

To appreciate the sensitivity gains from the application of the INEPT sequence, one should compare the results with those obtained from the usual direct observation of the low- γ species. This invariably means the spectrum obtained in the presence of proton broadband decoupling for which signal enhancement occurs by virtue of the ^1H –X NOE (Chapter 8). Thus, to make a true comparison, we need to consider the signal arising from polarisation transfer versus that from observation with the NOE, which for an XH pair is given by:

$$I_{\text{INEPT}} = I_0 \left| \frac{\gamma_{\text{H}}}{\gamma_{\text{X}}} \right| \quad I_{\text{NOE}} = I_0 \left(1 + \frac{\gamma_{\text{H}}}{2\gamma_{\text{X}}} \right) \quad (4.4)$$

where I_{INEPT} is the signal intensity following polarisation transfer, I_{NOE} is that in the presence of the NOE and I_0 represents the signal intensity in the absence of any enhancement. Notice, firstly, that the NOE makes a contribution that *adds* to the natural magnetisation and, secondly, because of this, the resulting signal intensity is also dependent upon the *sign* of the magnetogyric ratios, whereas polarisation transfer depends only on their magnitudes. The NOE therefore causes a *decrease* in signal intensity for those nuclei with a *negative* magnetogyric ratio, and may cause the observed signal to be inverted if the NOE is greater than the natural magnetisation or to become close to zero if comparable to it. Table 4.3 compares the theoretical *maximum* signal intensities that can be expected for polarisation transfer and the NOE from protons to heteronuclear spins in XH pairs. The degree of signal enhancement does not scale linearly with the number of attached source nuclei, n , used for polarisation transfer. In other words, the enhancement expected for an XH_2 group is not twice that for an XH group. Although greater enhancements do arise with

Table 4.3. Signal intensities for the X-spin in ^1H –X pairs arising from polarisation transfer (I_{INEPT}) and from direct observation with the maximum NOE (I_{NOE}). Intensities are given relative to those obtained by direct observation in the absence of the NOE (I_0)

X	^{13}C	^{15}N	^{29}Si	^{31}P	^{57}Fe	^{103}Rh	^{109}Ag	^{119}Sn	^{183}W	^{195}Pt	^{207}Pb
I_{INEPT}	3.98	9.87	5.03	2.47	30.95	31.77	21.50	2.81	24.04	4.65	4.78
I_{NOE}	2.99	–3.94	–1.52	2.24	16.48	–14.89	–9.75	–0.41	13.02	3.33	3.39

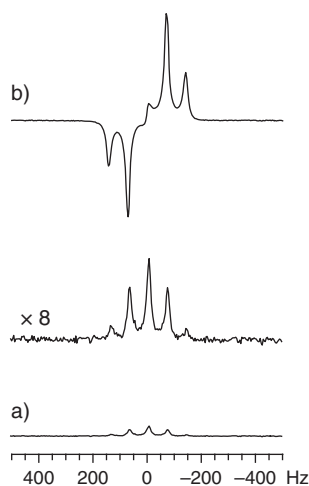


Figure 4.30. Signal enhancement of the ^{15}N spectrum of ammonium nitrate with INEPT. Direct observation using (a) the Ernst angle optimised for the nitrogen T_1 and (b) INEPT optimised for the proton T_1 . Both spectra were collected in the same total accumulation time.

more attached protons when the optimum refocusing delay is used, the gains over that for an XH group are only modest [33].

In practice, owing to experimental imperfections or to other relaxation processes reducing the magnitude of the NOE (CSA for the metals in particular), the figures in Table 4.3 may not be met, although they provide some guidance as to which method would be the most appropriate. The results for the higher- γ heteronuclei such as ^{31}P and ^{13}C are clearly comparable, whereas polarisation transfer provides far greater gains for the lower- γ species. A further benefit of the polarisation transfer approach not reflected in the figures of Table 4.3 is that the repetition rate of the experiment depends on the longitudinal relaxation times of the *protons*, since the populations of interest originate only from these spins. In contrast, the direct observation experiment, with or without the NOE, depends on the relaxation times of the X spins, which are typically very much longer. The opportunity for faster signal averaging provides another significant gain in sensitivity per unit time when employing polarisation transfer and in practice this feature can be as important or sometimes more important than the direct sensitivity gains from the transfer itself.

In short, polarisation transfer methods provide the greatest benefits for those nuclei that have low magnetogyric ratios and are slow to relax. It is also the preferred approach to direct observation for those nuclei with negative γ s where the NOE may lead to an overall signal reduction. Nitrogen-15 [36] (Fig. 4.30) and silicon-29 routinely benefit from polarisation transfer methods, as does the observation of metals, in particular transition metals, which often possess very low γ s and are extremely insensitive, despite their sometimes high natural abundance, some examples being ^{57}Fe , ^{103}Rh , ^{109}Ag and ^{183}W .

Despite these impressive sensitivity gains when directly observing the X spin, the more modern approach is to observe the X spin *indirectly* through the coupled proton when possible, which can be achieved through a number of heteronuclear correlation experiments. As these methods additionally employ proton observation, they benefit from a further theoretical gain of $(\gamma_{\text{high}}/\gamma_{\text{low}})^{3/2}$ over X-observe schemes (see Eq. (4.2)). These topics are pursued in Chapter 6, and should be considered as potentially faster routes to X-nucleus data.

Editing with INEPT

Returning to the selection of the Δ_2 period in the refocused INEPT sequence, it is apparent that whilst a period of $1/2J$ produces complete refocusing of doublets, the triplets (and quartets) remain anti-phase and will be absent from the spectrum recorded with proton decoupling (Fig. 4.31). Choosing $\Delta_2 = 1/2J$ therefore yields a sub-spectrum containing only methine resonances. This idea of editing heteronuclear spectra according to multiplicities is closely related to the editing with spin-echoes described in Section 4.3. Extending this idea to the selection of other carbon multiplicities, it is again convenient to define an angle $\theta = 180J\Delta_2$ degrees, from which the signal intensities in the decoupled experiment are:

$$\text{CH: } I \propto \sin \theta$$

$$\text{CH}_2: I \propto 2 \sin \theta \cos \theta$$

$$\text{CH}_3: I \propto 3 \sin \theta \cos^2 \theta$$

as presented graphically in Fig. 4.32. To differentiate all protonated carbons it is sufficient to record three spectra with Δ_2 adjusted suitably to give $\theta = 45^\circ$, 90° and 135° . The 90° experiment corresponds to $\Delta_2 = 1/2J$ mentioned above and hence displays methine groups only, $\theta = 45^\circ$ produces all responses whilst $\theta = 135^\circ$ has all responses again, but with methylene groups inverted. This process combines signal enhancement by polarisation transfer, with multiplicity determination through spectrum editing. The editing attainable here is superior to that provided by the basic spin-echo methods of Section 4.3 since

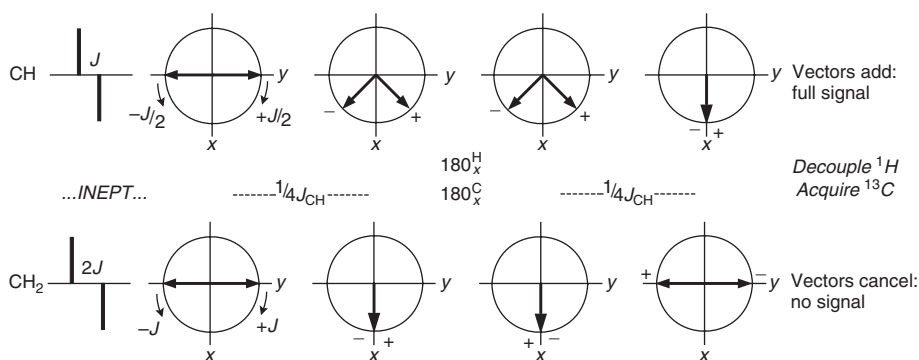


Figure 4.31. Editing with INEPT can be achieved with a judicious choice of refocusing delay, Δ_2 . A total delay of $1/2J_{\text{CH}}$ retains CH resonances but eliminates CH_2 (and CH_3) resonances.

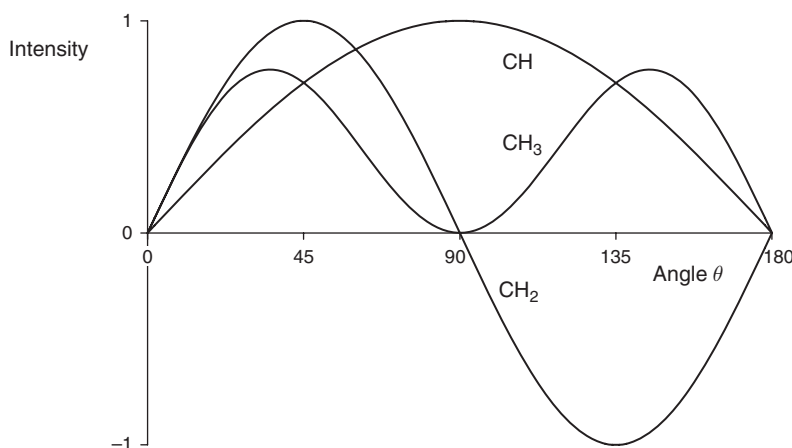


Figure 4.32. The variation of carbon signal intensities in the refocused INEPT experiment as a function of the evolution time Δ ($\theta = 180/\Delta^2$). Identical results are obtained for the DEPT experiment for which the angle θ represents the tip angle of the last proton pulse.

comparison of the three INEPT spectra allows one to determine the multiplicity of all resonances in the spectrum. However, in routine carbon-13 spectroscopy, this information tends not to be derived from INEPT but from the related DEPT sequence, which has better tolerance to experimental imperfections. Alas, DEPT, cannot be described fully by recourse to the vector model, and an understanding of INEPT goes a long way to help one appreciate the features of DEPT.

In summary, the INEPT sequence provides signal enhancement for all proton-coupled resonances in a single experiment by virtue of polarisation transfer. This also enables more rapid data collection since the repetition rate is now dictated by the faster relaxing protons and not the heteronucleus. By judicious choice of refocusing delays, refocused INEPT can also provide multiplicity editing of, for example, carbon-13 spectra. However, those nuclei that do not share a proton coupling cannot experience polarisation transfer and are therefore missing from INEPT spectra. The INEPT sequence is also used extensively as a building block in heteronuclear 2D sequences, as will become apparent in later chapters.

4.4.3. DEPT

The DEPT experiment [37] (Distortionless Enhancement by Polarisation Transfer) is the most widely used polarisation transfer editing experiment in carbon-13 spectroscopy, although its application is certainly not limited to the proton-carbon combination. It enables the complete determination of all carbon multiplicities, as does the refocused INEPT discussed above, but has a number of distinct advantages. One of these is that it directly produces multiplet patterns in proton-coupled carbon spectra that match those obtained from direct observation, meaning methylene carbons display the familiar 1:2:1 and methyl carbons the 1:3:3:1 intensity patterns; this is the origin of the term 'distortionless'. However, for most applications proton decoupling is applied during acquisition and multiplet structure is of no consequence, so the benefits of DEPT must lie elsewhere.

The DEPT sequence

The DEPT pulse sequence is illustrated in Fig. 4.33. To follow events during this, consider once more a ^1H - ^{13}C pair and note the action of the two 180° pulses is again to refocus chemical shifts where necessary. The sequence begins in a similar manner to INEPT with a $90^\circ(\text{H})$ pulse after which proton magnetisation evolves under the influence of proton-carbon coupling such that after a period $1/2J$ the two vectors of the proton satellites are anti-phase. The application of a $90^\circ(\text{C})$ pulse at this point produces a new state of affairs that has not been previously encountered, in which both transverse proton and carbon magnetisation evolve coherently. This new state is termed heteronuclear multiple-quantum coherence which, in general, cannot be visualised with the vector model, and without recourse to mathematical

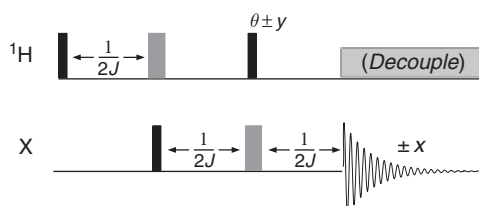


Figure 4.33. The DEPT sequence.

formalisms it is not possible to rigorously describe its behaviour. However, for these purposes we can imagine this multiple quantum coherence to be a pooling of both proton and carbon magnetisation, to be separated again at some later point.³

The heteronuclear multiple-quantum coherence now evolves for a further time period under the influence of the proton chemical shift but also, simultaneously, under the influence of the carbon chemical shifts until application of the proton θ pulse. However, it is *not* influenced by the proton–carbon coupling (this is another interesting property of multiple-quantum coherence, which need not concern us further at this time, see Chapter 5). To remove the effects of proton chemical shifts, a second evolution period is also set to $1/2J$ and a proton 180° pulse is applied between the two delays, coincident with the $90^\circ(\text{C})$ pulse. The action of the θ pulse is to transfer the heteronuclear multiple-quantum coherence into anti-phase transverse carbon magnetisation, that is, to regenerate observable magnetisation. The details of the outcome of this transfer process depend upon the multiplicity of the carbon resonance. In other words, methine, methylene and methyl groups respond differently to this pulse and this provides the basis of editing with DEPT. In the final $1/2J$ delay, the carbon magnetisation refocuses under the influence of proton coupling but also evolves according to carbon chemical shift, as it did in the second $1/2J$ period. Thus, the simultaneous application of a $180^\circ(\text{C})$ pulse with the proton θ pulse leads to an overall refocusing of the carbon shifts during the final $1/2J$ period. Carbon magnetisation is therefore detected without chemical shift-dependent phase errors, with or without proton decoupling. Phase alternation of the θ pulse, combined with data addition/subtraction by the receiver, leads to the cancellation of natural carbon magnetisation, as for INEPT. The net result is again polarisation transfer from protons to carbon, combined with the potential for spectrum editing.

Editing with DEPT

As was hinted at above, the key to spectrum editing with DEPT is the realisation that the phase and intensity of carbon resonances depends upon the proton tip angle, θ . In fact, the editing results for angle θ are analogous to those produced by refocused INEPT with $\Delta_2 = \theta/180J$, so the previous discussions relating to θ apply equally to DEPT, as does the graph of Fig. 4.32. However, with INEPT the editing delay Δ_2 must be chosen according to the spin coupling constant whereas with DEPT the editing is achieved through the variable pulse angle θ , which has no J dependence. This means the editing efficiency of DEPT tends to be superior to INEPT when a range of J values are encountered, and this is the principal advantage of DEPT in routine analysis. Evolution delays in DEPT do of course depend on J , although it turns out that the experiment is quite tolerant of errors in these settings.

The starting point for determining resonance multiplicities with DEPT is the collection of three spectra with $\theta = 45, 90$ and 135° , noting that the 90° experiment requires twice as many scans to attain the same signal-to-noise ratio as the others. The signs of the responses are then as summarised in Table 4.4. Knowing these patterns it is a trivial matter to determine multiplicities by direct comparison, whilst quaternaries can be distinguished by their appearance only in the direct carbon spectrum (or the DEPTQ variant described below). Example spectra are shown for the bicyclic terpene andrographolide **4.6** (Fig. 4.34). An extended approach is to combine these spectra appropriately to produce ‘sub-spectra’ that display separately CH, CH₂ and CH₃ resonances. Personally, I think it is always better to examine the original DEPT spectra directly since this is such a simple matter. The combining of spectra introduces greater potential for ‘artefact’ generation, and

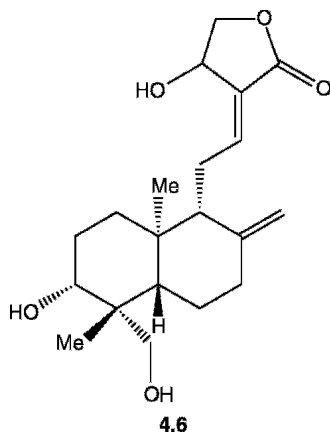


Table 4.4. Signs of multiplet resonances in DEPT and in DEPTQ spectra

	DEPT-45	DEPT-90	DEPT-135	DEPTQ
X	0	0	0	–
XH	+	+	+	+
XH ₂	+	0	–	–
XH ₃	+	0	+	+

³ The transverse magnetisation we observe directly in an NMR experiment is known as *single quantum coherence*. *Multiple quantum coherence*, however, cannot be directly observed because it induces no signal in the detection coil. For multiple quantum coherence to be of use to us, it must be transferred back into signal quantum coherence by the action of rf pulses. The concept of coherence is developed further in Chapter 5.

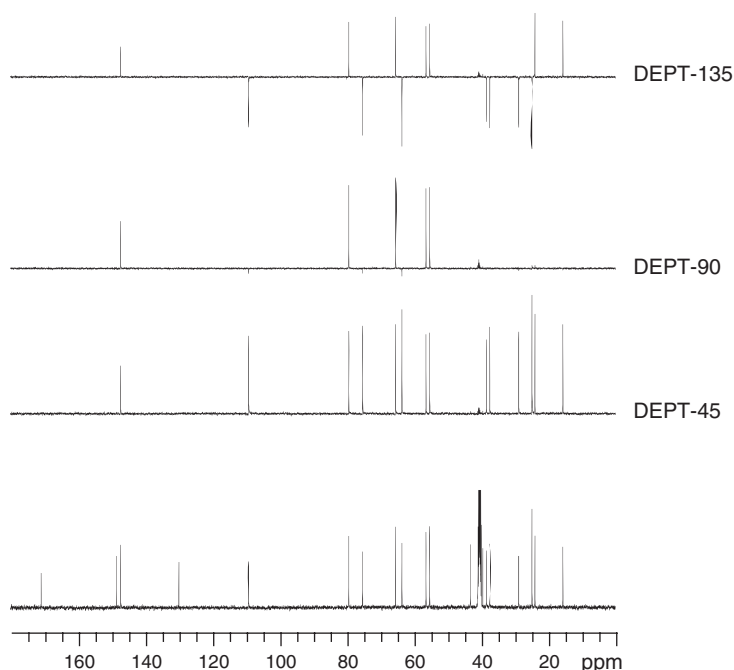


Figure 4.34. The conventional carbon and DEPT-edited spectra of the terpene andrographolide **4.6**.

it also becomes more difficult to identify what is going on if editing fails owing to the presence of an unusually high coupling constant or to instrument miscalibration, for example. In practice, the recording of a single DEPT spectrum with $\theta = 135^\circ$ is often sufficient to provide the desired information when methyls are absent or are easily recognised on account of their chemical shifts. Alternatively, the DEPTQ experiment has the further advantage of retaining quaternary centres, as described in the following section.

Errors in DEPT editing may arise from a number of sources. The most likely is incorrect setting of the proton pulses, especially the θ pulse used for editing, which may often be traced to poor tuning of the proton channel. Even small errors in θ can lead to the appearance of small unexpected peaks in DEPT-90; if θ is too small it approaches DEPT-45 whilst if it is too big it approaches DEPT-135. Usually, because of their low intensity, these spurious signals are easily recognised and should cause no problems. Even with correct pulse calibrations, errors can arise when the setting for the delay period is very far from that demanded by $^1J_{\text{CH}}$ [38] and in particular CH_3 resonances may still appear weakly in the CH sub-spectrum. A typical compromise value for $^1J_{\text{CH}}$ for determining Δ is ≈ 140 Hz, for which $\Delta = 3.6$ ms. Alkyne carbons in particular exhibit very high values of $^1J_{\text{CH}}$ (usually > 200 Hz), and may appear as weak signals displaying bizarre sign behaviour. Even more unexpected can be the appearance of the *quaternary* carbon of a terminal alkyne group, which experiences polarisation transfer from the alkyne proton two bonds away because of the exceptionally large $^2J_{\text{CH}}$ value (~ 50 Hz).

Optimising sensitivity

It was shown above that for the refocused INEPT experiment optimum sensitivity was obtained for a given multiplicity when the refocusing period was set according to the number of coupled protons and the associated coupling constant. When DEPT is used primarily as a means of enhancing the sensitivity of the heteronucleus rather than for spectrum editing, similar considerations apply. The difference here is that it is the θ proton pulse that must be optimised according to the number of spins in the XH_n group according to:

$$\theta_{\text{opt}} = \sin^{-1} \left(\frac{1}{\sqrt{n}} \right) \quad (4.5)$$

Notice the similarity with Eq. (4.3) except that the dependence on J is again not relevant for DEPT. For an XH group, θ_{opt} is 90° , whilst this angle decreases with higher multiplicities.

4.4.4. DEPTQ

The polarisation transfer sequences presented above provide signal enhancement of insensitive nuclei but suffer from a lack of responses from those centres that do not possess directly bound protons. Conversely, the spin-echo-based editing sequences do display such responses but gain enhancement only from the NOE and require signal averaging that is dictated by the typically slower-relaxing insensitive spins. As an alternative, the DEPTQ experiment [39] (DEPT retaining quaternary centres) operates in a similar manner to DEPT and so benefits from the advantages of polarisation transfer but also seeks to retain responses from quaternary centres and thus has obvious appeal for routine ^{13}C analysis of small molecules.

In the original DEPT method it was necessary to remove responses arising from direct carbon excitation by appropriate phase cycling because they would appear 90° out of phase with those arising from polarisation transfer (in other words they would appear confusingly as dispersive contributions) meaning also that quaternary signals were lost. The DEPTQ sequence (Fig. 4.35a) retains the same polarisation transfer elements as DEPT and the edited responses for all protonated centres remain the same. In addition, it incorporates a ^{13}C $90\text{-}1/2J\text{-}180$ element at the beginning of the sequence designed to excite quaternary centres and subsequently manipulate the behaviour of these such that they appear with the same phase response as CH_2 resonances and hence appear of opposite sign to those of CH and CH_3 groups (Table 4.4). For quaternary centres the complete sequence of ^{13}C pulses merely acts as two refocusing spin-echoes and the final spectrum retains all ^{13}C resonances with all the advantages of DEPT.

In practice this basic implementation of DEPTQ still suffers from limitations in the efficiency of responses arising from quaternary centres, meaning these are often weaker than observed in a simple pulse-acquire carbon experiment, especially at the far edges of the spectrum. This failing can be traced to three principal causes: (1) the use of a 90° ^{13}C excitation pulse, larger than would usually be suggested as the optimum Ernst angle, (2) the lack of potential NOE enhancements onto the quaternary centres, since the ^1H decoupling is gated off during the recovery period to allow for proton relaxation prior to polarisation transfer, and (3) losses due to off-resonance effects of the ^{13}C pulses, especially the 180° refocusing pulses. These issues have been addressed in a modified form of DEPTQ [40] illustrated in Fig. 4.35b. In this the first ^{13}C pulse tip angle is now reduced, requiring the incorporation of an additional ^{13}C 90° purging pulse immediately prior to data acquisition to remove phase errors in the quaternary responses. The proton recovery delay has also been extended and split in two parts initially maintaining ^1H decoupling to generate $^1\text{H}\text{-}^{13}\text{C}$ NOEs but then gating this off to allow for the necessary ^1H magnetisation recovery. Finally, the two ^{13}C 180° refocusing pulses are both applied as frequency-swept pulses (Chapter 10) which are effective over far greater bandwidths and thus seek to overcome the off-resonance losses associated with hard pulses. As a final modification, optional gradient pulses may be incorporated to provide for cleaner signal selection ($G_1 = G_2 = G_3$) or can be

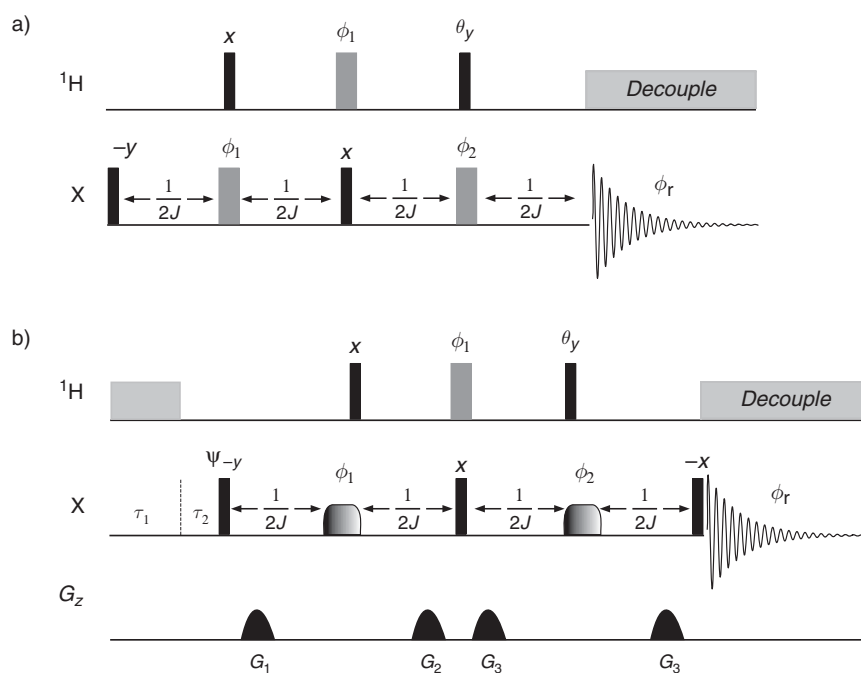


Figure 4.35. The DEPTQ sequences. (a) The original sequence, (b) the enhanced sequence in which the recovery delay is split in two (τ_1 and τ_2 , with and without ^1H decoupling, respectively) and the initial X pulse has reduced tip angle (ψ). The part-shaded pulses represent adiabatic 180° refocusing pulses. Phase cycling follows $\phi_1 = x, -x, y, -y$, $\phi_2 = x, -x, x, -x$ and $\phi_r = y, -y, -y$.

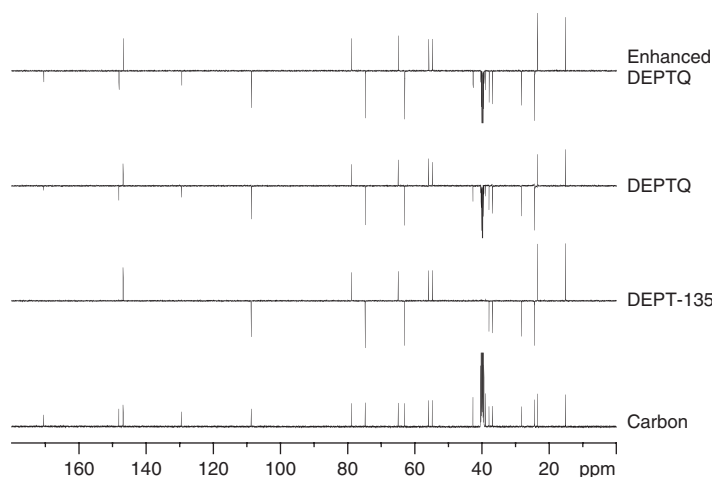


Figure 4.36. The carbon, DEPT-135, DEPTQ (distortionless enhancement by polarization transfer retaining quaternary centres) and enhanced DEPTQ spectra of the terpene andrographolide **4.6**.

used to select only protonated centres ($G_1 = 0$, $G_2 = G_3$) or only quaternaries ($G_1 = G_2 \neq G_3$); see Chapter 5 for a description of pulsed field gradient methodology.

A comparison of DEPT-135 with both the original and enhanced DEPTQ is made in Fig. 4.36 demonstrating the retention of quaternary centres and the improved performance achievable with the enhanced version. This method would now appear to be the optimal approach to spectrum editing with retention of all carbon centres and is attractive as a routine carbon-13 screening tool for the organic laboratory. The use of frequency-swept (adiabatic) 180° ^{13}C pulses is equally valid for the standard DEPT sequence, or indeed any using ^{13}C 180° pulses, and should also provide enhanced performance when large carbon bandwidths become problematic; see Chapter 10 for more information on these.

4.4.5. PENDANT

One further sequence that attempts to combine polarisation transfer with retention of unprotonated centres is PENDANT [41] (Polarisation Enhancement Nurtured During Attached Nucleus Testing) (Fig. 4.37). This relies on polarisation transfer from protons as for INEPT and indeed the sequence bears close similarity with the refocused-INEPT of Fig. 4.23b. From the point of view of protonated nuclei, PENDANT can, in essence, be understood by reference to this experiment. However, it also contains an additional $90^\circ(\text{X})$ pulse to elicit responses from non-protonated nuclei and lacks the usual phase cycling employed which suppresses such signals. Spectrum editing is achieved by judicious choice of the refocusing period, with $5/8J$ suggested as optimum for $1/2\Delta_2$. This should allow the simultaneous observation of both protonated and non-protonated carbons with the same form of editing as the DEPTQ experiment described above, although in practice quaternary carbons can still appear with rather low intensity. For the routine editing of carbon spectra, DEPTQ (especially in its enhanced form) would seem to be a more robust technique. The PENDANT sequence would appear to offer greater benefit in the transfer of polarisation through *long-range* couplings, particularly in $\text{X}-\text{H}_n$ systems where n is large ($3 < n < 9$), for which the $5/8J$ delays in the refocusing period becomes $1/16J$ for optimum results [42].

Before leaving these sections on the editing of X-nucleus spectra, some comments on the utility of such experiments in the modern NMR laboratory are required. As has already been mentioned, techniques based on proton observation are now used routinely for organic structure elucidation. Developments in this area have evolved techniques in which both 1D and more usefully 2D spectra are edited in such a way as to indicate X-nucleus multiplicities (Chapter 6). The higher sensitivity possible with these methods compared with direct X-nucleus observation, together with the additional correlation data provided by 2D techniques,

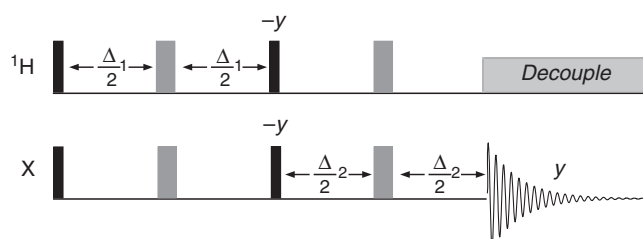


Figure 4.37. The PENDANT sequence. Phase cycling is not used since suppression of natural magnetisation is not required in this case.

have led to a reduced dependence on the traditional, more time-consuming X-nucleus observation and editing experiments, most notably where sample masses become limiting. However, for routine analysis of novel synthetic materials, and their associated characterisation to enable publication, 1D carbon observe techniques remain popular with synthetic chemists and are likely to remain so with the advent of cryogenic probes.

4.5. OBSERVING QUADRUPOLEAR NUCLEI

A characteristic feature of many quadrupolar nuclei is the broad lines they produce, due to rapid quadrupolar relaxation (Section 2.5.5). The rapid recovery of the spins following excitation means they can often be acquired under conditions of very fast pulsing with full excitation by a 90° pulse, which is clearly beneficial for signal averaging purposes. However, the corresponding rapid decay of an FID can make the direct observation of nuclei with linewidths of hundreds or thousands of hertz experimentally challenging. Following an rf pulse, a delay is required for the probe circuits and receiver electronics to recover before the NMR signal can be digitised. One particular concern is so-called *acoustic ringing* in the probehead [43] which is most severe for low-abundance, low-frequency nuclei at lower fields and which may take many tens of microseconds to decay. In the observation of spin- $\frac{1}{2}$ nuclei a pre-acquisition delay of this order can be used without problem but in cases of fast relaxing nuclei, such as ^{17}O or ^{33}S , this can lead to a loss of much of the FID before detection begins (resonance linewidths of 1 and 5 kHz correspond to relaxation times of only about 320 and 60 μs , respectively). Not only does this compromise sensitivity it also introduces substantial phase errors to the spectrum. The use of shorter delays is therefore essential but leads to a significant contribution to the spectrum in the form of broad baseline distortions from spectrometer transient responses. Numerous sequences to suppress this ring-down contribution have been proposed, [43] two of which are illustrated in Fig. 4.38b and c. In both cases suppression of the acoustic response from the 90° excitation pulses is achieved in two scans by inverting the NMR signal phase on the second transient with an additional 180° pulse, together with simultaneous inversion of the receiver phase [44] (Fig. 4.38a). Whilst the NMR signals are added by this process, the acoustic response remains unchanged in the two experiments and therefore cancels with receiver inversion. The remaining unwanted feature is now the acoustic response from the additional 180° pulse, which is suppressed in a slightly different manner in the two sequences. With ACOUSTIC [45] (Alternate Compound One-eighties USeD to Suppress Transients In the Coil, Fig. 4.38b) the whole experiment is repeated with inversion of the 180° pulse. This, in effect, inverts the associated acoustic response relative to the first experiment so adding the data from the two experiments cancels this component. The alternative RIDE sequence [46] (RIng-down DELay, Fig. 4.38c) instead repeats the whole process but with inversion of the NMR response by inverting the phase of both 90° pulses, and subtracts this second experiment from the first. Both sequences suffer from potential problem with off-resonance effects because of the use of 180° pulses, but

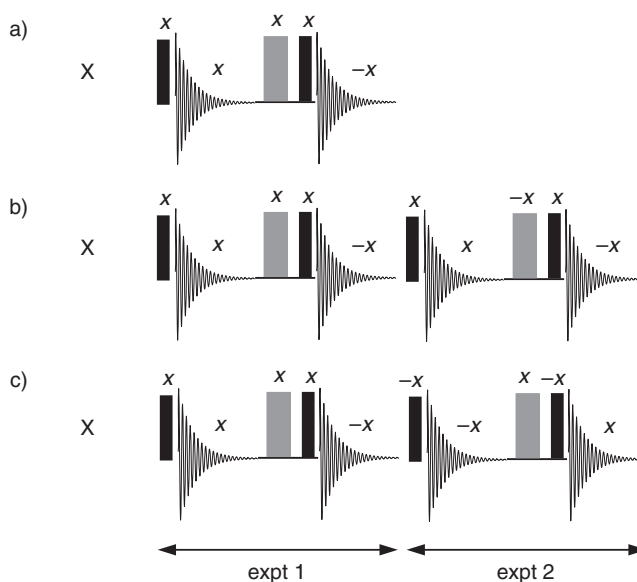


Figure 4.38. Sequences for the observation of quadrupolar nuclei with very broad lines for which acoustic ringing is a problem. Sequence (a) eliminates ringing associated with the 90° pulse whilst ACOUSTIC (b) and RIDE (c) further eliminate that associated with the 180° pulses.

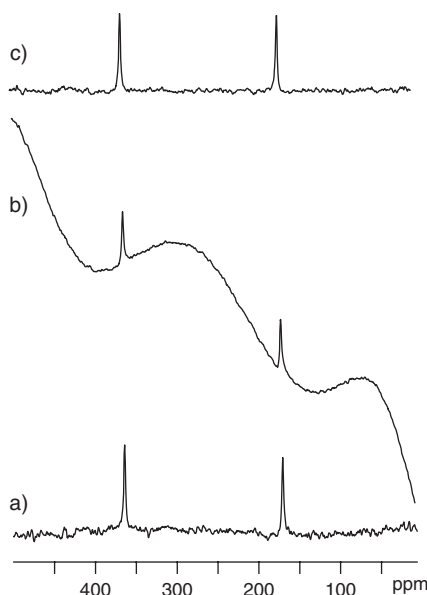


Figure 4.39. ^{17}O spectra of ethyl acetate recorded (a) with and (b) without the RIDE sequence. The severe baseline distortion in (b) arises from acoustic ringing in the probehead. Spectrum (c) was from the same FID of (b) but this had the first 10 data points replaced with backward linear predicted points, computed from 256 uncorrupted points. The spectra are referenced to D_2O and processed with 100 Hz line-broadening.

otherwise provide significant reductions in baseline distortions. Figure 4.39 illustrates the use of RIDE in the collection of ^{17}O spectrum of ethyl acetate, where the suppression of the baseline distortion is clear.

An alternative approach now available with modern processing software is to collect the distorted FID with the simple one-pulse-acquire sequence and to replace the early distorted data points with uncorrupted points generated through backward linear prediction. Figure 4.39c was produced from the same raw data as Fig. 4.39b, but with the first 10 data points of the FID replaced with predicted points. The baseline distortion is completely removed and there is no signal-to-noise loss through experimental imperfections, as occurs for the RIDE data set.

REFERENCES

- [1] R.R. Ernst and W.A. Anderson, *Rev. Sci. Instrum.*, 1966, **37**, 93–102.
- [2] D.E. Jones and H. Sternlicht, *J. Magn. Reson.*, 1972, **6**, 167–182.
- [3] E.D. Becker, J.A. Ferretti and P.N. Gambhir, *Anal. Chem.*, 1979, **51**, 1413–1420.
- [4] R. Freeman and H.D.W. Hill, *J. Magn. Reson.*, 1971, **4**, 366–383.
- [5] P. Waldstein and W.E. Wallace, *Rev. Sci. Instrum.*, 1971, **42**, 437–440.
- [6] J.S. Waugh, *J. Mol. Spectrosc.*, 1970, **35**, 298–305.
- [7] L. Barantin, A.L. Pap and S. Akoka, *Magn. Reson. Med.*, 1997, **38**, 179–182.
- [8] S. Akoka, L. Barantin and M. Trierweiler, *Anal. Chem.*, 1999, **71**, 2554–2557.
- [9] V. Molinier, B. Fenet, J. Fitremann, A. Bouchu and Y. Queneau, *Carb. Res.*, 2006, **341**, 1890–1895.
- [10] N. Michel and S. Akoka, *J. Magn. Reson.*, 2004, **168**, 118–123.
- [11] R.A. Hoffman and S. Forsén, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1966, **1**, 15–204.
- [12] W.A. Anderson and R. Freeman, *J. Chem. Phys.*, 1962, **37**, 85–103.
- [13] G. Massiot, S.K. Kan, P. Gonard and C. Duret, *J. Am. Chem. Soc.*, 1975, **97**, 3277–3278.
- [14] J.K.M. Sanders and J.D. Merish, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1982, **15**, 353–400.
- [15] F. Bloch and A. Siegert, *Phys. Rev.*, 1940, **57**, 522–527.
- [16] N.F. Ramsey, *Phys. Rev.*, 1955, **100**, 1191–1194.
- [17] J.P. Jesson, P. Meakin and G. Kneissel, *J. Am. Chem. Soc.*, 1973, **95**, 618–620.
- [18] R.W. Dykstra, *J. Magn. Reson.*, 1990, **88**, 388–392.
- [19] R.W. Dykstra, *J. Magn. Reson.*, 1992, **100**, 571–574.
- [20] R.W. Dykstra, *J. Magn. Reson. (A)*, 1993, **102**, 114–115.
- [21] D.W. Brown, T.T. Nakashima and D.L. Rabenstein, *J. Magn. Reson.*, 1981, **45**, 302–314.
- [22] C.L. Cocq and J.Y. Lallemand, *J. Chem. Soc., Chem. Commun.*, 1981, 150–152.
- [23] F.-K. Pei and R. Freeman, *J. Magn. Reson.*, 1982, **48**, 318–322.
- [24] S.L. Patt and J.N. Shoolery, *J. Magn. Reson.*, 1982, **46**, 535–539.
- [25] H. Bildsøe, S. Dønstrup, H.J. Jakobsen and O.W. Sørensen, *J. Magn. Reson.*, 1983, **53**, 154–162.
- [26] O.W. Sørensen, S. Dønstrup, H. Bildsøe and H.J. Jakobsen, *J. Magn. Reson.*, 1983, **55**, 347–354.
- [27] A.M. Torres, T.T. Nakashima and R.E.D. McClung, *J. Magn. Reson. (A)*, 1993, **101**, 285–294.
- [28] J. Ollerenshaw, T.T. Nakashima and R.E.D. McClung, *Magn. Reson. Chem.*, 1998, **36**, 445–448.
- [29] R. Freeman, *A Handbook of Nuclear Magnetic Resonance*, 2nd ed., Longman, Harlow, 1987.
- [30] G.A. Morris and R. Freeman, *J. Am. Chem. Soc.*, 1979, **101**, 760–762.
- [31] D.P. Burum and R.R. Ernst, *J. Magn. Reson.*, 1980, **39**, 163–168.

- [32] D.T. Pegg, D.M. Doddrell, W.M. Brooks and M.R. Bendall, *J. Magn. Reson.*, 1981, **44**, 32–40.
- [33] O.W. Sørensen and R.R. Ernst, *J. Magn. Reson.*, 1983, **51**, 477–489.
- [34] K.V. Schenker and W. von Philipsborn, *J. Magn. Reson.*, 1985, **61**, 294–305.
- [35] A. Mohebbi and O. Gonen, *J. Magn. Reson. (A)*, 1996, **123**, 237–241.
- [36] W. von Philipsborn and R. Müller, *Angew. Chem. Int. Ed.*, 1986, **25**, 383–413.
- [37] D.M. Doddrell, D.T. Pegg and M.R. Bendall, *J. Magn. Reson.*, 1982, **48**, 323–327.
- [38] M.R. Bendall and D.T. Pegg, *J. Magn. Reson.*, 1983, **53**, 272–296.
- [39] R. Burger and P. Bigler, *J. Magn. Reson.*, 1998, **135**, 529–534.
- [40] P. Bigler, R. Kümmerle and W. Bermel, *Magn. Reson. Chem.*, 2007, **45**, 469–472.
- [41] J. Homer and M.C. Perry, *J. Chem. Soc., Chem. Commun.*, 1994, 373–374.
- [42] J. Homer and M.C. Perry, *J. Chem. Soc., Perkin Trans. 2*, 1995, 533–536.
- [43] I.P. Gerothanassis, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1987, **19**, 267–329.
- [44] D. Canet, J. Brondeau, J.P. Marchal and B. Robin-Lherbier, *Org. Magn. Reson.*, 1982, **20**, 51–53.
- [45] S.L. Patt, *J. Magn. Reson.*, 1982, **49**, 161–163.
- [46] P.S. Belton, I.J. Cox and R.K. Harris, *J. Chem. Soc., Faraday Trans. 2*, 1985, **81**, 63–75.

Chapter 5

Correlations through the chemical bond I: Homonuclear shift correlation

Having discussed a number of methods that are considered routine 1D techniques in Chapter 4, this chapter leads into the world of two-dimensional NMR. Although many of the methods in Chapter 4 used through-bond interactions as a tool towards a specific goal, such as sensitivity enhancement through polarisation transfer, the two-dimensional methods in this and the following chapter exploit spin coupling to map specific through-bond interactions. It is quite likely that you have already made some use of two-dimensional methods in your research, most probably in the form of the COSY experiment, and it is with this technique that the chapter begins. Not only is this one of the most widely used 2D experiments, its simple form provides a convenient introduction to multi-dimensional experiments. Although the methods in this chapter are aimed at determining correlations between like spins (*homonuclear correlations*), a large part of the discussion is equally relevant to all two-dimensional NMR experiments, and many parts of this chapter provide the foundations for those that follow.

The principles underlying the generation of a two-dimensional spectrum were first presented in a lecture in 1971 [1], although it was a number of years later that the approach found wider application [2]. During the 1980s, the world of NMR, and consequently the chemist's approach to structural determination, was revolutionised by the development of numerous two-dimensional techniques, and nowadays many higher-dimensionality methods (3D and 4D) also exist. These methods utilising three or more dimensions have found greatest application in the hands of biological NMR spectroscopists studying macromolecules (a specialist area in its own right) and, at present, tend to be used rather less frequently within what may be considered main-stream organic chemistry. These methods therefore fall beyond the realms of this book but are covered in texts dedicated to biomolecular NMR [3, 4].

This chapter begins by introducing the principles that lie at the heart of all two-dimensional methods. The techniques themselves, summarised briefly in Table 5.1, begin with basic Correlation Spectroscopy (COSY), which maps nuclei sharing a mutual scalar coupling within a molecule, making the proton–proton COSY one of the workhorse techniques in organic structure elucidation. Later in the chapter, some variants of the basic COSY experiment that display a number of beneficial characteristics are presented and therefore also find widespread application. The last two sections cover somewhat different techniques. The first of these, Total Correlation Spectroscopy (TOCSY), provides an alternative to the COSY approach for establishing correlations within a molecule. This provides efficient transfer of information along a network of coupled spins, a feature that can be extremely powerful in the analysis of more complex spectra. The final method, INADEQUATE, establishes correlations between like spins of low natural abundance. This may be used, for example, to directly identify neighbouring carbon centres and in this form it is perhaps the ultimate experiment for defining a molecular skeleton, although, alas, it is also about the least sensitive.

The approach in this chapter is again a pictorial one. Although this avoids the need for becoming embroiled in formalisms that rigorously describe the behaviour of magnetisation during multi-pulse experiments, it has its limitations in that many of the techniques cannot be described completely by such a simplified approach. We have already come up against these limitations when attempting to understand the DEPT experiment in Chapter 4, where it was not possible to describe the behaviour of in terms of the classical vector model. An explanation of this technique was thus derived largely from the more readily understood INEPT sequence. Despite these limitations, it is still possible to develop some physical insight into how the experiments operate without resorting to esoteric mathematical descriptions. Section 5.4 introduces graphical formalisms, known as *coherence transfer pathways*, that provide a simple representation of the 'flow' of magnetisation during pulse experiments, which proves particularly enlightening for two-dimensional sequences and for those experiments that utilise pulsed field gradients for signal selection. Before any of this, however, we must first develop some feel for how two-dimensional spectra are generated.

Table 5.1. The principal applications of the main techniques described in this chapter

Technique	Principal applications
COSY-90	Correlating coupled homonuclear spins. Typically used for correlating protons coupled over two or three bonds but may be used for any high-abundance nuclide. The basic COSY experiment
DQF-COSY	Correlating coupled homonuclear spins. Typically used for correlating protons coupled over two or three bonds. Higher-resolution display than basic COSY. Additional information on magnitudes of coupling constants may be extracted from 2D peak fine structure. Singlets suppressed
COSY- β	Correlating coupled homonuclear spins. Typically used for correlating protons coupled over two or three bonds but may be used for any high-abundance nuclide. Reduced 2D peak structure over basic COSY. Vicinal and geminal coupling relationships can be differentiated in some cases
Delayed COSY	Correlating coupled homonuclear spins through small couplings. Often used to identify proton correlations over many bonds (>3), hence also known as long-range COSY
TOCSY	Correlating coupled homonuclear spins and those that reside within the same spin system but which may not share mutual couplings. Employs the propagation of magnetisation along a continuous chain of spins. Powerful technique for analysing complex proton spectra
INADEQUATE	Correlating coupled homonuclear spins of low natural abundance ($<20\%$). Typically used for correlating adjacent carbon centres at natural abundance but has extremely low sensitivity
ADEQUATE	Correlating coupled homonuclear spins of low natural abundance, primarily ^{13}C – ^{13}C , but employing ^1H excitation and detection for sensitivity improvement

5.1. INTRODUCING TWO-DIMENSIONAL METHODS

The first point to clarify when discussing two-dimensional techniques is the fact that the two dimensions refer to two *frequency* dimensions, whereas the so-called 1D methods have, of course, only one. In either case, there will also be a dimension representing signal *intensity*, although this is never usually included when describing the dimensionality of an experiment. The two frequency dimensions may represent any combination of chemical shifts or scalar couplings. More recently, methods have been developed where the idea of a two-dimensional representation has been adapted to include one frequency and one ‘other’ dimension. For example, hybrid HPLC-NMR methods are capable of producing 2D plots with the usual 1D NMR spectrum comprising one dimension and the chromatographic retention time in the other. Likewise, it is possible to disperse the spectra of solutes according to their diffusion properties, in which case the second dimension represents the self-diffusion coefficients (Chapter 9).

Conventional two-dimensional spectra find such wide utility in chemical research because they map out interactions within, or sometimes between, our molecules of interest. The interactions that can be probed may be broken down into three distinct categories, which relate to quite different physical phenomena: through-bond coupling, through-space coupling and chemical exchange. The first of these is considered in this and the following chapter under the division of homonuclear (between like nuclei, e.g. ^1H – ^1H) and heteronuclear (between unlike nuclei, e.g. ^1H – ^{13}C) couplings, and the experiments considered are principally aimed at the identification and subsequent piecing together of structural fragments within a molecule. Through-space coupling provides the basis for the nuclear Overhauser effect, which is most often employed to deduce molecular stereochemistry and conformation, as described in Chapter 8. Lastly, the mapping of chemical exchange pathways, which may be intra- or inter-molecular, will also be considered in Chapter 8 since the methods for studying both the NOE and the exchange are essentially identical.

5.1.1. Generating a second dimension

No matter what the nature of the interaction to be mapped, all two-dimensional sequences have the same basic format and can be subdivided into four well-defined units termed the *preparation*, *evolution*, *mixing* and *detection* periods (Fig. 5.1). The preparation and mixing periods typically comprise a pulse or a cluster of pulses and/or fixed time periods, the exact details of which vary depending on the nature of the experiment. The detection period is entirely analogous to the detection period of any one-dimensional experiment during which the spectrometer collects the FID of the excited spins. The evolution period provides the key to the generation of the second dimension. Before proceeding, briefly recall what happens during the acquisition of a one-dimensional FID and the processes that generate a single-frequency-domain spectrum. The detection process

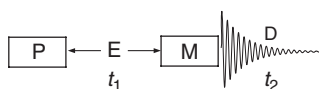


Figure 5.1. The general scheme for any two-dimensional experiment. P, Preparation; E, evolution; M, mixing and D, detection.

involves sampling (digitising) the oscillating FID at regular time intervals dictated by the Nyquist condition for an appropriate acquisition period. The collected data are then subject to Fourier transformation to produce the required frequency domain spectrum. In other words, the general requirement for creating a frequency dimension is the regular sampling of magnetisation as it varies in some way as a function of time. Extending this idea, one can conclude that to generate a spectrum with *two* frequency domains, f_1 and f_2 , it is necessary to sample data as a function of *two* separate time variables, t_1 and t_2 . Clearly one time domain and hence one frequency domain originate from the usual detection period (t_2 in Fig. 5.1), but how does the other arise?

To illustrate the required procedure, consider a simple pulse sequence in which both the preparation and mixing units of Fig. 5.1 are each single 90_x pulses (Fig. 5.2) acting on a sample that contains only a single, uncoupled proton resonance, say chloroform, with a chemical shift offset of ν Hz. Viewing events with the vector model (Fig. 5.3), the initial 90_x pulse places the equilibrium magnetisation in the x - y plane along the $+y$ axis, after which it will precess (or *evolve*) according to this chemical shift offset. After a time period t_1 , the vector moves through an angle of $360 \nu t_1$ degrees and is then subject to the second pulse. To appreciate the influence of this pulse, it is convenient to consider the vector as comprising two orthogonal components, one along the original y axis ($M_0 \cos 360 \nu t_1$) and the other along the x axis ($M_0 \sin 360 \nu t_1$). The second 90_x pulse places the y -component along the $-z$ axis whilst the x -component is unaffected and remains precessing in the transverse plane and produces the detected FID. Fourier transformation of this FID will therefore produce a spectrum containing a single resonance whose intensity depends upon the factor $\sin 360 \nu t_1$.

Performing this experiment with t_1 set to zero means the two 90_x pulses will add to produce a net 180_x pulse, which simply corresponds to inversion of the equilibrium vector. As there exists no transverse magnetisation, there is no signal to detect, and the spectrum contains only noise. Now imagine repeating the experiment a number of times with the t_1 interval increased by a uniform amount each time and the resulting FIDs stored separately. As t_1 increases from zero, the resulting signal intensity also increases as x -magnetisation has time to develop during the evolution period, reaching a maximum when it has evolved through an angle of 90° . Further increases in t_1 cause the x -component to diminish, pass through a null, then become negative and so on according to the sine modulation. Subjecting each of the acquired t_2 FIDs to Fourier transformation produces a series of spectra containing a single resonance whose intensity (or *amplitude*) varies as a function of time according to $\sin 360 \nu t_1$ (Fig. 5.4). With longer values of t_1 , relaxation effects diminish the intensity of the transverse magnetisation according to T_2^* , so the signal intensities show a steady (exponential) decay in addition to the *amplitude modulation*. The intensity of the resonance as a function of time therefore represents another FID for the t_1 time domain (referred to as an interferogram) that has been generated artificially or *indirectly* (Fig. 5.5). The frequency of the amplitude modulation corresponds to the chemical shift offset of the resonance in the rotating frame, and we say the magnetisation has been *frequency-labelled* as a function of t_1 . Subjecting this time-domain data to Fourier transformation, one would again expect this to produce a single resonance with a chemical shift of ν Hz in the f_1 frequency domain, just as for the f_2 domain. Generalising this process requires monitoring the intensity of *every data point* in the f_2 domain as a function of t_1 to produce a complete 2D data set. Following

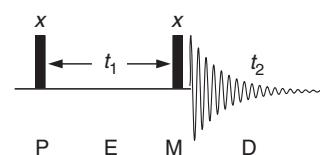


Figure 5.2. An illustrative two-dimensional sequence in which P and M are 90° pulses. This is also the basic COSY sequence.

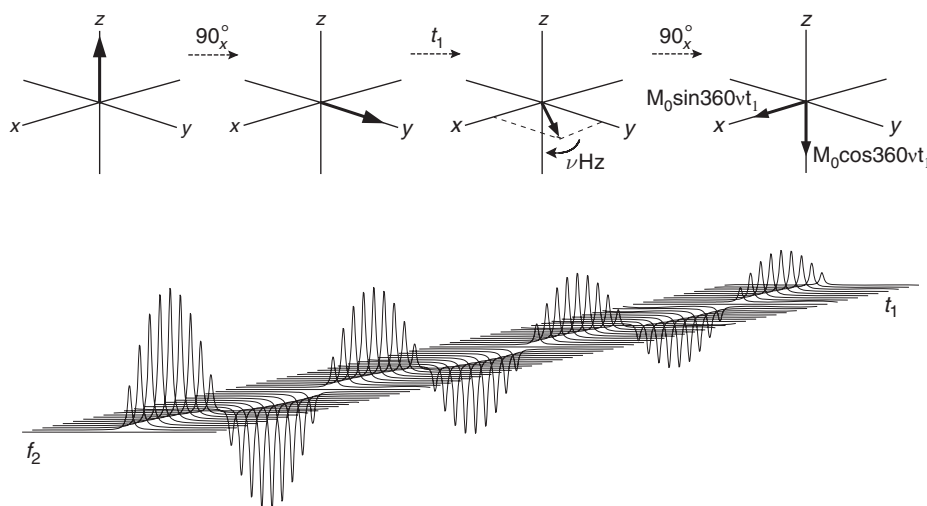


Figure 5.3. The action of the COSY sequence of Fig. 5.2 on a single, uncoupled proton resonance.

Figure 5.4. Amplitude modulation of a singlet resonance as a function of the evolution period t_1 . At longer values of t_1 , the signal intensity is diminished by spin relaxation.

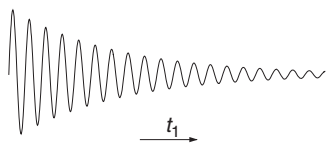


Figure 5.5. The variation in peak intensity of the amplitude-modulated resonance of Fig. 5.4 produces an FID (interferogram) for the t_1 domain.

Fourier transformation with respect to t_1 , another complete frequency domain is generated, which, in combination with the conventional domain, produces a two-dimensional spectrum displaying a single resonance at ν Hz in both dimensions (Fig. 5.6). Note that the labelling of the frequency axes as f_1 and f_2 follows from the ordering of the corresponding time domains in the pulse sequence, that is, t_1 followed by t_2 . The new artificial (t_1) time domain has been sampled discretely by recording a FID for each t_1 time point and storing each separately, analogous to the sampling of the t_2 data points described earlier. The repeated acquisition of FIDs with systematically incremented t_1 time periods (Fig. 5.7) is fundamental to the generation of *all* two-dimensional data sets. Indeed, this simple idea can be extended to produce a three-dimensional spectrum simply by having three independently incremented time periods, one detected 'directly' and two 'indirectly' (Fig. 5.8); although the technical details may be a little complex, the principle is quite straightforward. The philosophy behind the multi-dimensional approach is that one signal modulated as a function of one time variable (say t_1) is detected at some later point as a function of another (t_2 in the case of a 2D experiment). These general descriptions apply equally to spectra that contain many resonances. Repeating the above experiment for a sample containing just two uncoupled spins with offsets of ν_A and ν_X Hz results in a spectrum with two 2D singlets whose frequencies in both dimensions correspond simply to the respective chemical shift offsets of the resonances (Fig. 5.9).

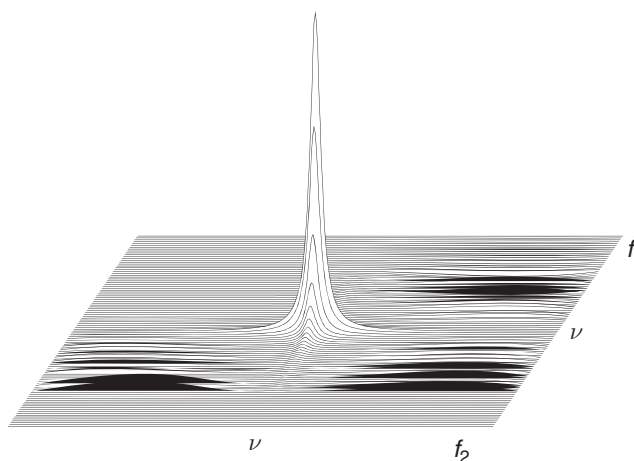


Figure 5.6. The two-dimensional spectrum resulting from the sequence of Fig. 5.2 for a sample containing a single uncoupled spin. One peak results with a shift of ν Hz in both dimensions.

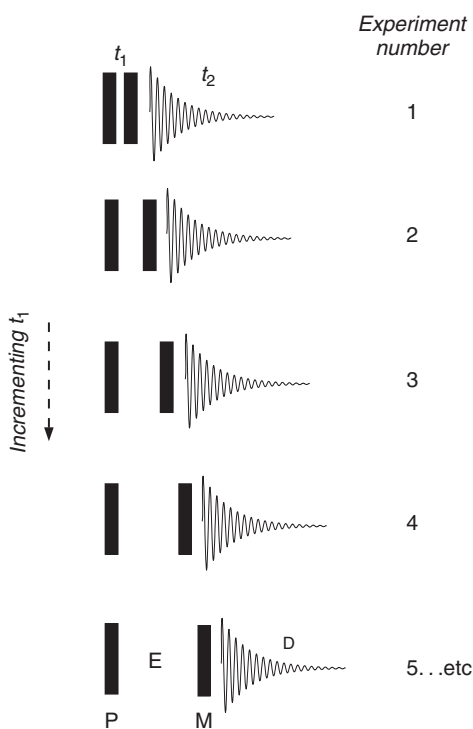


Figure 5.7. A generalised scheme for the collection of a two-dimensional data set. The experiment is repeated many times with the t_1 period incremented at each stage and the resulting FIDs stored separately. Following double Fourier transformation with respect to first t_2 and then t_1 , the two-dimensional spectrum results.

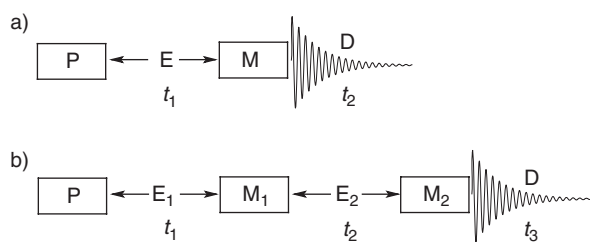


Figure 5.8. Schematic representations of (a) two-dimensional and (b) three-dimensional experiments. A 3D experiment is collected by independently varying the t_1 and t_2 intervals to generate the f_1 and f_2 domains, respectively, by analogy with a 2D experiment.

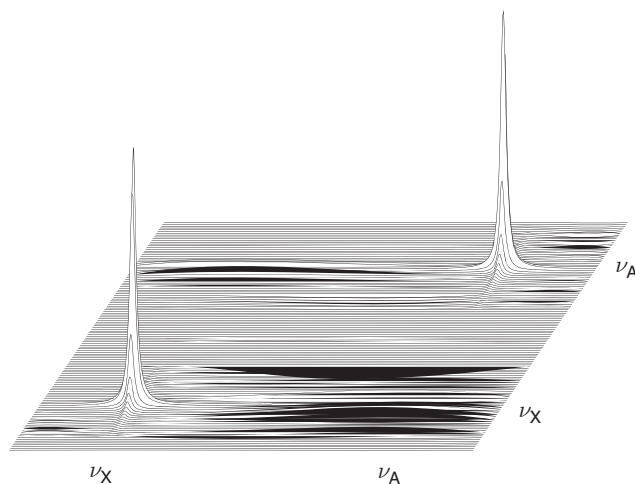


Figure 5.9. The two-dimensional spectrum resulting from the sequence of Fig. 5.2 for a sample containing two uncoupled spins, A and X, of offsets ν_A and ν_X . Each produces a 2D peak at its corresponding chemical shift offset in both dimensions.

Having gone to the trouble of generating a two-dimensional data set, the spectra of Figs 5.6 and 5.9 tell us nothing that we could not derive from the corresponding one-dimensional spectra, simply the chemical shifts of the participating nuclei. This provides no new information because the 2D peaks map (or correlate) exactly the same information in both dimensions ($\nu_1 = \nu_2$), in this case the chemical shifts. Two-dimensional spectra become useful when the peaks they contain correlate *different* information on the two axes, that is, when $\nu_1 \neq \nu_2$. This requires that magnetisation evolving at frequency ν_1 during the t_1 time period then evolves at a different frequency ν_2 in the detection (t_2) period. Clearly for this to happen, there must be some mechanism by which the magnetisation precession frequency changes during the sequence, and more specifically within the mixing period. The details of this period dictate the information content of the resulting spectrum according to the interaction that is exploited, that is coupling, NOE or chemical exchange. Just how the informative correlation peaks are produced will become apparent as various sequences are examined in the sections that follow.

Before moving on, a comment on the presentation of 2D spectra is required. The spectra of Figs 5.6 and 5.9 have been presented in the ‘stacked-plot’ mode to emphasise the similarity with 1D spectra and the presence of two frequency axes and one intensity axis. Although these may look aesthetically impressive, this form of presentation is of little use in practice. The usual way to present 2D spectra is via ‘contour plots’, in which peak intensities are represented by contours, as a mountain range would be represented on a map. Figure 5.10 shows the equivalent contour presentation of Figs 5.6 and 5.9, and unless stated otherwise, all 2D spectra from now on will make use of this contour mode.

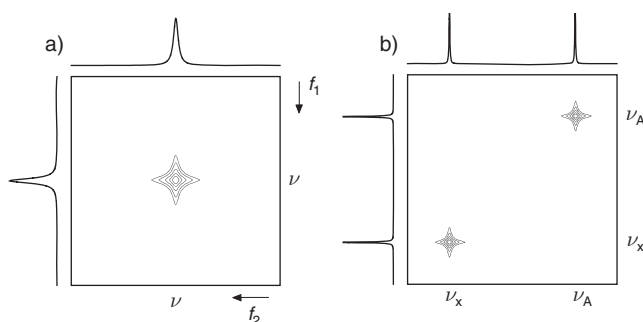


Figure 5.10. The equivalent contour plot representations of Figs 5.6 and 5.9. Alongside the f_1 and f_2 axes are the conventional 1D spectra for reference purposes.

5.2. CORRELATION SPECTROSCOPY (COSY)

It was stated in the opening remarks of this chapter that COSY was the first two-dimensional sequence proposed, and it is in fact given in Fig. 5.2, utilising just two 90° pulses. The sequence, which correlates the chemical shifts of spins that share a mutual J -coupling, is most often applied in proton spectroscopy although it is equally applicable to any high-abundance nuclide. It is without doubt the mostly widely used of all two-dimensional methods and is thus considered first. This section provides an introduction to the operation of the experiment and its principal features, after which you should be in a position to understand and interpret routine COSY spectra. Later sections look at more specific and useful features of the basic experiment and those of its cousins.

5.2.1. Correlating coupled spins

Moving on from the previous discussions concerning uncoupled spins, we now consider the more general case of spins that are scalar-coupled. We first note that the frequency labelling component of the experiment described above is in operation for all excited spins, so it is unnecessary to consider this further. Of interest now is the mixing part of the sequence, in this case the action of the pulse following t_1 . In short, this pulse transfers 'magnetisation' originally associated with one spin onto neighbouring spins to which it is coupled, this process being known formally as *coherence transfer* (Section 5.4), which is possible only because of the J -coupling. Coherence transfer has, in fact, already been described in Chapter 4, where it was presented in a slightly different guise as polarisation transfer from proton to carbon, which is nothing more than *heteronuclear coherence transfer*. In this, the original spin populations ('magnetisation') associated with the proton were ultimately transferred to carbon. In the heteronuclear case, events could be readily pictured by reference to population diagrams (SPI) and to the vector model (the INEPT sequence), because one could consider the action of pulses on the source proton and the target carbon quite independently. In the homonuclear case, things are rather more complicated because all spins experience the same pulses simultaneously and the simple picture of events tends to break down. Nevertheless, similar transfer processes are operative, and with COSY, we simply have the analogous *homonuclear coherence transfer* taking place between spins. The similarity can be further exemplified if one compares the COSY sequence of Fig. 5.2 with the basic INEPT sequence of Fig. 4.23a. Ignoring the refocusing 180° pulses in INEPT, the two sequences are largely identical, both with initial excitation being followed by a period of evolution, after which *both* coupled spins experience a 90° pulse, which elicits the transfer. The concept of coherence and coherence transfer is central to most NMR experiments, and this is further considered in Section 5.4.

The key point in all this is that magnetisation transfer occurs between coupled spins. To appreciate the outcome of this in the final COSY spectrum, consider the case of two J -coupled spins, A and X, with a coupling constant of J_{AX} and chemical shift offsets of ν_A and ν_X . The magnetisation associated with spin A will, after the initial 90° pulse, precess during t_1 according to its chemical shift offset, ν_A . The second 90° pulse then transfers some part of this magnetisation to the coupled X spin, whilst some remains associated with the original spin A (the reason for this segregation is described in Section 5.2.3). That which remains with A will then precess in the detection period at a frequency ν_A , just as it did during t_1 , so in the final spectrum, it will produce a peak at ν_A in both dimensions, denoted (ν_A, ν_A) . This peak is therefore equivalent to that observed for the uncoupled AX system of Fig. 5.9, and because it represents the same frequency in both dimensions, it sits on the diagonal of the 2D spectrum and is therefore referred to as a *diagonal peak*. In contrast, the transferred magnetisation will precess in t_2 at the frequency of the new 'host' spin X and will thus produce a peak corresponding to two different chemical shifts in the two dimensions (ν_A, ν_X) . This peak sits away from the diagonal and is therefore referred to as an off-diagonal or, more commonly, a *crosspeak* (Fig. 5.11). This is the peak of interest as it provides direct evidence of coupling between spins A and X. The whole process operates in the reverse direction also, that is, the same arguments apply for magnetisation originally associated with the X spin, giving rise to a diagonal peak at (ν_X, ν_X) and a crosspeak at (ν_X, ν_A) . Thus, the COSY spectrum is symmetrical about the diagonal, with crosspeaks on either side of it mapping the same interaction (Fig. 5.11).

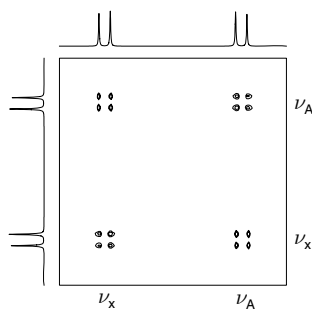


Figure 5.11. The COSY spectrum of a coupled, two-spin AX system. Diagonal peaks are equivalent to those observed in the 1D spectrum whilst crosspeaks provide evidence of a coupling between the correlated spins.

5.2.2. Interpreting COSY

The application of the COSY technique in spectral assignment is best illustrated by considering some real examples. The COSY spectrum of the azo-sugar **5.1**, a monomer for the synthesis of oligomeric carbopeptoids, demonstrates both the advantages of the technique and some of its limitations or potential pitfalls (Fig. 5.12). The goal is to assign all proton resonances in the spectrum of **5.1**. In this simple example, this could be accomplished through considered analysis of the multiplet fine structure of each resonance. However, this process fails when spectra display significant resonance overlap, and the greater dispersion available in two dimensions can often bypass such limitations. Furthermore, COSY has the added advantage of speed, by avoiding the need to employ time-consuming analysis of the 1D spectrum. Naturally, one may return to such levels of detail when assignments have been made and the gross structure identified to glean additional structural data, from coupling constants for example, but COSY can very rapidly provide evidence in support of a structure without the need for this. The first step in analysis is the matching of diagonal peaks to the equivalent resonances in the 1D spectrum, as this provides orientation for all subsequent assignments. Coupled spins are then correlated by stepwise movements, starting and finishing at a diagonal peak via an intermediate crosspeak. Assignments for **5.1** begin with the characteristic doublet of H2. Correlation to its vicinal partner in the sugar is made via either of the symmetrically related crosspeaks, which thus assigns H3 and so on. This trivial procedure provides the basis on which COSY spectra are analysed. The power and simplicity of the experiment is further illustrated by a second example, a carbopeptoid dimer **5.2** (Fig. 5.13). In this case, two discrete spin systems exist within the molecule on adjacent sugar rings, and the differentiation and assignment of these follows readily from the characteristic doublets of H2 in each. The two ring systems are traced in Fig. 5.13, above and below the diagonal in red (ring A) and black (ring B) for clarity. Further correlations from the H6 protons of ring B to the adjacent amide NH are not

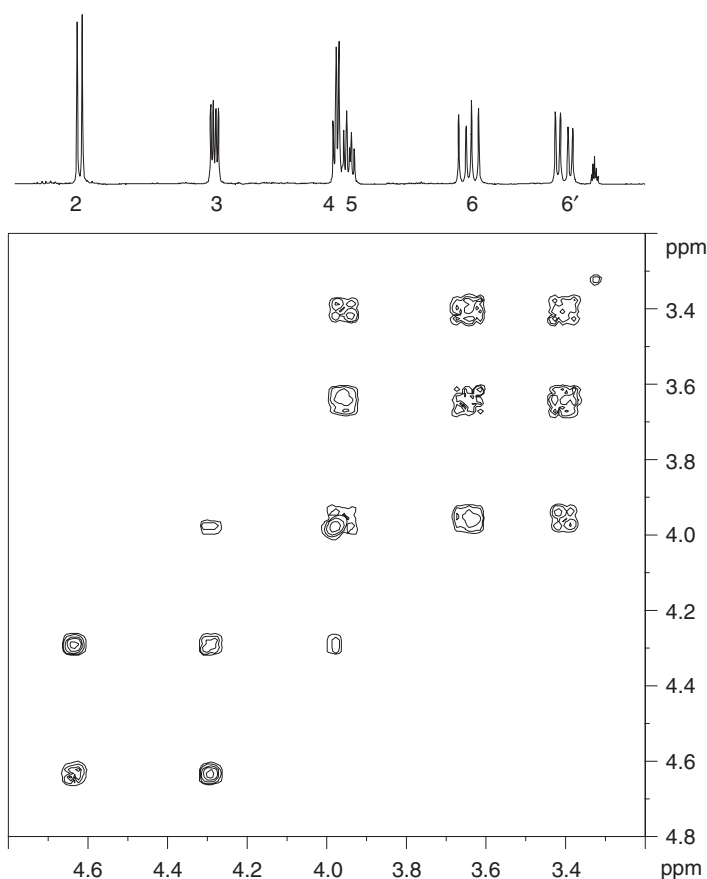
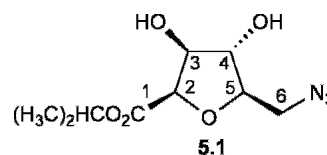


Figure 5.12. The 400-MHz COSY spectrum of the azo-sugar **5.1** in MeOD. Shown above is the conventional 1D spectrum.

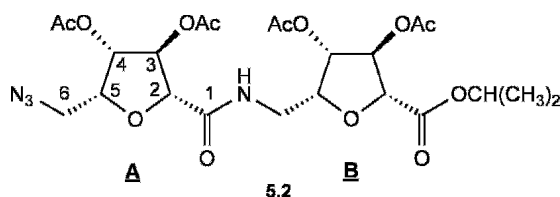
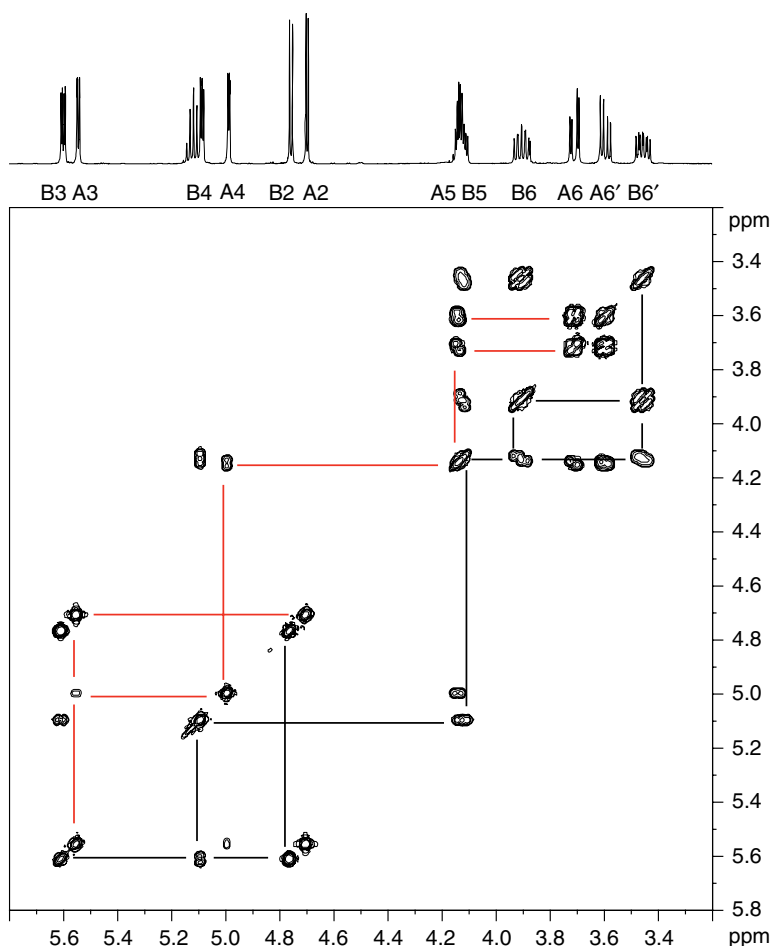


Figure 5.13. The 500-MHz COSY spectrum of the carbopeptoid dimer **5.2** in CDCl₃ shown with the 1D spectrum above. Traced in red above the diagonal are the assignments for sugar ring A and in black below the diagonal are those for sugar ring B (this plot excludes correlations to the amide proton of residue B). Both can be assigned starting from the H2 proton of each sugar. The rings are labelled from the N-terminus of the carbopeptoid and the sugar ring numbering follows IUPAC recommendations for carbohydrate nomenclature.



shown in this figure, but their presence readily differentiates rings A and B. Indeed, correlations from the lone amide resonance would also provide a suitable starting point to map ring B.

The COSY experiment, despite its simplicity, is, however, subject to a number of limitations and caveats that one should also be aware of. The correlation between H4 and H5 in Fig. 5.12 is almost lost due to the near degeneracy of these resonances, and when coupled spins overlap in this manner, the stepwise assignment process can break down since it may not be clear from which resonance subsequent correlations should be traced. Some caution is required in such instances to avoid incorrect assignments, and more sophisticated methods may ultimately be required to provide unambiguous results. With complex structures, one may be left with various groups of coupled spins identified from COSY spectra, which cannot be linked through homonuclear correlations. These pieces of the molecular ‘jigsaw’ require additional experimental data if they are to be joined together, such as from heteronuclear correlation experiments or NOE measurements. Most proton–proton couplings operate over two or three bonds, so the proton COSY spectrum typically identifies vicinal and geminal relationships. A further general limitation of COSY is the inability, *a priori*, to differentiate between these two types of correlation; in Fig. 5.12, the vicinal H5–H6 crosspeaks appear similar to the geminal H6–H6′ crosspeak. When analysing COSY, these two possible relationships should be borne in mind, particularly when diastereotopic geminal protons may be anticipated, notably in cyclic systems or those with stereogenic centres. A variant on the basic COSY sequence (COSY- β , Section 5.6.3) can, in favourable circumstances, differentiate between vicinal and geminal relationships, and this method also finds widespread use. Structural features, such as unsaturation or the well known *w*-geometry, can also enhance long-range coupling constants, and COSY can be surprisingly effective at revealing these correlations. As such, it is *not* safe to automatically assume *all* crosspeaks correspond to either vicinal or geminal coupling relationships. Geometrical factors can also cause vicinal couplings to be close to zero, in which case no COSY crosspeak is produced despite the presence of adjacent protons. Thus, the *absence* of a crosspeak between protons does *not* always exclude them from being adjacent in a structure.

The COSY experiment is not limited to proton spectroscopy but is suitable for establishing homonuclear correlations for any high-abundance nuclide, the most common examples

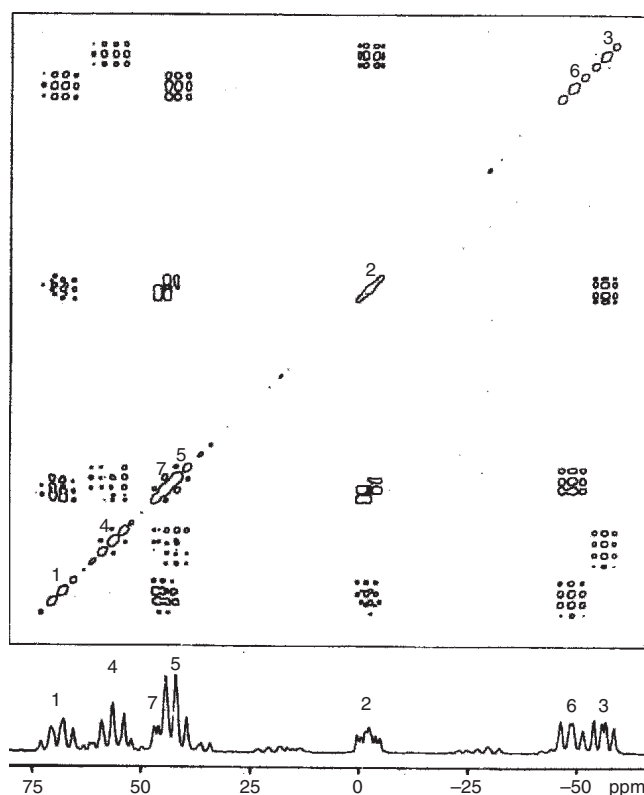
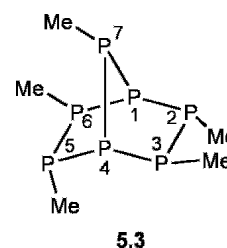


Figure 5.14. The ^{31}P - ^{31}P COSY spectrum of the P_7Me_5 cluster **5.3** with the ^{31}P 1D spectrum below (reproduced with permission from [5], Copyright Wiley-VCH Verlag GmbH & Co. KGaA.).

being ^{19}F , ^{31}P or ^{11}B . Figure 5.14 illustrates the ^{31}P - ^{31}P COSY spectrum of the P_7Me_5 cluster **5.3** for which the assignment procedure is exactly as described for the proton spectra above. In this case, single-bond P-P couplings are identified directly, along with a lone double-bond coupling between P^2 and P^7 , which arises from the sizeable $^2J_{\text{PP}}$ coupling.



5.2.3. Peak fine structure

The spectrum of Fig. 5.11 also reveals, within each diagonal peak and crosspeak, fine structure that is equivalent to the structure seen within the one-dimensional multiplets. Whether such fine structure is always resolved depends on the experimental settings used in the acquisition of the data. Although in the illustrative example of Fig. 5.11 the data were recorded so as to reveal these details, the fine structure in Figs 5.12 and 5.13 is not resolved, as is often the case in routine analysis. Interpretation of multiplet fine structure can, under the appropriate experimental conditions, provide valuable information, a topic addressed in Section 5.5.2 where we encounter a version of the COSY experiment better suited to this purpose. To realise the origin of this fine structure in the 2D spectrum, it is necessary to slightly modify the description of magnetisation transfer given above. It was stated that this transfer occurs between coupled spins but more precisely one should say it occurs between the *transitions* associated with these spins. Reference to the energy level diagram for a coupled two-spin system (Fig. 5.15) should clarify this. Magnetisation associated with, say, transition A_1 , which gives rise to one half of the A spin doublet, is redistributed to the other three transitions A_2 , X_1 and X_2 by the second 90° pulse of the COSY sequence. That associated with A_2 during the detection period gives rise to the fine structure *within* the diagonal peak, whilst that now present as X_1 and X_2 provides the structure of the crosspeak. The result is that, in addition to the modulation due to chemical shifts during t_1 and t_2 , there is additional modulation arising from J -couplings, and this produces the coupling fine structure in both dimensions.

Recall also that following the second pulse, 'some magnetisation remains associated with the original spin'. Thinking back to the discussions of polarisation transfer in the INEPT experiment, it was shown that the basic requirement for the transfer of polarisation was an anti-phase disposition of the doublet vectors of the source spin, which for INEPT was generated by a spin-echo sequence. Magnetisation components that were in-phase just before the second 90° pulse would not contribute to the transfer, hence the Δ period was optimised to maximise the anti-phase component. The same condition applies for

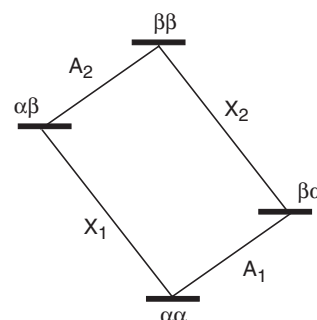
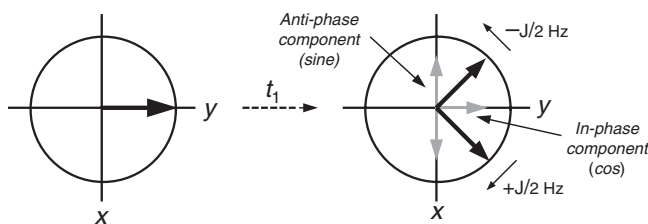


Figure 5.15. A schematic energy level diagram for the coupled two-spin AX system.

Figure 5.16. Coupling evolution during t_1 produces in-phase and anti-phase magnetisation components. Only the anti-phase component contributes to magnetisation transfer and hence to crosspeaks in the 2D spectrum.



magnetisation transfer between two protons as in the COSY experiment. This requires that the proton–proton coupling be allowed to evolve to give a degree of anti-phase magnetisation that may be transferred by the second pulse, whilst the in-phase component remains associated with the original spin (Fig. 5.16). The coupling evolution period for COSY is the t_1 period so that the amount of transferred magnetisation detected in t_2 is also modulated as a function of t_1 ($\sin 180Jt_1$); this is the modulation mentioned at the end of the last paragraph that ultimately characterises the crosspeak coupling fine structure in f_1 . Likewise, the amplitude of the in-phase, non-transferred component is also modulated in t_1 by the coupling ($\cos 180Jt_1$), and this produces the coupling fine structure of the diagonal peak in f_1 . Multiplet structure is further considered in Section 5.5.2 with the aim of extracting coupling constants from 2D crosspeaks.

5.3. PRACTICAL ASPECTS OF 2D NMR

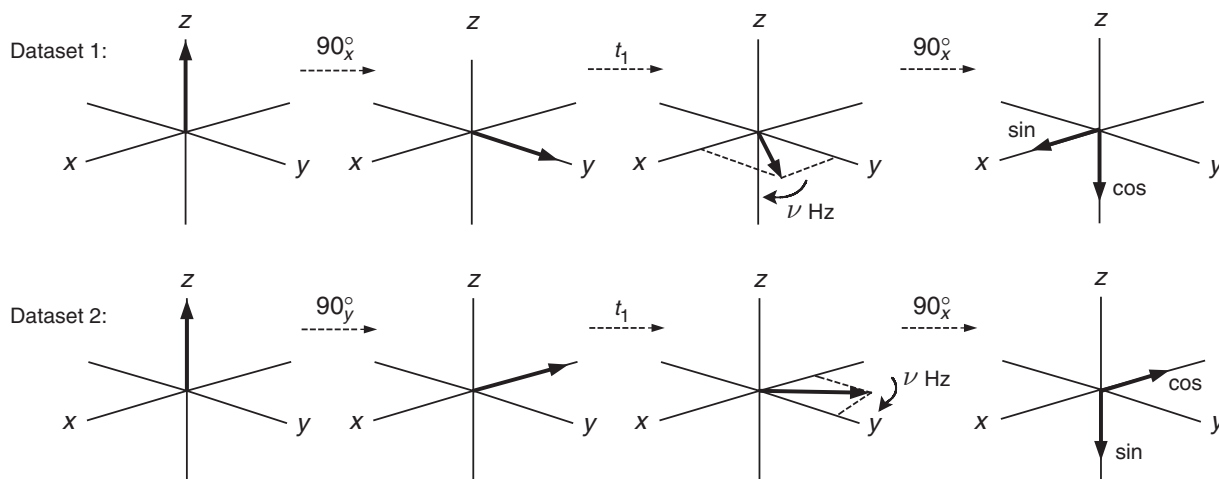
This section introduces the most important experimental aspects relating to two-dimensional data sets and again uses the COSY experiment to illustrate these. Many of the discussions are simply extensions of what has already been discussed in Chapter 3 for the 1D experiment and familiarity with this is assumed. No new concepts are introduced here, although a modified approach to experimental set-up and to data processing is essential if 2D experiments are to be successful.

5.3.1. 2D lineshapes and quadrature detection

All the two-dimensional spectra presented so far have made use of quadrature detection in both dimensions, enabling the transmitter frequencies for each to be placed at the centre of the spectrum. Quadrature detection for the f_2 data is achieved by either the simultaneous or sequential sampling schemes described in Section 3.2.4 and is therefore entirely analogous to that used for one-dimensional acquisitions. Quadrature detection in f_1 is also necessary for the same reasons. As with the 1D case, this demands some means of distinguishing frequencies that are higher than that of the reference from those that are lower when evolving during t_1 . In other words, it is again necessary to distinguish positive and negative frequencies in the rotating frame so that mirror-image signals do not appear on either side of $f_1 = 0$. There are two general approaches to this in widespread use, one providing the so-called *phase-sensitive* data displays, whilst the other providing data that it conventionally displayed in the *absolute-value* mode in which all phase information has been discarded. The first of these displays lineshapes in which absorption and dispersion modes are separated, meaning the preferred absorption-mode signal is available for a high-resolution display. The second approach is inferior in that it produces lineshapes in which the absorptive and dispersive parts are inextricably mixed, making it poorly suited to high-resolution work. However, since absolute-value spectra are rather easy to process and manipulate, they still find use in some routine work and in fully automated processes, so are also considered here.

Phase-sensitive presentations

As for 1D data, f_1 quadrature detection requires two data sets that differ in phase by 90° to be collected, thus providing the necessary sine and cosine amplitude-modulated data. Since the f_1 dimension is generated artificially, there is strictly no reference rf to define signal phases, so it is the phase of the pulses that bracket t_1 that dictate the phase of the detected signal. Thus, *for each t_1 increment*, two data sets are collected, one with a 90_x preparation pulse (t_1 sine modulation) and the other with 90_y (t_1 cosine modulation), both stored separately (Fig. 5.17). These two sets are then equivalent to the two channel data collected with *simultaneous* acquisition, which produces the desired frequency discrimination when subject to a complex FT (also referred to as a hypercomplex transform in relation to 2D data). The rate of sampling in t_1 or, in other words, the size of the t_1 time increment, is dictated by the f_1 spectral width and is subject to the same rules as for the simultaneous sampling of one-dimensional data. This



method is derived from the original work of States, Haberkorn and Ruben [6, 7] and is therefore often referred to in the literature as the *States* method of f_1 quad detection.

An alternative approach is analogous to the method of *sequential* sampling introduced in Section 3.2.4. As in the 1D approach, the aim is to avoid both positive and negative frequencies arising by shifting the *apparent* frequency range from $\pm 1/2SW_1$ to 0 to $+SW_1$ Hz by making the evolution frequencies during t_1 appear faster than they actually are. As for the States method above, there is no reference rf phase to shift for this artificial domain, so the equivalent effect is achieved by incrementing the phase of the preparation pulse by 90° for each t_1 increment and sampling the data twice as fast as for the States method (by halving the t_1 increment). Only one data set is acquired for each t_1 period, but twice as many t_1 increments are collected, so the total AQ_{t_1} , and hence the digital resolution, is equal for both methods. This approach to f_1 quad detection [8] is now referred to as Time-Proportional Phase Incrementation (*TPPI*).

The States and TPPI methods produce equivalent data sets [9] although they differ subtly in the appearance of aliased signals and the artefacts known as axial peaks, which are described more fully below. It turns out that an effective way to deal with axial peaks is to beneficially combine these two approaches to yield the so-called *States-TPPI* method of quadrature detection [10] as described in Section 5.3.2. Selecting between these related approaches is often left to the experimentalist, but in general, the preferred method is States-TPPI. The most significant point from all this is the two-dimensional lineshapes that these methods produce. All methods involve the detection of a signal that is *amplitude-modulated* as a function of the t_1 evolution period (Fig. 5.4), and this is the general requirement for producing spectra that have absorption and dispersion parts separated (*pure-phase* spectra), thus allowing a *phase-sensitive* presentation. Separate real and imaginary parts of the data exist for both the f_1 and f_2 dimensions, again analogous to the real and imaginary parts of a 1D spectrum. This gives rise to four data quadrants (Fig. 5.18) with only the (real, real) data set being presented to the user as the final 2D spectrum, the others being retained for phase correction. It is usual for the displayed spectrum to contain absorption-mode lineshapes in both dimensions (Fig. 5.6) wherever possible, since the double-absorption lineshape affords the highest possible resolution (Fig. 5.18, RR

Figure 5.17. The States method of f_1 quadrature detection requires two data sets to be acquired per increment to generate separate sine- and cosine-modulated data sets.

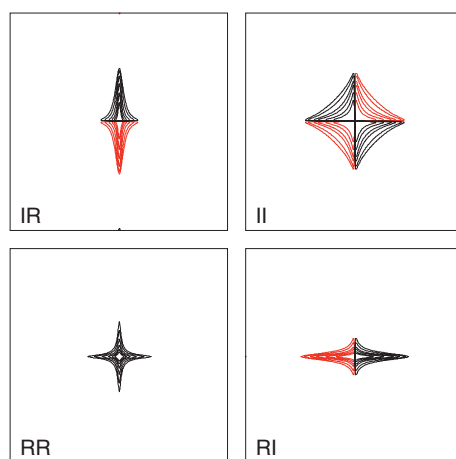


Figure 5.18. The four quadrants of a phase-sensitive data set. Only the RR quadrant is presented as the 2D spectrum, and this is phased to contain absorption-mode lineshapes in both dimensions to provide the highest resolution (R = real, I = imaginary). Positive contours are in black and negative in red.

quadrant). The phase information contained within crosspeaks can also provide additional information in some circumstances, and this is especially true for the phase-sensitive COSY experiment.

Finally, we note an alternative approach to quadrature detection known as *echo-antiecho* selection [11], which is applicable only to pulsed field gradient selected 2D methods and which now finds widespread use. As this involves a quite different procedure, it will not be considered further here but will be introduced in Section 5.5.2 after field gradients have been described.

Signal phases in phase-sensitive COSY

When discussing two-dimensional spectra, it is important to consider the relative phases and lineshapes for all resonances in the 2D plot. For the COSY spectrum, as explained above, the diagonal peaks arise from the in-phase component of magnetisation produced by evolution under spin–spin coupling. This is not transferred to the coupled spin by the mixing pulse, and diagonal peaks therefore have in-phase multiplet fine structure in both dimensions. The crosspeaks, in contrast, arise from the transferred anti-phase component, so have anti-phase multiplet structure in both dimensions. The initial phase of these two sets of signals also differs by 90° , since the diagonals arise from magnetisation that is cosine-modulated whilst the crosspeaks arise from magnetisation that is sine-modulated. Thus, in the final spectrum, the phases of the diagonal and crosspeaks will also differ by 90° , and since the crosspeaks are of most interest, these are phased so as to have (anti-phase) absorption-mode lineshapes in both dimensions, meaning the diagonal peaks possess (in-phase) double-dispersion lineshapes (Fig. 5.19). The unfortunate presence of the dispersion-mode lineshapes is an unavoidable consequence of the COSY sequence with the wide tails of the diagonals potentially masking peaks that sit close by. One popular variant of the experiment [double-quantum-filtered (DQF)-COSY] removes the dispersive contribution that is favoured for high-resolution work. The anti-phase character of COSY crosspeaks can also be troublesome when couplings are small relative to resonance linewidths, since cancellation of the lines occurs and the crosspeak disappears. This is an important factor in determining whether correlations due to small couplings can be detected with this experiment, a topic addressed in Section 5.6.2.

Aliasing in two dimensions

Resonances that fall outside the chosen spectral width will be characterised with incorrect frequencies and so will appear aliased in the 2D spectrum. For symmetrical data sets such as COSY, the two spectral widths are chosen to be the same, so aliasing will occur in both dimensions, and the position of the aliased signal can usually be predicted from the quadrature detection scheme used for each dimension. Thus, in analogy with 1D spectra, simultaneous or States sampling causes signals to be *wrapped* around, appearing at the far end of the spectrum, whilst sequential or TPPI sampling causes signals to be *folded* at the near end. Some confusion can be introduced if different sampling schemes are used for the two dimensions, for example, simultaneous sampling in f_2 but TPPI in f_1 [12]. Deliberately aliasing signals can be a useful trick in acquiring 2D data since reduced spectral widths imply reduced acquisition times and smaller data sets, or alternatively, greater resolution. For example, it is often feasible to eliminate lone phenyl groups from

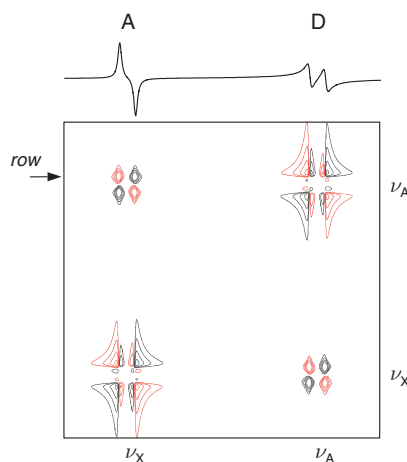


Figure 5.19. The phase-sensitive COSY for a coupled two-spin AX system. Diagonal peaks have broad, in-phase double-dispersion lineshapes (D) whereas crosspeaks have narrow, anti-phase double-absorption lineshapes (A), as further illustrated in the row extracted from the spectrum.

COSY spectra and concentrate on the aliphatic region only as this is where the interesting correlations most often lie. With modern digital filters, aliased signals in f_2 can be eliminated, although those aliased in f_1 remain since there is no equivalent filtration in the indirect dimension.

Absolute-value presentations

Prior to the advent of the above methods that allowed the presentation of phase-sensitive displays, 2D data sets that were *phase-modulated* as a function of t_1 rather than amplitude-modulated were collected. Phase modulation arises when the sine- and cosine-modulated data sets collected for each t_1 increment are combined (added or subtracted) by the steps of the phase cycle, meaning each FID per t_1 increment contains a mixture of both parts. Here it is the sense of phase precession that allows the differentiation of positive and negative frequencies. This method is inferior to the phase-sensitive approach because of the unavoidable mixing of absorptive and dispersive lineshapes, so it is generally suitable only for routine, low-resolution work.

The selection of only one of the two possible mirror-image data sets in f_1 is now achieved by suitable combination of the sine and cosine data sets. *Addition* of the two data sets within the phase cycle (Table 5.2) selects those signals that have the *same* sense of precession in t_1 as they have during t_2 (say, both positive), and these are referred to as *P-type* signals. *Subtraction* within the phase cycle selects those signals that have the *opposite* sense of precession during t_1 and t_2 (say, negative in t_1 and positive in t_2) and are referred to as *N-type* signals. To clarify this point, remember the two senses of precession we speak of here simply represent the two possible signals that would be detected on either side of $f_1 = 0$ if one were *not* using quadrature detection. By employing this, it becomes possible to select only one of these to appear in the final spectrum whilst cancelling the mirror image. The information content of the P-type or N-type spectra is equivalent, only their appearance differs by virtue of reflection about $f_1 = 0$. One significant difference arises from the fact that with N-type selection, signals that have opposite senses of precession in the two time periods are chosen. This may be thought of as being analogous to a spin-echo where vectors move in opposite senses on either side of the refocusing pulse. A similar effect arises during t_2 with N-type selection whereby echoes also occur, known as *coherence transfer echoes* (Fig. 5.20). For this reason, N-type selection is also referred to as *echo selection* and P-type signals as *anti-echo selection*, since the refocusing effect does not arise for signals that precess in the same sense. Refocusing of field inhomogeneity with echo selection together with the fact that P-type signals are subject to more severe distortions means that N-type selection is preferred when this method of quad detection is used. Conventionally, these spectra are then presented with the diagonal running from bottom left to top right, as in earlier figures.

The greatest drawback with data collected with phase modulation is the inextricable mixing of absorption and dispersion-mode lineshapes. The resonances are said to possess a *phase-twisted* lineshape (Fig. 5.21a), which has two principal disadvantages. Firstly, the undesirable and complex mix of both positive and negative intensities and secondly, the presence of dispersive contributions and the associated broad tails that are unsuitable for high-resolution spectroscopy. To remove confusion from the mixed positive and negative intensities, spectra are routinely presented in absolute-value mode, usually after a *magnitude calculation* (Fig. 5.22).

$$\mathbf{M} = (\text{real}^2 + \text{imaginary}^2)^{1/2}$$

where $\mathbf{M}$ represents the resulting spectrum. To improve resolution, severe window functions are also employed to eliminate the dispersive tail from the lineshape. Although a number of shaping functions are suitable [13], the most frequently used are the *sine-bell* and the *squared sine-bell* (Section 3.2.7), which are simple to use as they have only one variable, the position of the maximum. When used unshifted, that is, when the half-sine wave has a maximum at the centre of the acquisition time, the resulting peaks possess no dispersive component, thus reproducing the desired absorption lineshape (Fig. 5.22c). The sine-bell shape is also beneficial in enhancing the crosspeak intensities relative to the diagonal. Since the crosspeaks arise from sine modulations, as described above, they initially have zero intensity, which

Table 5.2. COSY phase cycles to select the N-type (echo) or P-type (anti-echo) signals in f_1

	N-type			P-type		
	Pulse 1	Pulse 2	Acquire	Pulse 1	Pulse 2	Acquire
Cycle 1	<i>x</i>	<i>x</i>	<i>x</i>	<i>x</i>	<i>x</i>	<i>x</i>
Cycle 2	<i>y</i>	<i>x</i>	$-x$	<i>y</i>	<i>x</i>	<i>x</i>

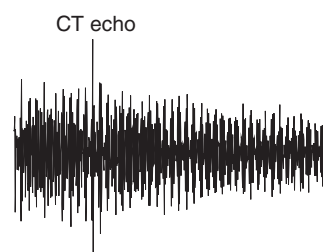


Figure 5.20. The coherence transfer echo is apparent in an FID taken from an N-type COSY data set.

Figure 5.21. A stacked plot illustration of (a) the phase-twisted lineshape and (b) the double-absorption lineshape. Clearly the resolution in (b) is far superior, and for this reason phase-sensitive methods are preferred.

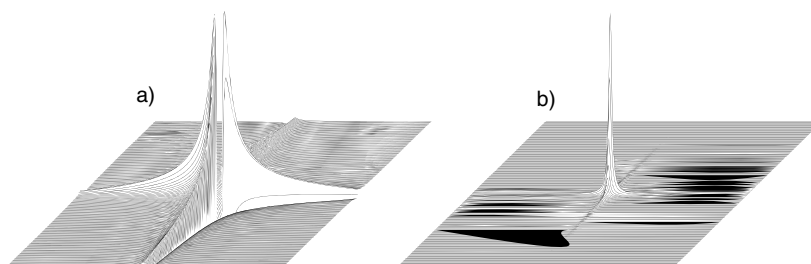
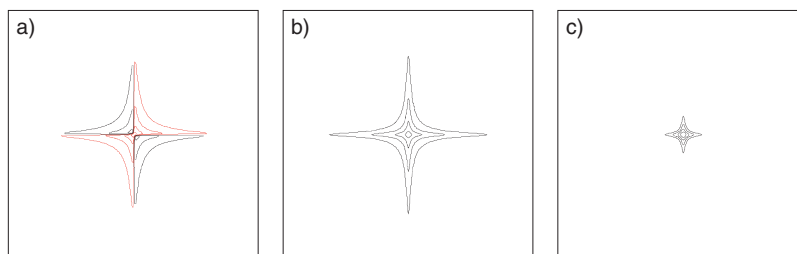


Figure 5.22. Contour plots of (a) the phase-twist lineshape, (b) the same following magnitude calculation, and (c) the same following resolution enhancement with an unshifted sine-bell window and magnitude calculation.



builds within the acquisition time. Diagonal peaks are cosine-modulated, so they begin with maximum intensity and are therefore attenuated by the window function. However, the attenuation presents a problem for signals with differing relaxation times (or in other words, linewidths) as these will be attenuated to different extents and, in particular, broader lines will be notably reduced in intensity by the early part of the sine-bell. The moral when interpreting absolute-value data processed in this way is to be wary of differing crosspeak intensities and not simply to associate these with smaller coupling constants. The final problem with the use of these extreme resolution enhancing window functions is that much of the early signal is attenuated and the later noise-enhanced, leading to a potential loss of sensitivity. It is possible to shift the function maximum to earlier in the FID to help improve this, with the inevitable re-introduction of some dispersive component. A better approach is to acquire phase-sensitive data which does not demand the application of such severe window functions. Despite this, when sensitivity is not a limiting factor, the magnitude experiment does have the advantage of not requiring phase corrections to be made, so it is well suited to routine acquisitions and to automated methods, especially when implemented as the COSY- β variant described in Section 5.6.3.

5.3.2. Axial peaks

Axial peaks are experimental artefacts that arise from magnetisation that was longitudinal (aligned along the z axis) during the t_1 evolution period, which is subsequently returned to the transverse plane by the mixing pulse and detected in t_2 . Since this magnetisation does not evolve during t_1 , it is not frequency-labelled and therefore has zero f_1 frequency. The peaks can be associated with all resonances in the spectrum and therefore appear as a band of signals across the spectrum at $f_1 = 0$ Hz. For the States or absolute-value methods, this occurs at the centre of the f_1 dimension (Fig. 5.23) and, unless eliminated, may interfere with genuine

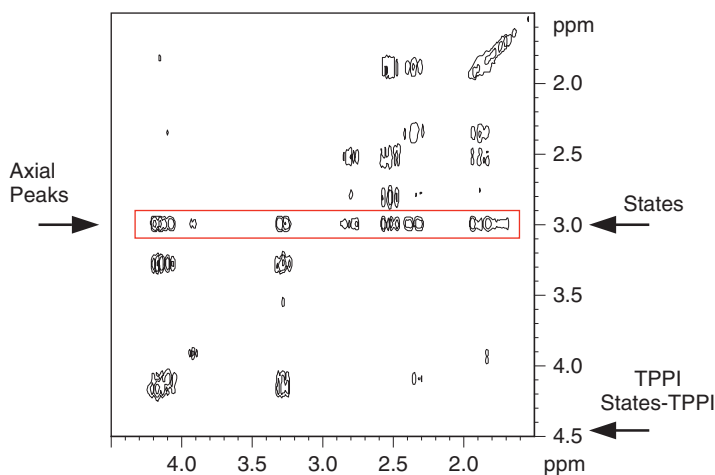


Figure 5.23. Axial peaks in a COSY spectrum form a band of signals along f_2 (boxed) at either the midpoint of f_1 (States or absolute-value data sets) or the high-frequency edge of the spectrum (TPPI or States-TPPI data sets).

crosspeaks. If TPPI is used for quadrature detection in f_1 , the peaks are shifted towards one edge of the spectrum, which, as described above, is where $f_1 = 0$ appears to be, and although aesthetically undesirable, are less of an interference. This advantage has led to the combined approach to quadrature detection known as the *States-TPPI* method [10], in which the original States procedure is modified such that axial peaks arise at the edge and not at the centre of the f_1 dimension. This is achieved by simply incrementing the phase of the pulse prior the t_1 period by 180° on each t_1 increment, combined with inversion of the receiver phase. For the desired magnetisation components, this simultaneous inversion has no net influence, and signal modulation remains as for the States procedure whilst the magnetisation associated with axial peaks does not experience the initial pulse and so accumulates the receiver-phase increment. This is then equivalent to the TPPI procedure *for the axial peaks only* and hence they appear advantageously at the edge of the spectrum.

The longitudinal magnetisation responsible for axial peaks arises from both experimental imperfection and relaxation during t_1 . Thus, if the preparation sequence does not place all magnetisation in the transverse plane, which for COSY means the initial pulse is not exactly 90° , some residual z -component remains. Elimination of these unwanted peaks is achieved simply by repeating the experiment with the receiver phase inverted. The phase of the axial peaks will be unchanged, so will cancel when the two data sets are subtracted by the phase cycle. This procedure also has the benefit of cancelling any offset of the FIDs that arises from receiver imperfections. To avoid simultaneous cancellation of the desired signals, the phase of the preparation pulse is also inverted to ensure that it matches the receiver phase, leading to the two-step phase cycle of Table 5.3.

5.3.3. Instrumental artefacts

Although there exist a whole host of artefacts that can arise in 2D spectra according to the details of the experiment, the two most likely to be encountered are briefly considered here. Both arise from instrumental imperfections, and how significant they are to you will be somewhat dependent on your instrument and its performance, but in any case it is useful to be able to recognise these artefacts if and when they appear. Nevertheless, the digital architectures of modern NMR instruments contribute significantly to the systematic reduction of many sources of instability and can reduce, or even eliminate, certain artefacts more common to older instrument designs.

f_2 quadrature artefacts

Artefacts appearing along f_2 rows are in many instances the exact parallel of those that may be observed in 1D acquisitions, and, not surprisingly, the same solutions apply, including their elimination by digital processing methods. If likely to arise, mirror-image quadrature artefacts arising from the imbalance of the receiver quadrature channels may again be suppressed by integration of the 4-step CYCLOPS routine (Section 3.2.5) into the existing phase cycle. This involves incrementing the phase of all pulses in the sequence together with the receiver phase in steps of 90° and leads to a four-fold increase in the duration of the phase cycle. On modern instruments, the intensity of quadrature artefacts are so small (or even non-existent with digital detection schemes) that the addition of CYCLOPS can often be avoided and time savings made when sensitivity is not limiting.

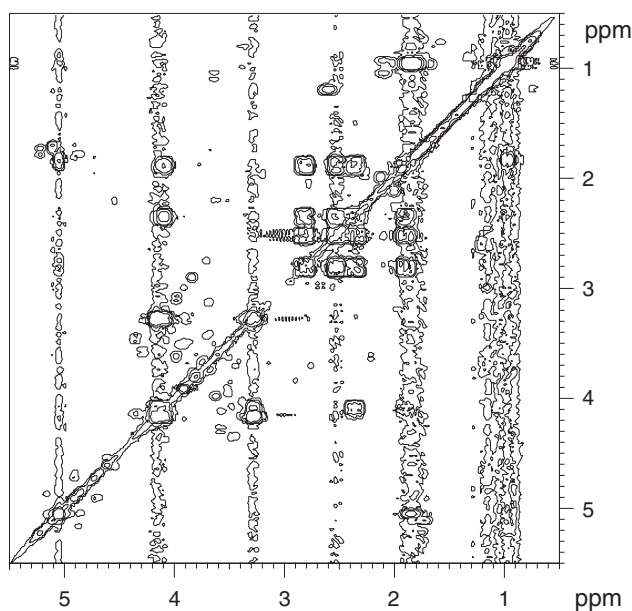
t_1 noise

Digitisation of data during a 2D experiment is subject to the same thermal noise arising from the probe head and preamplifier as in a 1D experiment, and this contributes to the noise baseplane observed in the 2D spectrum. There also exists a particularly objectionable artefact associated with 2D experiments, referred to as t_1 noise (note the t_1 here refers to the evolution period and should not be confused with the T_1 relaxation time constant). This appears as bands of noise parallel to the f_1 axis where an NMR resonance exists, and it is sometimes this that limits the observation of peaks in the spectrum rather than the true thermal noise. Indeed, it appears that the very earliest work on 2D NMR was unpublished due to excessive t_1 noise present in the spectra. Generally speaking, this is caused by

Table 5.3. The basic two-step phase cycle for the elimination of axial peaks

	Pulse 1	Pulse 2	Acquire
Scan 1	x	x	x
Scan 2	$-x$	x	$-x$

Figure 5.24. Bands of noise parallel to the f_1 axis (t_1 noise) often appear in 2D spectra.



instrument instabilities that lead to random fluctuations in signal intensities and phase from one FID to the next over the course of the 2D experiment. Since these fluctuations relate to perturbations of the NMR resonance, the bands of t_1 noise characteristically appear only at the f_2 shifts of resonances and have intensities proportional to the corresponding resonance amplitude (Fig. 5.24). Thus, stronger bands of noise are associated with intense, sharp peaks, most notably singlets.

The instrumental instabilities that contribute to t_1 noise are numerous [14, 15], including irreproducible rf pulse phase and amplitude, instability in the field-frequency lock and field homogeneity, and so on. These factors are in the hands of the instrument manufacturers, who have made steady progress in reducing various sources of instrumental instabilities over the years. In preparing a 2D acquisition, there are a number of steps that will also help minimise these artefacts. The same arguments apply to any experiment that uses difference spectroscopy to retain selected signals and cancel others (e.g. the NOE difference experiment in Chapter 8), so they are not exclusive to the world of 2D NMR. Use of a strong, sharp lock signal will provide optimum field-frequency regulation, and the use of auto-shim routines on the lower-order shims for long-term acquisitions should also help maintain lineshape reproducibility. Stability of the sample environment is also important. Magnetic field stability is, of course, essential, so movement of any magnetic materials anywhere in the vicinity of the instrument is highly undesirable; this could even include motion (human or otherwise) above or below the magnet or in the lab next door. Temperature stability should also be maintained with a suitable temperature control unit, and equilibration of the sample prior to running the experiment may be necessary, especially for aqueous solutions. Finally, it is recommended **not** to spin samples during 2D acquisitions. This typically causes additional modulation of the detected signal as the sample rotates, notably from an imperfect tube or a ‘wobbling’ sample spinner, further contributing to t_1 noise.

Symmetrisation

Unwanted t_1 noise in spectra can also be reduced by a number of post-processing strategies. One of the most common procedures for absolute-value COSY experiments is symmetrisation [16]. This involves replacing symmetrically related data points on either side of the diagonal with the lesser of the two values, thus retaining crosspeaks but suppressing unsymmetrical artefacts. This must be applied with caution, however, as it may enhance artefacts that have coincident symmetry, such as regions of t_1 noise associated with intense singlets, giving them the appearance of genuine correlation peaks; confusion can usually be avoided by comparison with the non-symmetrised spectrum. This method is not generally suitable for phase-sensitive data as it may introduce distortion of crosspeak structure and can lead to reduced sensitivity, so this symmetrisation finds rather limited use in modern NMR.

Significant reductions in the level of t_1 noise can also be obtained in certain experiments where signal selection is achieved by pulsed field gradients, although homonuclear experiments such as COSY show little benefit in this respect, more notable gains being apparent

for proton-detected heteronuclear correlation experiments. Alternative post-processing methodologies such as reference deconvolution [17–20] (Section 3.4.6) have also provided impressive reductions in t_1 noise [21, 22], and the algorithms required for this are to be found in some commercial processing software.

5.3.4. 2D data acquisition

The success of any NMR experiment is, of course, crucially dependent on the correct setting of the acquisition parameters. In the case of 2D experiments, one has to consider the parameters for each dimension separately, and we shall see that the most appropriate parameter settings for f_2 are rarely optimum for f_1 . Likewise, one has to give rather more thought to the setting up of a 2D experiment than is usually required for 1D acquisitions to make optimum use of the instrument time available and data storage space. Once again, the general considerations below will be applicable to all 2D experiments, although we restrict our discussion at this stage to COSY.

At the most basic level, one has to address three fundamental questions when setting up an experiment: (1) will the defined parameters enable (or limit) the detection of the desired information and exclude the unwanted? (2) how long will it take and do I have enough time? and (3) how much storage space is required for the data and is this available? Ideally we would wish to acquire data sets rapidly, and we would like these to be as small as possible for the speed of processing and the ease of handling yet still be able to provide the information we require; so we set up our experiment with these goals in mind.

Firstly, spectral widths, which should be the same in both dimensions of the COSY experiment, should be kept to minimum values with transmitter offsets adjusted so as to retain only the regions of the spectrum that will provide useful correlations. It is usually possible to reduce spectral widths to well below the 10-ppm or so proton window observed in 1D experiments. The use of excessively large windows leads to poorer digital resolution in the final spectrum or requires greater data sizes, neither of which is desirable. The spectral widths in turn define the sampling rates for data in t_2 , in exact analogy with 1D acquisitions, and the size of the t_1 increment, again according to the Nyquist criteria. The acquisition time (AQ_t), and hence the digital resolution ($1/AQ_t$), for each dimension is then dictated by the number of data points collected in each. For t_2 , this is the number of data points digitised in each FID, whilst for t_1 this is the number of FIDs collected over the course of the experiment. The appropriate setting of these parameters is a most important aspect to setting up a 2D experiment, and the way in which one thinks about acquisition times and digital resolution in a 2D data set is, necessarily, quite different from that in a 1D experiment. As an illustration, imagine transferring the typical parameters used in a 1D proton acquisition into the two dimensions of COSY. The acquisition time might be 4 s, corresponding to a digital resolution of 0.25 Hz/pt, with no relaxation delay between scans. On, for example, a 400-MHz instrument, with a 10 ppm spectral width, this digital resolution would require 32K words to be collected per FID. The 2D equivalent, with States quad detection in f_1 and with axial-peak suppression, requires four scans to be collected for each t_1 increment. The mean acquisition time for each would be 6 s (t_2 plus the mean t_1 value), corresponding to 24 s of data collection per FID. If 16K t_1 increments were to be made for the f_1 dimension (two data sets are collected for each t_1 increment remember), this would correspond to a total experiment time of about 4.5 days. Furthermore, the size of the resulting data matrix would be a little over 1000 million words, and with a typical 32-bit-per-word computer system, this requires some 4 GB of disk space! I trust you will agree that 4 days for a basic COSY acquisition is quite unacceptable, let alone the need for such disk space per experiment; so acquiring data with such high levels of digitisation in both dimensions is clearly not possible.

The key lies in deciding on what level of digitisation is required for the experiment in hand. The first point to notice is that adding data points to extend the t_2 dimension leads to a relatively small increase in the overall length of the experiment, so we may be quite profligate with these (although they will lead to a corresponding increase in the size of the data matrix). Moreover, adding t_1 data points requires that a complete FID of potentially many scans is required *per increment*, which makes a far greater increase to the total data collection time. Thus, one generally aims to keep the number of t_1 increments to a minimum, which is consistent with resolving the correlations of interest and increasing t_2 as required when higher resolution is necessary. For this reason, the digital resolution in f_2 is often greater than that in f_1 , particularly in the case of phase-sensitive data sets. The use of smaller AQ_{t1} is, in general, also preferred for reasons of sensitivity since FIDs recorded for longer values of t_1 will be attenuated by relaxation and so will contribute less to the overall signal intensity. The use of small AQ_{t1} is likely to lead to truncation of the t_1 data, and it is

then necessary to apply suitable window functions that force the end of the data to zero to reduce the appearance of truncation artefacts.

For COSY in particular, one of the factors that limits the level of digitisation that can be used is the presence of intrinsically anti-phase crosspeaks, since too low a digitisation will cause these to cancel and the correlation to disappear (see Section 5.6.2 for further discussions). The level of digitisation will also depend on the type of experiment and the data one expects to extract from it. For absolute-value COSY, one is usually interested in establishing where correlations exist, with little interest in the fine structure within these crosspeaks. In this case, it is possible to use a low level of digitisation consistent with identifying correlations. As a rule of thumb, a digital resolution of J to $2J$ Hz/pt (AQ of $1/J$ to $1/2J$ s) should enable the detection of most correlations arising from couplings of J Hz or greater. Thus for a lower limit of, say, 3 Hz, a digital resolution of 3–6 Hz/pt (AQ of ~ 300 –150 ms) will suffice. The AQ_{t1} is typically half that for t_2 in this experiment, with one level of zero-filling applied in t_1 so that the final digital resolution is the same in both dimensions of the spectrum (as required for symmetrisation).

For phase-sensitive data acquisitions, one is likely to be interested in using the information contained within the crosspeak multiplet structures, and a higher degree of digitisation is required to adequately reflect this, a more appropriate target being around $J/2$ Hz/pt or better (AQ of $2/J$ s or greater). Again, digitisation in t_2 is usually two or even four or eight times greater than that in t_1 . In either dimension, but most often in t_1 , this may be improved by zero-filling, although one must always remember that it is the length of the time-domain acquisition that places a fundamental limit on peak resolution and the effective linewidths after digitisation, regardless of zero-filling. The alternative approach for extending the time domain data is to use forward linear prediction when processing the data (Section 3.2.3). The rule as ever is that high resolution requires long data-sampling periods.

Having decided on suitable digitisation levels and data sizes, one is left to choose the number of scans or transients to be collected per FID and the repetition rates and hence relaxation delays to employ. The minimum number of transients is dictated by the minimum number of steps in the phase cycle used to select the desired signals. Further scans may include additional steps in the cycle to suppress artefacts arising from imperfections. Beyond this, further transients should be required only for signal averaging when sensitivity becomes a limiting factor. Since most experiments are acquired under 'steady-state' conditions, it is also necessary to include 'dummy' scans prior to data acquisition to allow the steady state to establish. On modern instruments that utilise double buffering of the acquisition memory, dummy scans are required only at the very beginning of each experiment to make a negligible increase to the total time required. On older instruments that lack this feature, it is necessary to add dummy scans for each t_1 increment, and these may then make a significant contribution to the total duration of the experiment. The repetition rate will depend upon the proton T_1 s in the molecule, and since the sequence uses 90° pulses, the optimum sensitivity is achieved by repeating every $1.3T_1$ s.

Returning to the example of 400 MHz acquisition discussed above, we can apply more appropriate criteria to the selection of parameters. Table 5.4 compares the result from above with more realistic data, and it is clear that under these conditions, COSY becomes a viable experiment, requiring only hours or even minutes to collect, rather than many days. The introduction of PFGs to high-resolution spectroscopy (Section 5.6) allows experiments to be acquired with only one transient per FID where sensitivity is not limiting, thus further reducing the total time required for data collection. The data storage requirements in these realistic examples are also well within the capabilities of modern computing hardware and are likely to become increasingly less significant as this develops further. Although illustrated for COSY spectra, the general line of reasoning presented here is applicable to the set-up of any 2D experiment. These issues are briefly considered with reference to different classes of techniques in the following chapters describing other 2D methods.

Table 5.4. Illustrative data tables for COSY experiments

Experiment	Spectral width/ppm	$N(t_2)$	$N(t_1)$	Hz/pt (t_2)	Hz/pt (t_1)	Experiment time	Raw data-set size
(a) Phase-sensitive	10×10	32K	32K	0.25	0.25	4.5 days	1000 M words
(b) Phase-sensitive	6×6	2K	1K	2.3	2.3	55 min	2 M words
(c) Absolute value	6×6	1K	256	4.6	4.6	22 min	0.25 M words

Scenario (a) transplants acquisition parameters from a typical 1D proton spectrum into the second dimension leading to unacceptable time requirements, whereas (b) and (c) use parameters more appropriate to 2D acquisitions. All calculations use phase cycles for f_1 quad detection and axial-peak suppression only and for (b) and (c), a recovery delay of 1 s between scans. A single zero-filling in f_1 was also employed for (b) and (c).

5.3.5. 2D data processing

The general procedure for processing any 2D data set is the same and essentially follows that outlined in the flow chart of Fig. 5.25. The details of the parameters selected for each stage will depend upon the technique used, the acquisition conditions and the nature of the sample being studied, so only the general principles that are to be considered during data processing are considered here. All of these are extensions of the ideas already introduced in Chapter 3 for the handling of 1D data, and this section assumes familiarity with these (Sections 3.2.3 and 3.2.8). Processing techniques have even greater importance for two-dimensional work where the operator is able to tailor the appearance of the final spectrum to a large degree according to the chosen parameters. The choice of window functions has a major impact on the final spectrum, and this selection differs markedly for phase-sensitive and absolute-value data sets, so initially we restrict discussion to the phase-sensitive case. These functions are applied typically with the following three goals in mind:

1. To ensure truncation of the time-domain data and thus attenuate truncation artefacts;
2. To enhance resolution in the spectrum; and
3. To provide optimum signal-to-noise.

Owing to the short acquisition times used, data truncation typically occurs in both dimensions. Particularly when zero-filling is applied, as is nearly always the case in t_1 , the window function must force the FID to zero if truncation wiggles are to be avoided. The longer t_2 acquisition time often means zero-filling is not required in this dimension, and similar considerations apply to those for handling a 1D FID. Generally some enhancement of resolution without degradation of signal-to-noise is of primary concern for which any of the resolution enhancements functions of Section 3.2.8 can be used, the most popular being the Lorentz–Gauss transformation or the shifted sine-bell or squared-sine-bell. The latter two guarantee forcing the decay to zero, so are also suitable for the shaping of t_1 data prior to zero-filling, which is typically applied at least once. In either dimension, excessively strong enhancement leads to the appearance of negative-going excursions about resonances, and trial and error is generally the best approach for achieving optimum results. Often the first FID of a 2D data matrix can be used as a convenient test 1D spectrum to visualise the effects different window functions will have on the final f_2 data. Modern software now allows interactive changes of shaping parameters along with a display of the final spectrum making such experimenting rather straightforward. An alternative to the zero-filling of t_1 data for high signal-to-noise data sets is the extension of t_1 interferograms by linear prediction to typically two or four times their original size. This not only extends the decay but also reduces the need for apodisation of highly truncated data, both of which

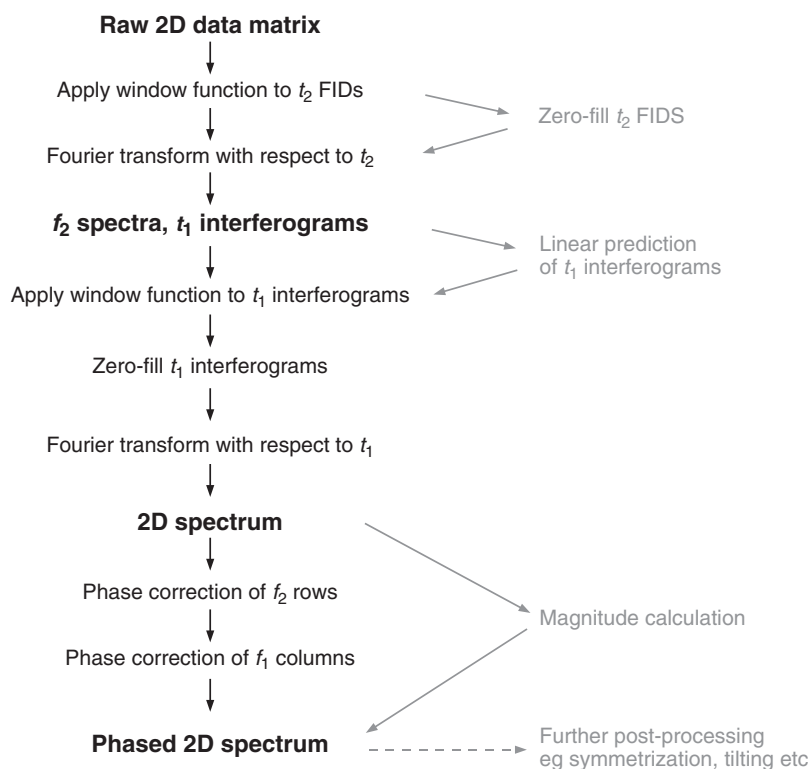


Figure 5.25. The typical scheme followed in the processing of a two-dimensional data set. The items shown to the right in grey are additional or alternative procedures that may be executed.

contribute to improved resolution in f_1 . Further zero-filling beyond this may also be applied to improve the digitisation of the final spectrum if desired.

Absolute-value data sets (typically presented following a magnitude calculation) demand stronger resolution enhancement functions in both dimensions to suppress the undesirable dispersion-mode contributions to the phase-twisted lineshapes. This is most often achieved with the sine-bell or squared-sine-bell functions. These are well suited to the anti-phase peaks that give rise to COSY correlations since these start with zero intensity (being initially anti-phase they have no net magnetisation) and grow during the course of the FID. However, most 2D experiments produce in-phase resonances that provide full intensity at the start of the FID and if used non-shifted, these functions lead to attenuation of much of the signal and can severely degrade sensitivity (try processing a 1D proton spectrum with a non-shifted sine-bell!).

Phase correction

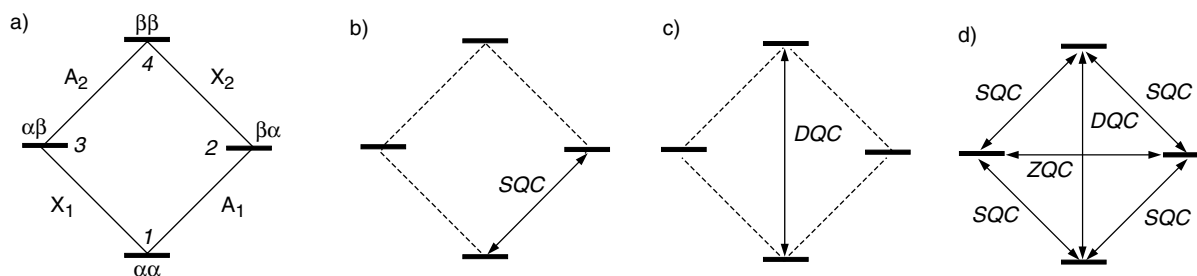
Phase correction of a 2D spectrum is undertaken independently for the two frequency domains. Where the first FID of a 2D matrix contains signals, this can be processed and phased as for a 1D spectrum and the resulting zero- and first-order phase constants applied to the f_2 data. The frequency-independent phase correction for f_1 will depend on the details of the phase cycling used in the pulse sequence but should be close to 0° or 90° . In theory, a frequency-dependent (first-order) phase correction in f_1 should not be necessary since the time delays prior to data sampling that produce these errors do not arise for the artificially generated time domain. In practice, some small correction is often required for both domains for optimum results, and this is undertaken interactively as for a 1D spectrum. Thus, one aims to observe signals at either end of the spectrum on which to make the corrections. This involves independently selecting f_2 rows (or f_1 columns) from the 2D spectrum. To provide the necessary frequency distribution of resonances, two or more from each dimension are usually required, and the resulting phase constants are applied to all other rows (or columns).

Presentation

It has already been stated that the most informative and convenient way to present 2D data is as a contour plot, in which the horizontal and vertical axes represent the frequency dimensions and the peak intensity is indicated by appropriate contours. The lower contour setting defines the cut-off below which no signals are presented. Typically this will be set some way above the baseplane noise level but if set too high, it may eliminate potentially useful information such as weak correlations arising from small couplings. In some instances, a cut-off level suitable for revealing weak signals will be too low for more intense ones and may cause the appearance of artefacts such as t_1 noise, which does not make for an aesthetically appealing plot. In such situations, it may be worthwhile producing multiple plots with differing settings. Producing expansions of different regions can also be beneficial, especially in crowded situations or where the details of crosspeak structures are to be analysed. Such analysis is best performed interactively on screen if possible, where the use of cursors and crosshairs is more accurate than that of pencil and ruler. In situations where both positive and negative contours must be displayed and differentiated, different colours can be employed or different line styles if monochrome output is demanded.

5.4. COHERENCE AND COHERENCE TRANSFER

The notion of coherence has already been touched upon in the introduction to COSY in this chapter and has been briefly mentioned in Chapter 4. This lies at the foundation of every NMR experiment, and to appreciate many of the topics that follow in this book, it is useful to develop some feel for this and the notion of coherence transfer. Coherence is a generalisation of the notion of transverse magnetisation yet it is important to appreciate some specific features of coherence, which may be illustrated with reference to the energy level diagram of a coupled two-spin AX system (Fig. 5.26a). Following the application of a 90° pulse on this system at equilibrium, transverse magnetisation exists, which, according to the vector model, may be represented as four vectors in the rotating frame, one for each of the transitions in Fig. 5.26a. These give rise to the four lines of the two doublets in the resulting spectrum. These transitions are termed *single-quantum* transitions because they are associated with a change in magnetic quantum number, M , of 1 ($\Delta M = 1$). The magnetisation associated with each is therefore referred to as a *SQC* (*single-quantum coherence*), which is said to possess a *coherence order*, p , of 1. SQC is the conventional transverse



magnetisation detected in any NMR experiment as this is able to induce a voltage in the NMR detector. In this respect, it is useful to consider the difference between a transition excited by a 90° pulse, as above, and one that is saturated by weak rf irradiation. Both situations correspond to equal α and β populations and hence to zero population differences across the transitions, yet only the first produces a detectable signal. The saturated transition has no *net* magnetisation to detect because the spins are distributed about the x - y plane with random phase. The spins excited by the pulse, however, are bunched together along one axis in the transverse plane and so produce a net component that is detectable; they have *phase coherence* imposed on them by the pulse (Fig. 5.27). In general, we may distinguish a single-quantum coherence as a phase coherence between two states that have $\Delta M = \pm 1$.

Now imagine the application of a selective 90° pulse to the two-spin system at equilibrium but to just one of the transitions, say A_1 . This then creates a single-quantum coherence for this transition alone (Fig. 5.26b). Next imagine applying a selective 90° pulse to the transition X_2 and hence equalising the populations across this transition. This process transfers some of the spins associated with the $\beta\alpha$ state to the $\beta\beta$ state, and some part of the phase coherence originally associated with A_1 is now associated with the 1–4 transition ($\alpha\alpha$ – $\beta\beta$) (Fig. 5.26c). This is a new form of coherence not previously considered in any detail. It corresponds to a change in magnetic quantum number of 2 ($\Delta M = 2$), so it is termed two-quantum or more usually *double-quantum coherence* (DQC) ($p = 2$), which cannot be readily represented with the vector model notation used thus far. In the COSY experiment, a similar state of affairs arises through the use of non-selective 90° pulses applied simultaneously to all transitions. Thus, during t_1 , the single-quantum coherences (transverse magnetisation) generated by the first 90° pulse evolve under the influence of spin–spin coupling to give the anti-phase magnetisation components required for *coherence transfer*, as described in Section 5.4. On application of the second 90° pulse, the coherence originally associated with the A_1 single-quantum transition is distributed amongst the other transitions within the spin system (X_1 , X_2 , A_2 , 1–4 and 2–3) whilst some part remains with A_1 (Fig. 5.26d). The first two again correspond to SQC but now of the X spin, so this coherence transfer from A to X (Fig. 5.28) gives rise to the COSY crosspeak. Coherence remaining with A_1 or that transferred to A_2 ultimately produces the diagonal peak multiplet that we have already seen. The 1–4 coherence is the DQC described above, whilst the 2–3 coherence corresponds to a change in magnetic quantum number of 0, so it is termed ZQC (*zero-quantum coherence*) ($p = 0$). Longitudinal magnetisation, although not a state of coherence, also has $p = 0$ since it does not correspond to transverse magnetisation. All coherences for which $p \neq \pm 1$ are referred to as *multiple-quantum coherences*. Quantum mechanical selection rules dictate that only those coherences corresponding to $\Delta M = \pm 1$ are able to induce a signal in the detection coil or in other words, *only* single-quantum coherences *may be observed directly* since all others have no *net* magnetisation associated with them. Unfortunately, simply stating this fact leaves a slightly uncomfortable air of mystery surrounding the ‘invisible’ multiple-quantum coherences. A simple physical picture of these states views them as combinations of pairs of evolving vectors that are *always*

Figure 5.26. Schematic energy level diagrams for a coupled two-spin AX system (see text). SQC, single-quantum coherence; DQC, double-quantum coherence and ZQC, zero-quantum coherence.

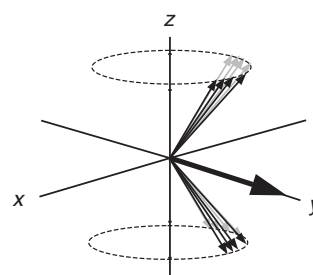


Figure 5.27. Net transverse magnetisation is produced from the bunching of individual magnetic moments, which gives rise to an observable NMR signal. These spins are said to possess *phase coherence*, and because only single-quantum spin transitions ($\alpha \leftrightarrow \beta$) are involved in generating this state, it is termed *single-quantum coherence*.

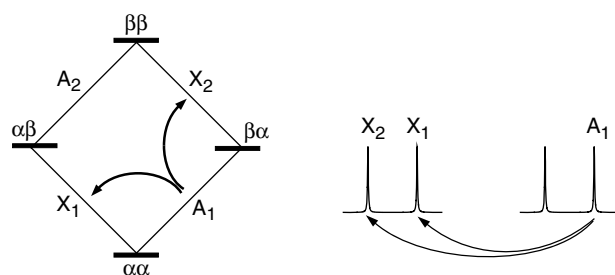


Figure 5.28. The coherence transfer process responsible for generating crosspeaks in correlation spectroscopy, illustrated for the A_1 transition. Coherence of spin A during t_1 of a 2D sequence becomes coherence of spin X during t_2 , thus correlating the two spins in the resulting spectrum.

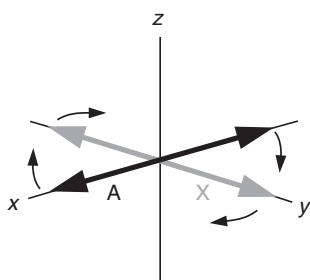


Figure 5.29. A simplified picture of multiple-quantum coherence in an AX system views this as being composed of groups of evolving anti-phase vectors that have zero net magnetisation and hence can never be directly observed.

anti-phase and hence never produce observable *net* magnetisation (Fig. 5.29). The evolution frequency of these states is dictated by the chemical shifts of the participating nuclei and the coherence order. Whereas single-quantum coherences evolve in the rotating frame according to the chemical shift offset of the spin from the transmitter, double-quantum coherence evolves according to the *sums* of the chemical shift offsets of the two spins ($\nu_A + \nu_X$) and zero-quantum coherences evolve according to their offset *differences* ($\nu_A - \nu_X$). In a system containing more than two coupled spins, higher orders of multiple-quantum coherence may also be excited, so, for example, at least three coupled spins are required for the generation of triple-quantum coherence, and so on. Although again these higher orders cannot be observed *directly*, their presence may be detected *indirectly* if they are reconverted to single-quantum coherences prior to detection. For example, adding a third pulse to COSY would regenerate single-quantum coherence from the otherwise invisible multiple-quantum coherences that were generated; an example of this is found in the double-quantum filtered COSY described shortly. Just as spin-spin relaxation produces a loss of observable signals by destroying the phase coherence of transverse (single-quantum) magnetisation, multiple-quantum coherences are also dephased by such relaxation processes and similarly have a finite lifetime in which they can be manipulated.

It should also be noted that a coherence order has sign associated with it, so single-quantum coherence may have an order of $p = +1$ or $p = -1$, whereas double-quantum coherences will have $p = \pm 2$ and so on. The positive and negative signs represent hypothetical vectors that precess at the same rate but in opposite senses in the rotating frame. For single-quantum coherence, these simply correspond to the two sets of mirror-image signals that would be observed on either side of the reference frequency in the absence of quadrature detection, so they have a realistic physical manifestation. By using quadrature detection, when collecting data, we choose to keep one of these sets of signals and eliminate the other, to avoid possible confusion.

5.4.1. Coherence transfer pathways

The existence of the different levels of coherence during any pulse sequence may be illustrated by means of coherence level diagrams. These were originally introduced to NMR for the purpose of designing pulse sequence phase cycles [23–25] but can also provide an extremely effective yet simple graphical means of following the flow of magnetisation through a pulse sequence. This formalism proves to be especially powerful when considering the pathway taken by magnetisation from the start of a 2D pulse sequence to its arrival at the detector. It also provides a means of following multiple-quantum coherences, which cannot be represented by the vector model, thus overcoming some of the limitations of this without resorting to mathematical formalisms. The coherence level diagram for a single-pulse 1D acquisition is presented in Fig. 5.30. A single pulse generates only coherences with $p = \pm 1$, and these are represented in this figure as solid and dashed lines. As stated above, one of these pathways is selected by the hardware quadrature detection whilst the other is rejected, and purely by convention, the $p = -1$ pathway is retained (note that in the original paper [23], the opposite convention was chosen). Coherence level diagrams are used to indicate the pathway followed by the desired magnetisation that is selected by the phase cycle or pulsed field gradients in use. All other possible pathways (of which there could be very many) are not shown and are assumed to be eliminated, thus the dashed pathway of Fig. 5.30 would not be usually presented.

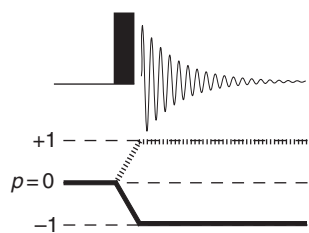


Figure 5.30. The coherence level diagram for a single-pulse 1D experiment. The solid line represents the coherence transfer pathway of the detected magnetisation. The dotted line represents the mirror-image pathway that is rejected by the quadrature detection scheme.

To introduce the idea of coherence transfer pathways for 2D experiments, the COSY sequence is again considered, and for simplicity, the absolute-value P- and N-type COSY experiments described in Section 5.3.1 are described. The desired pathways for both these experiments are shown in Fig. 5.31 with the only difference between these two being the relative sense of precession of (single-quantum) coherence in the t_1 and t_2 time periods, this being the same for the P-type and opposite for the N-type signals. This difference is then clearly illustrated by the coherence transfer pathways followed, both of which begin with equilibrium longitudinal magnetisation that has coherence order zero. Since coherence order $p = -1$ is selected in t_2 , the corresponding coherence in t_1 is therefore $p = -1$ for the P-type signal but $p = +1$ for the N-type. These pathways are selected by the phase cycle described earlier and are illustrated in Table 5.2. During the t_1 period, and in general any period of free precession under the influence of chemical shifts and/or couplings, the coherence orders remain unchanged; only rf pulses are able to alter coherence levels (although relaxation will ultimately regenerate longitudinal magnetisation and hence coherence level zero).

For the phase-sensitive COSY experiment, both $p = \pm 1$ pathways must be preserved during t_1 . However, for a pure-phase 2D spectrum, the signal detected in t_2 must be amplitude-modulated as a function of t_1 . Such a signal can be obtained only if both of the counter-rotating coherences are retained since the retention of only one of these unavoidably leads to a

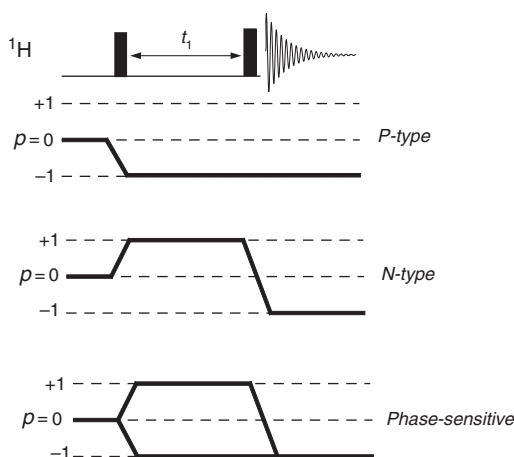


Figure 5.31. The coherence level diagrams and coherence transfer pathways for (a) P-type, (b) N-type and (c) phase-sensitive COSY.

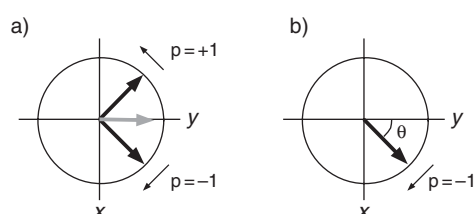


Figure 5.32. (a) Amplitude modulation requires both counter-rotating vectors ($p=\pm 1$ coherences) be retained during t_1 . The resultant signal (greyed) is modulated in amplitude along the y axis as the vectors precess. (b) Phase modulation results if only one vector ($p=1$ or $p=-1$) is retained; the phase angle θ varies as the vector precesses.

phase-modulated signal (Fig. 5.32) and hence the phase-twist lineshape, as for the P- or N-type COSY. However, the presence of both pathways implies the detection of the NMR resonance plus its mirror (quadrature) image in f_1 , so the use of a suitable quadrature detection scheme (States or TPPI) to eliminate one set of these responses at the data processing stage is essential. The need to retain both pathways during t_1 for the acquisition of pure-phase spectra is fundamental to all 2D experiments. This has prime importance when utilising pulsed field gradients for coherence pathway selection.

5.5. GRADIENT-SELECTED SPECTROSCOPY

It may be argued that the most significant fundamental development in experimental methodology in high-resolution NMR during the 1990s was the introduction of pulsed field gradients, which extend the scope of numerous NMR techniques [26–28]. The concept of applying magnetic field *gradients* across a specimen or a sample has long been used in (nuclear) magnetic resonance imaging [29], and although the potential for their use in high-resolution NMR was realised some time ago [30, 31], technical limitations precluded their widespread use for some years [32]. Nowadays, pulsed field gradients find application in numerous 1D and multi-dimensional NMR techniques as a means of selecting those signals deemed interesting and suppressing those which are not and have proved hugely beneficial to many of the routine methods used within structural organic chemistry. This section introduces the general principles behind the use of pulsed field gradients (PFGs), and although nestled in this introduction to two-dimensional NMR techniques, the discussions are quite general and applicable to all gradient-selected experiments. Gradient-based methods have not been collected together within a separate chapter but are presented alongside the more conventional equivalents throughout the book. This more properly reflects the way in which the gradient methods have steadily permeated the NMR world and may now be considered standard techniques as more instruments are supplied with the appropriate hardware incorporated. Furthermore, the majority of methods that use PFGs are often simple variations of the original non-gradient equivalent (hence the use of terms such as *gradient-enhanced* or *gradient-accelerated* experiments in the literature) and thus require no greater understanding to enable one to apply the experiment and interpret the data. In fact the advantages endowed by PFGs over traditional methods largely relate to the practicalities of data collection, often allowing the collection of superior-quality data in shorter times.

The term ‘traditional’ in this context refers to those experiments that make use of phase cycling to select the desired signals and suppress all others. The notion of phase cycling has already been encountered in previous sections; the point to recall at this stage is that this procedure involves the repetition of a pulse sequence with the phases of the rf pulse(s) adjusted

on each transient and the data from each combined such that the desired signals add constructively whilst those that are unwanted cancel. All phase-cycling procedures are thus based on difference spectroscopy and as a result are subject to problems of imperfect subtraction of the undesired signals, which leads to artefacts in the final spectrum. The dependence on a difference procedure also means that, on each transient, data is collected which is ultimately discarded; this clearly does not make optimum use of the receiver's dynamic range. These methods also require that a full phase cycle be completed to achieve the required selection regardless of signal strength, thus placing a fundamental limit on how quickly an experiment can be collected. Experiments that make use of PFGs for signal selection do not suffer from these limitations because *only the desired signal* is collected *on each transient*, thus leading to cleaner spectra that are free from difference artefacts and, when sensitivity is not limiting, to faster data collection because one is not limited by the number of steps in a phase cycle.

5.5.1. Signal selection with PFGs

Defocusing and refocusing with PFGs

Before subjecting any sample to NMR analysis, one of the operations that must be performed is that of shimming the magnetic field to remove inhomogeneities. This ensures that all spins in the same chemical environment experience the same applied field and hence possess the same Larmor frequency regardless of their location within the sample. If, however, one were to deliberately impose a linear magnetic field *gradient* (known as a B_0 gradient) across the sample, say along the z axis, chemically equivalent spins would now experience a different applied field according to their position in the sample, in this case, along its length. This may be understood by considering the sample to be composed of microscopically thin disks in the x - y plane with each experiencing a different local magnetic field according to their physical location (Fig. 5.33). If the field gradients were imposed for a discrete period of time as a *gradient pulse* (Fig. 5.34a), then during this, the spins, having been previously excited with an rf pulse, precess with differing frequencies and will thus rotate through differing angles (Fig. 5.33b). Provided the gradient pulse is of sufficient strength and duration, the *net* magnetisation remaining, which is the sum of the transverse magnetisation from all disks in

Figure 5.33. The action of field-gradient pulses. (a) In a homogeneous magnetic field, all chemically similar spins possess the same Larmor frequency within every microscopically thin disk throughout the sample causing their individual magnetic moments to add and to produce a sharp resonance. (b) After the action of a linear field gradient of strength B_g for a duration τ_g , the spins have evolved through different angles according to their local magnetic fields, adding to give zero net magnetisation. (c) Application of a second, identical gradient but in the opposite direction refocuses the individual vectors once more and recovers the resonance. The magnetisation is said to have been first defocused and then refocused by the action of the gradients.

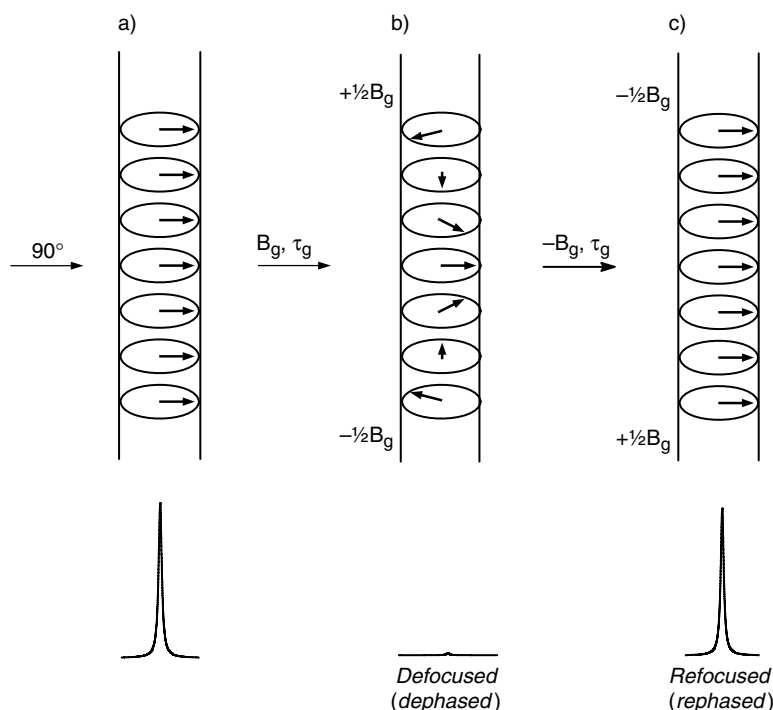
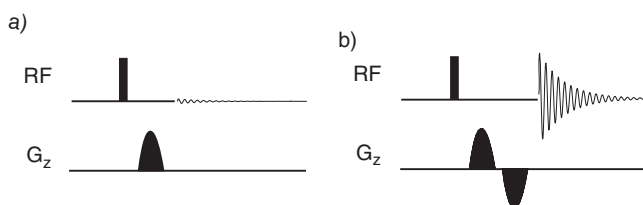


Figure 5.34. The pulse sequence representation of (a) a single z axis field-gradient pulse and (b) two z axis gradient pulses applied in opposite directions. Gradient pulses typically have a shaped rather than square profile, such as the sine profile illustrated here (see Section 5.5.4). Rf identifies the radiofrequency pulse channel.



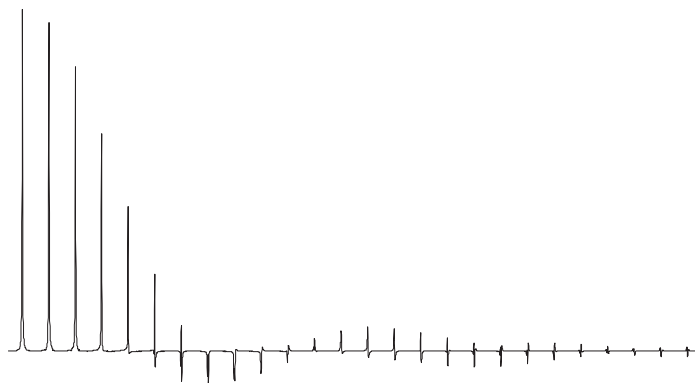


Figure 5.35. The destruction of an NMR resonance with a pulsed field gradient. The sequence of Fig. 5.34a was employed with a progressively stronger gradient for each experiment.

the sample, will be zero, and there would be no detectable NMR signal. The gradient pulse is said to have *defocused* (or *dephased*) the magnetisation. The effect of applying such a sequence with increasing gradient strengths is shown in Fig. 5.35.

Although there is now no *detectable* signal, magnetisation is not lost from the transverse plane and may be recovered. Applying a second gradient pulse immediately after the first, of equal intensity and duration but in the opposite sense (now along the $-z$ axis, Fig. 5.34b), causes the dephasing of the first gradient to be exactly ‘unwound’ by the action of the second (Fig. 5.33c), and the magnetisation vectors from each disk would *refocus* (or *rephase*) to produce a *gradient echo* and an observable net signal (Fig. 5.36). The concept of defocusing all unwanted signals and *selectively* refocusing only those that one desires lies at the heart of every gradient-selected experiment, but to understand how this refocusing can be made selective, we must consider the action of the gradient pulse on transverse magnetisation in a slightly more formal context.

Selective refocusing

In the absence of a field-gradient pulse, the Larmor frequency, ω , of a spin depends upon the applied static field, B_0 , such that $\omega = \gamma B_0$. When the gradient pulse is applied, there is an additional spatially dependent field, $B_{g(z)}$, associated with the gradient giving rise to a spatially dependent Larmor frequency, $\omega_{(z)}$:

$$\omega_{(z)} (\text{rad/s}) = \gamma(B_0 + B_{g(z)}) \quad (5.1)$$

If the gradient is applied for a duration τ_g s, the magnetisation vector rotates through a spatially dependent phase angle, $\Phi_{(z)}$, of

$$\Phi_{(z)} (\text{rad}) = \gamma(B_0 + B_{g(z)})\tau_g = \gamma B_0\tau_g + \gamma B_{g(z)}\tau_g \quad (5.2)$$

The first term in the final expression simply represents the Larmor precession in the absence of the field gradient, which is constant across the whole sample and as such shall not be considered further at this point, except to note that this precession has bearings on the practical implementation of field gradients, as described later. The second term represents the spatially dependent phase caused by the gradient pulse itself, and it is this quantity that is of interest. Generalising the above expression that is only applicable to transverse single-quantum magnetisation, it is necessary to include a term that represents the coherence order, p , of magnetisation, so that

$$\Phi_{(z)} = p\gamma B_{g(z)}\tau_g \quad (5.3)$$

This modification reflects the fact that a p -quantum coherence dephases at a rate proportional to p . Thus, double-quantum coherences dephase twice as fast as single-quantum coherences, yet zero-quantum coherences are insensitive to field gradients. When the coherence involves different nuclear species, allowance must be made for the magnetogyric ratio and the coherence order for each, such that

$$\Phi_{(z)} = B_{g(z)}\tau_g \sum p_I \gamma_I \quad (5.4)$$

It is therefore apparent that the degree of defocusing caused by a gradient is dependent upon the coherence order and the magnetogyric ratio of the spins involved, and these two features provide the key to signal selection with field gradients. The purpose of gradients in the majority

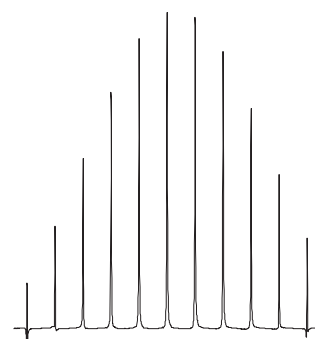


Figure 5.36. The recovery of an NMR resonance in a gradient echo. The sequence of Fig. 5.34b was employed with the strength of the first gradient fixed and that of the second varying from 90 to 110% of the first.

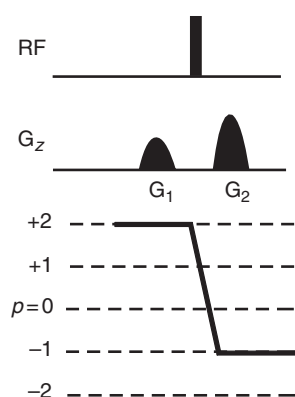


Figure 5.37. An illustration of signal selection by pulsed field gradients. Using two gradients with ratio $G_1:G_2$ of 1:2 selects only the coherence transfer pathway shown, leaving all others defocused and unobservable.

of experiments is the selective refocusing of magnetisation that has followed the desired coherence transfer pathway during the experiment whilst leaving all other pathways defocused and hence unobservable. Although PFGs themselves are unable to alter coherence orders, rf pulses can, and it is a combination of rf pulses to generate the appropriate coherence orders together with the pulsed field gradients to select these that ultimately provides desired result.

This general process is illustrated for a homonuclear spin system in the scheme of Fig. 5.37 in which only DQC existing prior to the pulse is to be retained. Thus, prior to the rf pulse, $p=2$ coherence exists, which experiences a gradient of strength B_{g1} and duration τ_1 and thus obtains a spatially dependent phase

$$\Phi_1 = 2\gamma B_{g1}\tau_1 \quad (5.5)$$

Following the rf pulse, the coherence is transformed into observable single-quantum magnetisation of order $p=-1$, which experiences a second pulsed gradient of strength B_{g2} and duration τ_2 , which encodes a phase

$$\Phi_2 = -\gamma B_{g2}\tau_2 \quad (5.6)$$

For coherence following a defined transfer pathway to be refocused and hence observable, its *total spatially dependent phase from all gradient pulses must be zero*

$$\sum \Phi_i = \sum \gamma B_{gi}\tau_i = 0 \quad (5.7)$$

or in other words, the refocusing induced by the last gradient must exactly undo the defocusing caused by all earlier gradients in the coherence transfer pathway. Thus, in this illustration, Φ_2 must be equal $-\Phi_1$ for this condition to be satisfied. This may be achieved by either altering the duration of the second gradient relative to the first, whilst keeping the amplitude the same, or altering the amplitude and retaining the duration. Nowadays it is uniform practice to use gradient pulses of the same duration throughout a pulse sequence and to alter their amplitudes. Hence, setting $B_{g2}=2B_{g1}$ in this example (remembering that the coherence order has sign as well as magnitude) selects the desired pathway (Fig. 5.37). In contrast, single-quantum magnetisation originating from, for example, any triple-quantum coherence ($p=3$) that may have originally existed acquires a net phase of

$$\Phi = (3\gamma B_{g1}\tau_1) + (-1\gamma 2B_{g1}\tau_1) = \gamma B_{g1}\tau_1 \quad (5.8)$$

and hence remains dephased and does not contribute to the detected signal. The gradient pair has therefore selected only the desired pathway. For *homonuclear* systems with gradients of equal duration, it is sufficient to consider only the *coherence orders* and *ratios of gradient amplitudes* involved in the selection of the coherence transfer pathway, as illustrated in the COSY examples below. For *heteronuclear* systems, *magnetogyric ratios* of the participating spins must also be included, as will be illustrated in Chapter 6. Since gradients within a sequence are assumed to have the same shape and duration in all following discussions, a gradient pulse will simply be denoted as G_i in shorthand form from now on, which is taken to represent a pulse of strength B_{gi} and duration τ_i .

Gradient-selected COSY

To illustrate the incorporation of PFGs into conventional experiments, the basic COSY sequence is again used as an example. It has already been shown that for the absolute-value COSY experiment, those signals to be retained in the final COSY spectrum follow the coherence transfer pathway illustrated in Fig. 5.38, with N-type selection being the favoured option. For signal selection, it is sufficient to phase-encode coherences before the second 90° pulse and decode these after the pulse, immediately prior to detection. For

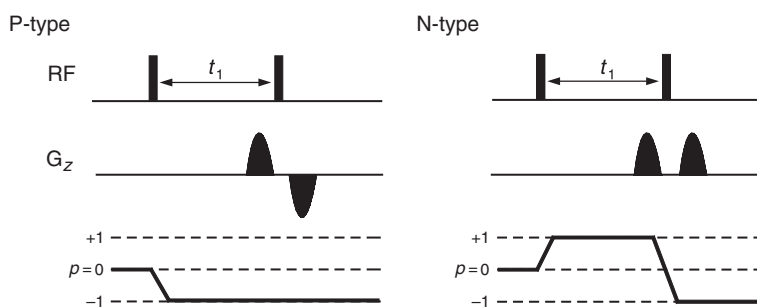


Figure 5.38. The necessary gradient combinations to select P- and N-type COSY data, shown with the corresponding selected coherence transfer pathway.

N-type signals, the desired coherence orders are +1 and -1 at these points and thus the phase encoding induced by each gradient may be represented using shorthand notation as

$$\Phi_1 = +1G_1 \quad \Phi_2 = -1G_2 \quad (5.9)$$

Since one is dealing with a *homonuclear* spin system in COSY, the magnetogyric ratios need not be included as these are constant throughout, so the only variables are the gradient strengths. Hopefully it is quite apparent that for these two expressions to cancel one another, one simply selects $G_2 = G_1$, that is, both gradients should have the same strength applied in the same direction. Conversely, for P-type selection (coherence orders -1 on either side of the pulse), one must select $G_2 = -G_1$, that is, similar gradients applied in opposite directions. In either case, the desired pathway is selected *in a single scan* without the need for phase cycling because gradients that refocus N-type signals cannot simultaneously refocus P-type; hence quadrature detection in f_1 is inherent with this scheme. Axial peaks are also suppressed by this gradient selection because, by definition, these arise from magnetisation that was along the z axis during t_1 and was therefore unaffected by the first gradient. When this magnetisation is made transverse by the second 90° pulse, the final gradient dephases it and it is simply not observed. Thus, the original four steps of the phase-cycled absolute-value COSY (two for f_1 quad detection and two for axial-peak suppression) can be replaced by a single step with PFGs, allowing the 2D data set to be collected with only one transient per t_1 increment, leading to a fourfold reduction in time. This assumes that sensitivity is sufficient to allow the collection of only one scan per increment, which is the case in many routine applications. As an educational exercise, try running a gradient COSY sequence first with N-type and then with P-type selection as described above and compare the results. One should simply be the mirror image of the other about the midpoint of f_1 .

5.5.2. Phase-sensitive experiments

One problem with the gradient approach described above for COSY is that it necessarily precludes the acquisition of high-resolution phase-sensitive data sets by selecting only one pathway in t_1 . Since this leads to phase-modulated data, it provides only phase-twisted lineshapes. Recall that phase-sensitive acquisitions require both $p = \pm 1$ pathways to be retained during t_1 , but this is impossible when a gradient is placed within the evolution period since only one of these pathways may be refocused prior to detection. The most general solution to collecting phase-sensitive data is to avoid placing gradients within t_1 altogether, as this then allows conventional States or TPPI f_1 quadrature detection to be employed. The details of how this is done depend on the sequence in use, and examples of this approach will be illustrated for various techniques in this and in the following chapters. There also exists an ingenious approach to f_1 quadrature detection [11], which provides absorption-mode phase-sensitive data and allows gradients to be placed conveniently within the evolution time. The procedure involves collecting both P- and N-type data sets alternately and storing them *separately* for each t_1 value. The two pathways are selected typically by inverting the sign of the final refocusing gradient, as for the P *versus* N selection above. These data sets contain mirror-image signals in the f_1 dimension when processed conventionally, so mirroring one data set about $f_1 = 0$ and adding it to the second will retain only one set of signals and hence provide the required f_1 frequency discrimination. This procedure also exactly cancels the dispersive contributions otherwise apparent within the phase-twist lineshapes of each data set and thus produces the favoured absorption-mode lineshapes. The data handling required for this procedure is now found within the conventional processing software and is often referred to as the *echo-antiecho* approach.

However, this is not all that is required to produce pure-phase spectra. Following Eq. (5.2), the evolution of chemical shifts under the influence of the static B_0 field during the application of a gradient pulse was ignored for convenience as this played no part in determining signal selection. Such shift evolution during the gradient pulse (which is typically of the order of milliseconds) leads to appreciable frequency-dependent phase errors in spectra if not eliminated. These errors are of no consequence for absolute-value presentations and hence were not considered for the COSY examples above, but they prove disastrous for phase-sensitive data sets. To avoid these failings, gradient pulses are applied either within the existing J -evolution (Δ) periods or must be placed within a spin-echo to refocus the chemical shift evolution (Fig. 5.39). Examples of both these approaches are to be found in the techniques that follow.

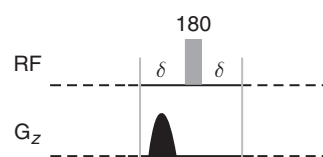


Figure 5.39. Gradient pulses are often applied within spin-echoes to refocus chemical shift evolution occurring during the pulse and thus remove phase errors from spectra.

5.5.3. PFGs in high-resolution NMR

This section summarises some of the key features of pulsed field gradients when applied to high-resolution NMR spectroscopy, highlighting some of the benefits they provide and also warning of potential limitations. Many of these features will be made apparent when describing the gradient-selected techniques in the remainder of the book, so this merely provides an overview of these topics. Broadly speaking, the role of PFGs in high-resolution NMR experiments may be grouped into the following three classes:

1. *Selection of a coherence transfer pathway*: The examples discussed above have already provided an illustration of how gradients may be used in this manner, the aim being to select magnetisation that has followed one desired pathway and suppress those that has followed any other. This application represents the most widespread use of gradients in organic NMR spectroscopy, the resulting experiments providing alternatives to the conventional phase-cycled equivalent.
2. *Suppression of a solvent resonance*: The ability of PFGs to completely destroy an NMR resonance makes them ideally suited for methods of solvent suppression, where the aim is to selectively remove the solvent resonance but retain all others. The most widespread use of these methods is in the studies of molecules in *protonated* water where gradient-based suppression methods prove most effective (Chapter 10).
3. *Purging of unwanted magnetisation*: Purging in this context means the elimination of responses that arise from experimental imperfections and the like, such as inaccurate pulse widths. For example, the use of an imperfect 180° inversion pulse on $+z$ -magnetisation will leave an unwanted residual component in the transverse plane. This may be destroyed by the application of a single purging z gradient (sometimes referred to as a 'homospoil' pulse), ensuring that it will not contribute to the final spectrum. The use of purging z gradients requires all wanted magnetisation to sit along the z axis and so be invariant to the gradient pulse. One of the earliest applications of a gradient pulse within a high-resolution experiment was as a purge pulse in a 2D exchange sequence [33].

Advantages of field gradients

The principal benefits arising from the use of field gradients for coherence pathway selection as opposed to conventional phase cycling may be summarised thus:

Quality: On each transient, only the desired signal is detected, avoiding the need for signal addition/subtraction in the steps of a phase cycle. Difference artefacts are thus not encountered, and the t_1 noise often associated with these in 2D spectra is reduced, providing cleaner spectra.

Speed: As there is no requirement to complete a phase cycle, experiment times are dictated by sensitivity and resolution considerations only. When sensitivity is not limiting, 2D experiments can be acquired with a single transient per t_1 increment, leading to significant time savings.

Dynamic range: As only the desired signal is ever detected, all others being destroyed in the probe head, optimum use can be made of the receiver's dynamic range and greater receiver gains can be employed. This compensates to some degree for the sensitivity losses sometimes associated with gradient selection (see below).

Signal suppression: Very high signal suppression ratios can be achieved. The elimination of protons bound to carbon-12 in proton-detected ^1H - ^{13}C correlations becomes trivial (suppression ratio of $\approx 1:100$) and even the selection of natural-abundance ^1H - ^{13}C - ^{13}C fragments becomes feasible [34], requiring suppression ratios of 1:10,000 (see Section 5.9). Likewise the suppression of large solvent resonances becomes very much easier with gradient methods [35, 36] (Chapter 10).

Ease of use: In contrast to the use of rf pulses, the accurate calibration of gradient strengths is not required, and the absolute strengths used are not critical for the success of the experiment, provided they are sufficient to dephase unwanted magnetisation. More important is the fact that gradient *ratios* must be precise and the gradients reproducible. These demands can be quite readily met with appropriate instrument design, making gradient-selected techniques easy to implement and experimentally robust.

Limitations of field gradients

Despite their undoubted advantages, PFGs have a number of fundamental limitations that should also be appreciated. These have important consequences for the design of gradient experiments, and although one may not be concerned with experiment design, these points help explain some of the features that are common to many of the gradient-selected sequences.

Sensitivity: When used for coherence selection, gradients are able to refocus only one of two $\pm p$ coherence orders. In some applications, this means that only one-half of the available signal is detected in contrast to phase-cycled experiments in which both pathways may be retained. Experiments that use PFGs for coherence selection may therefore exhibit lower sensitivity than the phase-cycled equivalent by a factor of typically 2 or $\sqrt{2}$ depending on the precise experimental details [28, 37]. Purging gradients, however, do not cause such a sensitivity loss.

Quadrature detection: As described above, some care is required when employing gradients in phase-sensitive experiments. This requires either that gradients not be placed within time-domain evolution periods or that the echo–antiecho approach be used.

Phase errors: Chemical shifts also evolve during the application of a gradient pulse due to the static B_0 field. Therefore, gradient pulses are generally applied within a spin-echo to refocus this evolution and thus remove phase errors when phase-sensitive displays are required.

Diffusion losses: The detected signal can be attenuated by losses due to diffusion. The refocusing condition requires that the refocusing gradient undoes the spatially dependent phase caused by all previous gradients. If a molecule was to move along the direction of the gradient (along the length of the NMR tube for a z gradient) between the dephasing and rephasing pulses, the gradient strengths would not be perfectly matched and incomplete refocusing will result, leading to a loss of signal intensity (it is exactly this phenomenon that is used to study diffusion in solution by NMR; see Chapter 9). This will be more of a problem for small, fast-moving molecules in low-viscosity solvents, which can be best avoided by keeping the defocusing and refocusing gradient pulses close together in the sequence. The degree of signal attenuation also depends on the *square* of the gradient strengths, so the use of weaker gradients, which must still be sufficient to dephase magnetisation, will also help minimise such losses.

5.5.4. Practical implementation of PFGs

As any user of an NMR spectrometer will know, the simplest way to generate a field gradient through a sample is to offset one of the shim currents from its optimum. In principle, it is possible to generate the gradient needed by momentarily driving the Z-shim at maximum current, and some spectrometers are provided with a so-called ‘homospoil’ capability to allow this. However, such a set-up is suitable only for providing a basic purge gradient since it produces only relatively weak fields, offers no control over amplitude and would be plagued by the dreaded eddy currents described below. For performing the full range of gradient-selected experiments, a probe equipped with a dedicated gradient coil surrounding the usual rf coils is required, together with an appropriate gradient amplifier. The field gradient across the sample is then generated by applying a current to the gradient coils. Inverting the sign of a gradient corresponds to reversing the applied current. Typical maximum gradient strengths are around 0.5 T/m (50 G/cm) for routine work, although in practice the actual gradient strengths used will often be somewhat less than this. A method for calibrating gradient strengths is described in Section 3.5.2.

As simple as this scheme may sound, it is plagued by a number of technical problems that would make the collection of high-resolution data impossible if left unchecked, and it is these problems that slowed the introduction of field gradients in high-resolution NMR for so long. The worst of these are the eddy currents that are generated on application of the gradient pulse. These are currents in the conducting components that surround the sample, such as the probe case, magnet bore and even the shim coils themselves, which in turn generate further spurious magnetic fields within the sample. Since these can last for hundreds of milliseconds, they prevent the acquisition of a high-resolution spectrum. The most effective way to suppress the generation of eddy currents is the use of the so-called actively shielded gradient coils, which are now used in all commercial gradient probes. These consist of a second gradient coil surrounding the first and driven by the same current. The outer coil is designed in such a way that the field it generates *outside of the active sample region* cancels that produced by the inner coil. The net result is a field gradient within the sample but not outside of it, and hence no eddy currents are stimulated in surrounding structures. Since eddy currents are caused by the rapid change in the local magnetic field on application of the gradient, they can also be attenuated by using a shaped gradient profile with a gentle rise and fall rather than a simple rectangular pulse that has very steep leading and trailing edges. Commonly used gradient profiles are a half-sine wave or a truncated Gaussian. With these steps, it is possible to acquire an unperturbed spectrum within tens of *microseconds* of the gradient pulse; a method for gauging gradient recovery periods is also given in Section 3.5.2.

One final point regarding field gradients is worthy of note. Since a gradient pulse induces a spatially dependent phase for all magnetisation in the sample, this also leads to

the destruction of the deuterium lock signal itself, a rather alarming sight when first encountered. How then does the spectrometer retain the field-frequency stability over extended experiments? The key point stems from the fact that the regulation system integrates the lock error signal over a period of typically many seconds, so it is little effected by the brief disturbances caused by the gradient pulse. The signal presented to the operator is not exactly that used for the field-frequency regulation and is integrated over much shorter time periods, so it presents a more dramatic picture of events to the operator (which itself can be a useful indicator of whether the gradient system is operating!).

This then finishes this part of the chapter that has essentially laid the foundations for understanding 2D (or more generally n D) NMR. Throughout this, the basic COSY sequence, either in its absolute-value or phase-sensitive forms, has been used as an illustrative 2D sequence. Not only is COSY a simple sequence, but the spectra are rather easy to interpret and require relatively little explanation. Beyond this basic sequence, there are various other COSY experiments that provide the chemist with new or modified information. These are, in fact, more widely used in the laboratory than the experiment described thus far, and it is these that are now addressed before progressing to consider other homonuclear correlation techniques.

5.6. ALTERNATIVE COSY SEQUENCES

There are an enormous range of experiments that are, in essence, variations on the simple COSY sequence described above. In this section, we take a look at a selection of these and examine the benefits that these modified sequences provide the practising chemist. Many of the COSY variants proposed in the literature offer little gain over the simpler sequences whilst being rather more complex to execute or process, or are beneficial for a rather limited class of compounds and lack wide applicability. Although the experiments presented below represent only a fraction of the proposed sequences, they have established themselves over the years as the most widely used and informative methods.

5.6.1. Which COSY approach?

Before moving on, we briefly examine the key characteristics of the different experiments and consider why these might be of interest to the research chemist. Table 5.5 summarises the most significant attributes, some of which have already been introduced

Table 5.5. A summary of the characteristic features of the principal COSY experiments and of the related TOCSY experiment

Sequence	Advantages	Potential drawbacks
Absolute-value (magnitude-mode) COSY-90	Simple and robust, magnitude processing well suited to automated operation	Phase-twisted lineshapes produce poor resolution, which require strong resolution enhancement functions. Crosspeak fine structure not usually apparent
Phase-sensitive COSY-90	High-resolution display due to absorptive lineshapes. Crosspeak fine structure apparent; J measurement possible	Diagonal peaks have dispersive lineshapes that may interfere with neighbouring crosspeaks. Requires high digital resolution to reveal multiplet structures
Phase-sensitive DQF-COSY	High-resolution display due to absorptive lineshapes. Crosspeak fine structure apparent; J measurement possible. Diagonals also have absorptive lineshapes. Singlets suppressed	Theoretical sensitivity loss by a factor of 2 relative to the COSY-90 variant. Requires high digital resolution to reveal multiplet structures
COSY- β	Simple and robust. Magnitude processing well suited to automated operation. Simplification of crosspeak structures reduces peak overlap. Vicinal and geminal couplings can be distinguished in some cases from tilt of peaks	Usually requires magnitude-mode presentation as phase-sensitive variant has mixed-phase lineshapes
Delayed COSY	Enhances detection of small- and long-range couplings (< 2 Hz) such as between protons in allylic systems or those in w -relationships	Requires magnitude-mode presentation. Crosspeaks due to larger couplings can be significantly attenuated
Relayed COSY	Provides two (or more)-step transfers and can reduce ambiguities arising from crosspeak overlap	Typically has low sensitivity and responses show mixture of lineshapes, so magnitude-mode presentations may be required. TOCSY preferred
TOCSY	Provides multi-step (relayed) transfers to overcome ambiguities arising from crosspeak overlap. High sensitivity. In-phase lineshapes can provide correlations even in the presence of broad resonances	Number of transfer steps associated with each crosspeak not known, <i>a priori</i> . In-phase lineshapes tend to mask crosspeak fine structure and may preclude J measurement

whilst others are expanded in the sections that follow. Although the TOCSY experiment is not strictly a member of the COSY family, its information content is so closely related to that of COSY that it has also been included in the table.

Pre-empting what is to follow, some general recommendations can be made at this stage. The COSY experiments with the greatest information content are the phase-sensitive versions. For solving spectra of any complexity, the most useful, by virtue of its high resolution and informative crosspeak structure, is the phase-sensitive DQF-COSY. As described below, the theoretical loss in sensitivity is not as detrimental as it may at first sound, and this method is widely used in structural elucidation. To genuinely benefit from the information contained within crosspeak structures, it requires high digital resolution, which invariably corresponds to longer experiments, and phasing of the resulting two-dimensional spectrum can be something of a black art to the uninitiated. For these reasons, it is not so well suited to the rapid sample throughput demanded in busy organic laboratories. For establishing correlations in relatively well-dispersed spectra, the absolute-value COSY offers faster turnover and simplicity of data handling, so it may be favoured when fully automated generation of spectra is required. The absolute-value COSY- β variant, where the mixing pulse β is typically 45 or 60°, provides the most compact peak structure for both diagonal and crosspeaks and is best suited for routine use. In the study of complex spectra where considerable peak overlap occurs, the total correlation or TOCSY experiment can be extremely informative. It provides additional correlation information by relaying magnetisation along networks of coupled spins. This is particularly favoured in the analysis of peptides and oligosaccharides, for example, in which the molecules are composed of discrete monomer units, which themselves represent isolated spin networks. This method has all but replaced the older and inferior relayed COSY experiment that elicits stepwise transfers between coupled spins. The delayed (or long-range) COSY experiment tends to be reserved for addressing specific questions regarding the presence of small couplings and is typically less used.

5.6.2. Double-quantum filtered COSY (DQF-COSY)

Previous sections have already made the case for acquiring COSY data such that it may be presented in the phase-sensitive mode. The pure-absorption lineshapes associated with this provide the highest possible resolution and allow one to extract information from the fine structure within crosspeak multiplets. However, it was also pointed out that the basic COSY-90 sequence suffers from one serious drawback in that diagonal peaks possess dispersion-mode lineshapes when crosspeaks are phased into pure-absorption mode. The broad tails associated with these can mask crosspeaks that fall close to the diagonal, so there is potential for useful information to be lost. The presence of dispersive contributions to the diagonal may be (largely) overcome by the use of the DQF variant of COSY [38], and for this reason DQF (double-quantum filtered)-COSY is the experiment of choice for recording phase-sensitive COSY data.

The DQF sequence

The DQF-COSY sequence (Fig. 5.40) differs from the basic COSY experiment by the addition of a third pulse and the use of a modified phase cycle or gradient sequence to provide the desired selection. Thus, following t_1 frequency labelling, the second 90° pulse generates multiple-quantum coherence, which is not observed in the COSY-90 sequence since it remains invisible to the detector. This may, however, be reconverted into SQC by the application of the third pulse and hence subsequently detected. The required phase cycle or gradient combination selects only signals that existed as DQC between the last two pulses, whilst all other routes are cancelled, hence the term DQF-COSY.

The rules for filtering multiple-quantum coherences of order p are simple; all pulses prior to the p -quantum coherences must be cycled in steps of $180/p$ degrees with

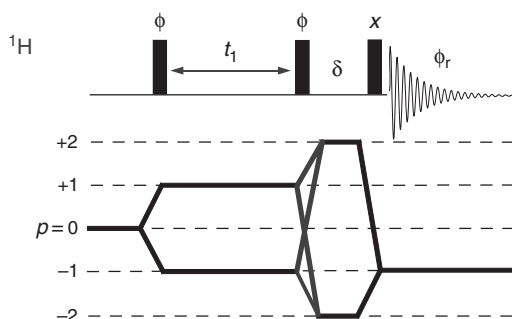


Figure 5.40. The DQF-COSY experiment and coherence transfer pathway. The pulses are phase-cycled as described in the text to select the pathway shown with quadrature detection observing the $p = -1$ magnetisation. The period δ allows for rf phase changes and is typically of only a few microseconds.

Table 5.6. A suitable phase cycle for double-quantum filtration

ϕ	ϕ_r
x	x
y	$-x$
$-x$	x
$-y$	$-x$

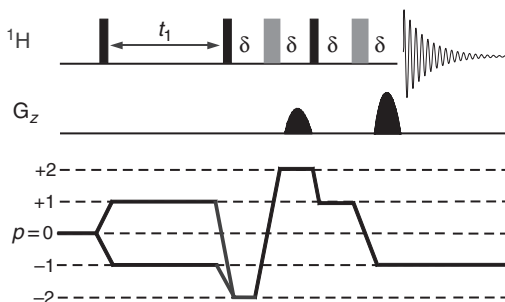
Phase ϕ is used for all pulses prior to the double-quantum coherence (DQC) and ϕ_r for the receiver. The phase of the final pulse remains unchanged.

associated alternation of the receiver phase on each step, [39], so a suitable phase-cycle (although not the only one) for a DQF would be that presented in Table 5.6. More effective filtration can be obtained through gradient selection [40]. Coherence order +2 within the filter may be selected by using gradient amplitude ratios of +1:2 on either side of the last 90° pulse, as in the example of Fig. 5.37 above, whilst order -2 is retained with ratios of +1:–2. Thus, it is possible to select either the +2 or -2 pathway, *but not both at once*. This is in contrast to the phase-cycled experiment in which both pathways are retained, so the gradient experiment detects only *one-half* of the available signal and thus has a two-fold poorer sensitivity relative to its phase-cycled cousin. This fundamental limitation of being able to refocus only one of the two possible $\pm p$ coherence order pathways on each transient is one of the disadvantages of using PFGs for coherence selection, but this must be balanced against the cleaner suppression of unwanted signals. Since these gradients are not required within t_1 in this sequence, conventional quadrature detection (States or TPPI) can be employed. However, simply inserting the appropriate gradients before and after the final pulse would introduce large phase errors to the spectrum because of chemical shift evolution during the gradient pulses. This must therefore be refocused if phase-sensitive data are required; so both gradients are applied within spin-echoes, producing the sequence of Fig. 5.41 for the gradient-selected, phase-sensitive DQF-COSY experiment.

The result from the filtration step, and the principal reason for its use, is that the diagonal peaks now possess anti-phase absorption-mode lineshapes, as do the crosspeaks, which are unaffected by the filtration. Strictly speaking, for spin systems of more than two spins, the diagonal peaks still possess some dispersive contributions; but these are now anti-phase, so they cancel and tend to be weak and rarely problematic. The severe tailing previously associated with diagonal peaks, therefore, is removed, providing a dramatic improvement in the quality of spectra (Fig. 5.42).

An additional benefit from the filtration is that singlet resonances do not appear in the resulting spectrum because they are unable to create DQC, so they cannot pass through the filter. Since sharp singlets produce the most intense t_1 noise bands, their suppression alone can be beneficial. This filtration could also be used to suppress large-solvent resonances that would otherwise dominate the spectrum. Although some success may be achieved through phase cycling, with suppression ratios of the order of a few hundred, far greater suppression can be provided by gradient selection, where suppression ratios can reach 10,000:1. Editing through filtration is exemplified in the 1D DQF spectrum of the peptide Leu-enkephalin **5.4** in CD_3OD (Fig. 5.43), produced with the 1D sequence of Fig. 5.44. The singlet solvent resonances are removed whereas those from all coupled spins are retained and appear with the characteristic anti-phase structure.

Figure 5.41. The gradient-selected DQF-COSY experiment and coherence transfer pathway. No phase cycling is needed as the required pathway is selected with gradient ratios of 1:2. Both gradient pulses are applied within spin-echoes for phase-sensitive presentations. Note that only one pathway is retained from the double-quantum filter.



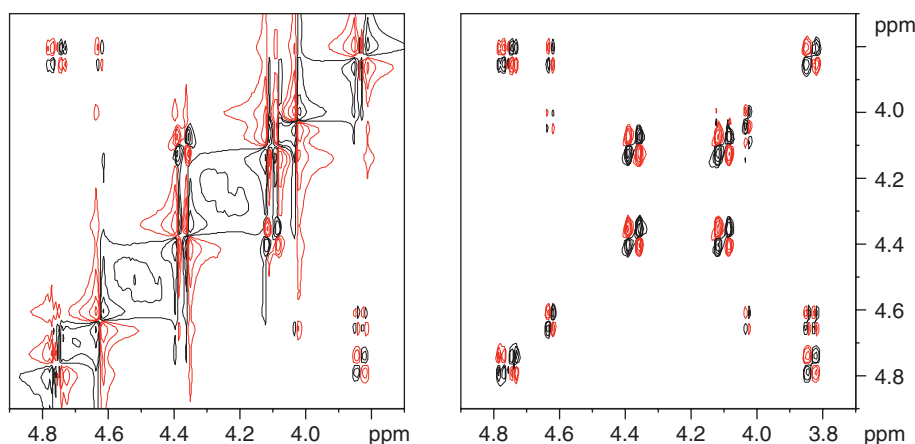


Figure 5.42. The double-quantum filtered COSY spectrum (right) provides greater clarity close to the diagonal peaks than does the basic phase-sensitive COSY (left) as it does not suffer from broad, dispersive diagonal peaks.

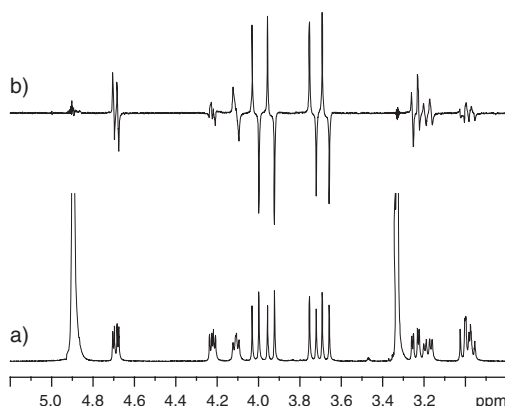
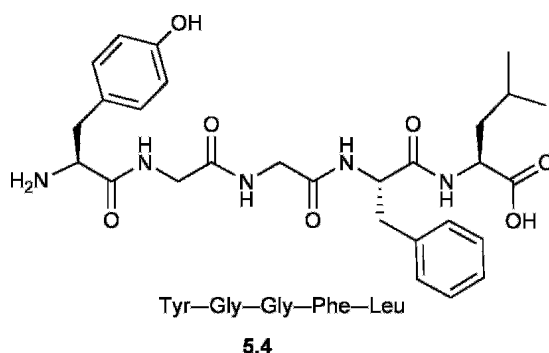


Figure 5.43. 1D double-quantum filtration of the spectrum of the peptide Leu-enkephalin **5.4** in CD_3OD . The singlet resonances of the solvent, truncated in the conventional 1D spectrum (a), have been filtered out in (b). The remaining peaks in (b) display the characteristic anti-phase multiplet structure (which may be masked by magnitude calculation if desired).

The potential disadvantage of using double-quantum filtration is the theoretical reduction in signal-to-noise by a factor of 2 due to losses associated with the generation of DQC. However, the benefits arising from the removal of the dispersive contributions to the diagonal usually compensates for the reduction in sensitivity, hence the DQF-COSY is widely employed.

Interpreting multiplet structure

A major part of 1D spectrum analysis consists of measuring coupling constants within multiplets to gain as much information as possible on relationships between spins within the molecule. Although this is at least plausible for well-resolved multiplets, the task becomes rapidly more difficult and the data obtained less reliable for resonances that overlap. In such cases, it would be desirable to use the greater dispersion available in the 2D spectrum to reveal coupling patterns from otherwise intractable regions, and it has already been shown that COSY crosspeak structure reflects that of the corresponding 1D multiplets. This section examines how one interprets the data within crosspeaks with the ultimate aim of extracting coupling constants.

To do this, it is first necessary to differentiate between active and passive couplings within a crosspeak. The *active* coupling is that which gives rise to the crosspeak that correlates the coupled spins. As described previously, the coupling responsible for the



Figure 5.44. The 1D double-quantum filter. The sequence is derived from the 2D experiment by replacing the variable t_1 period with a fixed spin-echo optimised to produce anti-phase vectors ($\Delta = 1/2J$) as required for the generation of double-quantum coherence. Signal selection is then as for the 2D experiment, and gradient selection may be implemented as in Fig. 5.41.

crosspeak appears as an anti-phase splitting in both dimensions and so produces the basic anti-phase square array from which all COSY crosspeaks are ultimately derived (Fig. 5.45). *Passive* couplings are those to *all other spins* and appear in the spectrum as in-phase splittings. These replicate the anti-phase array of the active coupling without introducing further changes of sign. These features and the basic principles behind analysing multiplet structures may be illustrated by reference to the crosspeaks of a three-spin AMX system in which $J_{AM} > J_{AX} > J_{MX}$ (Fig. 5.46). Consider first the MX crosspeak, in which the anti-phase square pattern of the active J_{MX} coupling is clearly apparent in the upper left corner. This whole pattern is then reproduced with the same phase by the larger passive couplings J_{AX} in f_2 and J_{AM} in f_1 to produce the final 4×4 structure. This stepwise splitting is the precise equivalent to that used in the analysis of 1D multiplets via coupling 'trees'. The f_2 structure of the AX multiplet arises from the large anti-phase J_{AX} coupling being further split by the smaller passive J_{MX} coupling to yield the $++--$ structure. In f_1 , the active coupling is now the smaller of the two, so it is simply repeated to yield the $+-+-$ pattern. Finally, the AM multiplet is dominated by the very large active J_{AM} coupling in both dimensions, which initially yields a large square array, with each part then split by the smaller passive couplings to spin X. The rules for interpreting these structures therefore exactly parallel those for interpreting 1D multiplets, aside from the distinction between active and passive couplings. When coupling occurs between one spin and n equivalent spins, the resulting peak structure may be derived by imagining only one of the n spins to be active and all others passive. One can then construct the anti-phase analogue of the familiar Pascal's triangle for coupling with equivalent spins (Fig. 5.47). Thus, for an AX_3 system, the crosspeak displays a doublet for X and a $+1+1-1-1$ quartet for A (Fig. 5.48).

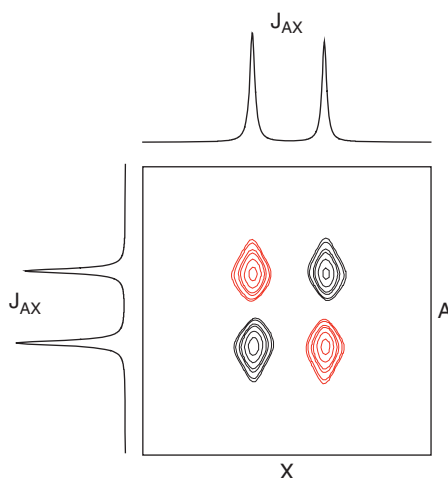


Figure 5.45. The anti-phase square coupling pattern reflects the active coupling, J_{AX} , between two correlated spins A and X. This pattern provides the basis for all COSY crosspeak structures.

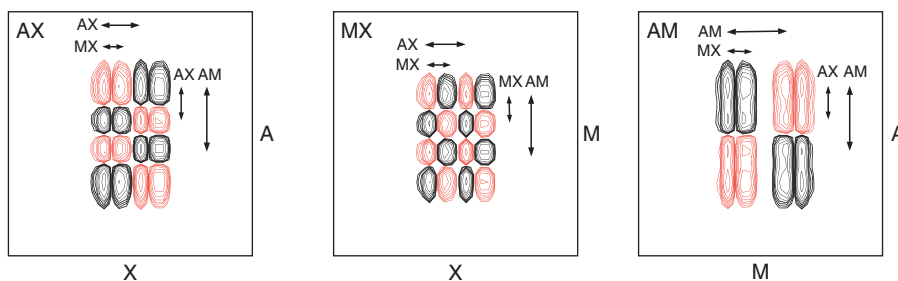


Figure 5.46. The crosspeak structures from the phase-sensitive COSY spectrum of a three-spin AMX system. The arrows indicate splittings due to the labelled couplings. The spectrum was simulated with $J_{AM} = 18$, $J_{AX} = 12$ and $J_{MX} = 6$ Hz, and the final digital resolution was 2.3 Hz/pt in each dimension.

n	a)	b)
0	1	1
1	1 1	1 -1
2	1 2 1	1 0 -1
3	1 3 3 1	1 1 -1 -1
4	1 4 6 4 1	1 2 0 -2 -1
5	1 5 10 10 5 1	1 3 2 -2 -3 -1
6	1 6 15 20 15 6 1	1 4 5 0 -5 -4 -1

Figure 5.47. (a) The conventional Pascal's triangle for 1D multiplets and (b) the anti-phase equivalent for predicting COSY multiplet structures from coupling to n equivalent spins.

In realistic systems, multiplet structures may derive from very many couplings, and resolution of all of these may not be possible. This leads to overlap within the multiplet with associated line cancellation and/or superposition. In general, crosspeak structures tend towards the simplest pattern as neighbouring lines with like sign merge. Often in such cases, smaller couplings may not be resolved, as is nearly the case for the passive MX and AX couplings within the AM crosspeak of Fig. 5.46. When analysing fine structure in detail, it is often advantageous to examine both sets of equivalent crosspeaks on either side of the diagonal since these may have differing appearances according to the different digitisation levels used, meaning a coupling may be unresolved in one dimension but quite apparent along the other.

Measuring coupling constants

Provided multiplets structures are sufficiently resolved and free from overlap with other peaks, it is, in principle, possible to measure coupling constants directly from these [41]. Some caution is required, however, since one needs to consider whether cancellation within the multiplet could lead to an erroneous measurement. For example, if the central lines of a double doublet were to interfere destructively and cancel, the measured splitting would then be greater than the true coupling constant. Digital resolution must also be adequate to properly characterise the coupling constant, with the highest resolution being found in the f_2 dimension. To improve the accuracy of the measurement, it is advantageous to extract 1D traces from the 2D spectrum and to subject these to *inverse* Fourier transformation (*iFT*), zero-filling (or still better, linear prediction) and then Fourier transformation to reproduce the 1D row with increased digital resolution. This process is illustrated in Fig. 5.49 for the $\alpha\beta$ coupling constants in the tyrosine residue of the peptide Leu-enkephalin 5.4. Above the 2D crosspeaks is the 1D trace through these which has been treated as described above and zero filled to produce the same digital resolution as the conventional 1D spectrum shown on top (0.4 Hz/pt). From the anti-phase and in-phase splittings, the two active $\alpha\beta$ couplings and the passive $\beta\beta$ coupling are readily measured as 6.1, 8.3 and 14.0 Hz.

The measurement of coupling constants in this manner is, however, limited by finite linewidths, which in 2D spectra of small- to mid-sized molecules are usually dictated by the digital resolution of the spectrum rather than natural linewidths. Under such conditions, lines that are in-phase tend to merge together to produce a single maxima, which may mask the coupling, whereas anti-phase lines remain separated but may produce apparent splittings that are greater than the true couplings. As coupling constants become smaller, the splitting of anti-phase lines in fact tends to a *minimum* of ≈ 0.6 times the linewidth [42] (Fig. 5.50), after which further reductions in the true separation act only to reduce peak intensities until complete cancellation occurs and the crosspeak is lost. One may conclude therefore that it is not possible to measure reliably coupling constants that are significantly smaller than the observed linewidths after digitisation. A rule of thumb is that couplings should be greater than 1.5 times the *digitised* linewidth for measurements to be made, and spectra recorded for the measurement of coupling constants are typically acquired with higher digital resolution in f_2 where one can afford to be profligate with data points. These

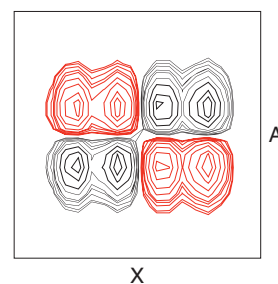


Figure 5.48. The COSY crosspeak for an AX_3 group illustrating the structure predicted from Pascal's triangle.

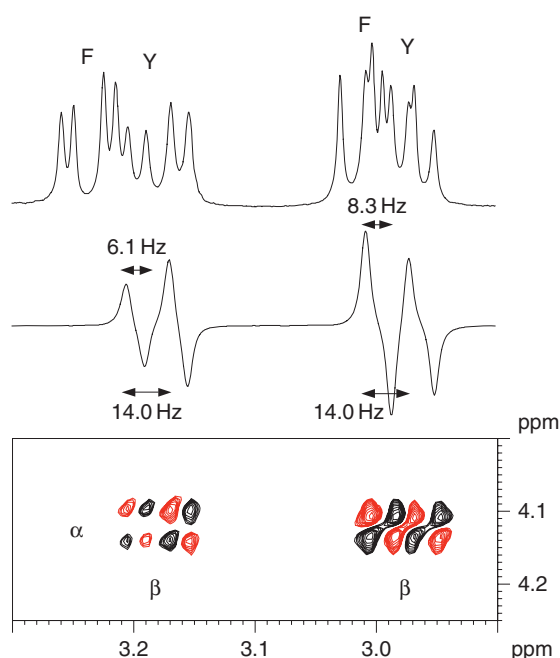
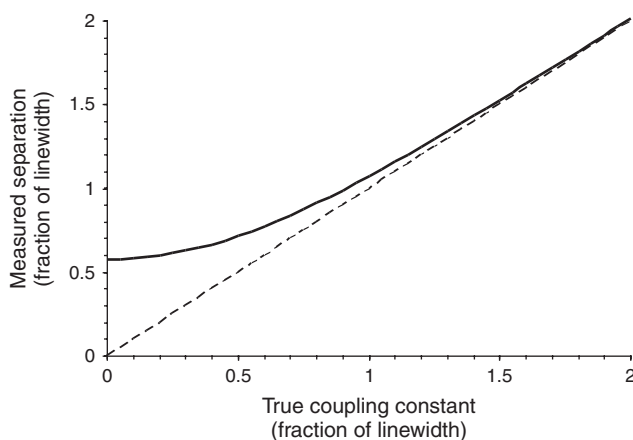


Figure 5.49. Sections from the DQF-COSY spectrum of the pentapeptide Leu-enkephalin 5.4. The 2D crosspeaks and the f_2 1D trace taken through these correspond to correlations within the tyrosine (Y) residue. The upper trace is taken from the conventional 1D spectrum in which the β -proton resonances partially overlap with those of phenylalanine (F). The original 2D data had an f_2 resolution of 1.8 Hz/pt but the 1D trace was treated as described in the text to yield a final resolution of 0.4 Hz/pt.

Figure 5.50. Peak separations of anti-phase lines as a function of coupling constant and linewidth. As coupling constants become relatively smaller, the measured splitting (solid line) deviates from the true value (dashed line), producing measurements that are too large.



considerations also indicate that crosspeaks will disappear from COSY spectra if linewidths are large or if digital resolution is low relative to the active coupling constant. Thus, crosspeaks due to small couplings or to broad lines are more likely to be missing. This can be a serious problem for very large molecules that have naturally broad resonances, and in such cases, the TOCSY experiment provides superior results since crosspeaks have only in-phase structure. More sophisticated methods also exist, which enable the measurement of coupling constants in complex multiplets. For example, the DISCO method [43–45] of post-processing relies on the addition and subtraction of rows from COSY spectra to produce simplified traces containing fewer signals, thus making measurements more reliable. Details of the various methods available shall not be addressed here but have been reviewed [41].

Even when the data at hand precludes the measurement of coupling constants, analysis of the relative magnitudes of active and passive couplings within multiplets can prove enlightening when interpreting spectra. For example, it is possible in favourable cases to determine the relative configuration of protons within substituted cyclohexanes from consideration of the crosspeak structures. Since *axial–axial* couplings in chair conformers are typically far greater than *axial–equatorial* or *equatorial–equatorial* couplings ($ax–ax \approx 10–12$ Hz, $ax–eq/eq–eq \approx 2–5$ Hz), they appear as large anti-phase splittings within crosspeak multiplets, indicative of the diaxial relationship between the correlated spins. This is illustrated in Fig. 5.51, which shows a region of the DQF-COSY spectrum of andrographolide **5.5**,

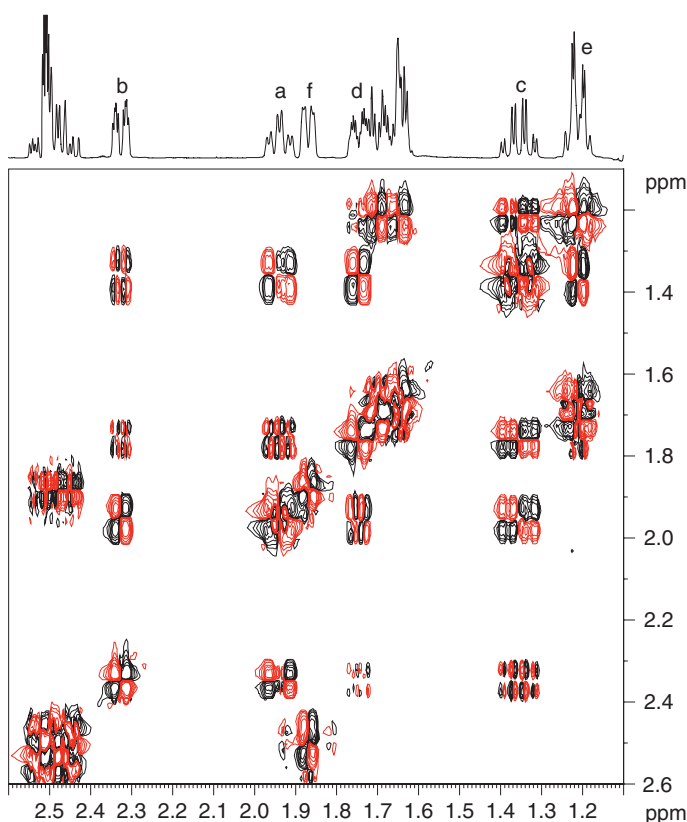
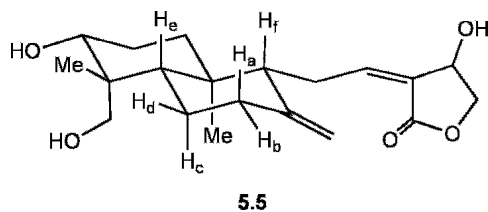


Figure 5.51. A region of the DQF-COSY spectrum of andrographolide **5.5**. The data were collected under conditions of high f_2 resolution (1.7 Hz/pt) to reveal the coupling fine structure within the crosspeaks.



an hepato-protective agent found in traditional Indian herbal remedies. Three of the cross-peaks of proton H_C display large active couplings consistent with either geminal (H_d) or diaxial (H_a , H_e) relationships, whilst the much smaller active coupling to H_b limits these to being *ax-eq* or *eq-eq*. Such detail can be extracted from the 2D map even when multiplets are buried or are too complex for direct analysis in the 1D spectrum. For example, the correlations of H_d identify its geminal partner and indicate three vicinal *ax-eq* or *eq-eq* relationships with H_e , H_a and H_b . This type of information, which can be determined reliably only from phase-sensitive presentations, can often be used to good effect in stereochemical assignments, particularly when used in conjunction with the nuclear Overhauser effect.

Higher-order multiple-quantum filters

Using the same sequences as for DQF-COSY, but with modified phase cycles or gradient combinations, it is possible to filter for higher orders of multiple-quantum coherence between the last two pulses; so, for example, a gradient ratio of 1:3 in the sequence of Fig. 5.41 selects triple-quantum coherence only. The triple-quantum-filtered (TQF) COSY experiment eliminates responses from all singlets and two-spin systems leading to potential simplification. More generally, a p -quantum filter can be used to remove peaks arising from spin systems with fewer than p coupled spins [39, 46]. Such experiments have potential utility in the study of molecules containing well-defined spin systems that are isolated from each other in the molecule, notably amino acids in peptides and proteins [47]. However, the great disadvantage of higher-order filtering is the reduction in sensitivity by a factor of 2^{p-1} for a p -quantum filter. Although a loss of a factor of 2 for the DQF-COSY relative to its unfiltered cousin might be tolerable, a factor of 4 for the TQF-COSY may make it untenable. Largely for this reason, the TQF-COSY is rarely used and higher-order filtering is essentially unheard of in routine work. The rules for interpreting multiplet fine structure in COSY recorded with high-order filtration also require some modification [47, 48].

5.6.3. COSY- β

A common modification of the basic COSY sequence is one in which the 90° mixing pulse is replaced with one of the shorter tip angle, β , usually of 45° or 60° (Fig. 5.52). These experiments are typically acquired and presented as absolute-value experiments since, strictly speaking, the use of a pulse angle less than 90° does not produce purely amplitude-modulated data.

The use of a reduced mixing pulse largely restricts coherence transfer between transitions that are *directly* connected, or in other words, those that share an energy level, for example, A_1 and X_2 in Fig. 5.53. Coherence transfer between *remotely* connected transitions, for example, A_1 and A_2 , is attenuated. This results in a reduction in intensity of certain lines *within* the multiplet structures of both diagonal and crosspeaks, which is particularly noticeable when spins experience more than one coupling. Diagonal peaks have a somewhat smaller 'footprint', increasing the chances of resolving crosspeaks that sit close to this, whilst crosspeaks themselves also have a simplified structure that can take on a distinctive 'tilted' appearance (Fig. 5.54). In favourable cases, this tilting effect can be used to differentiate between peaks arising from active couplings of opposite sign, such as geminal or vicinal couplings. Consider the situation of a three-spin AMX system in which all spins couple to each other. The AM crosspeak, for example, will have a positive slope and its tilt will be approximately parallel to the diagonal if the two *passive* couplings, J_{AX} and J_{MX} , have the *same* sign. In contrast, the peak will display a negative slope and lie anti-parallel to the diagonal if the signs of the passive couplings differ. In proton spectroscopy, vicinal couplings are typically positive and geminal couplings negative, meaning a crosspeak between a proton and its *geminal* partner will possess a *positive slope* whereas that with the *vicinal* partner will display a *negative slope*. This effect is apparent in the COSY- 45° spectrum of **5.1** in Fig. 5.54. Couplings to additional spins further split the crosspeak

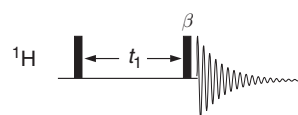


Figure 5.52. The COSY- β experiment. The β mixing pulse is usually set to 45° or 60° .

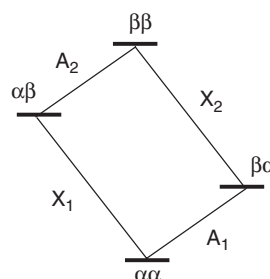
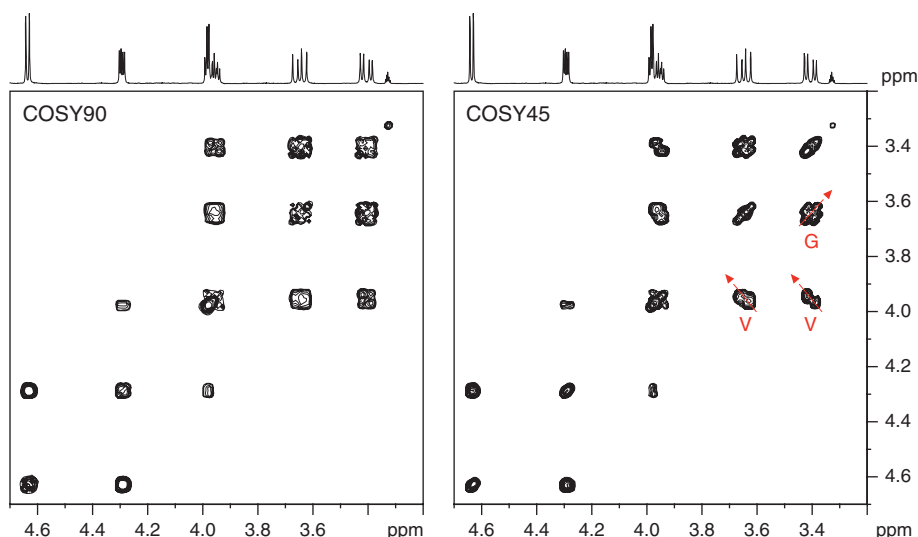


Figure 5.53. The schematic energy level diagram for a two-spin AX system.

Figure 5.54. The absolute-value COSY-45 spectrum of **5.1** compared with the equivalent COSY-90 spectrum. The tilting apparent on some COSY-45 crosspeaks can indicate whether the active coupling arises from protons sharing geminal (G) or vicinal (V) relationships, which produce peaks with positive and negative slopes, respectively (see text).



structure and in such cases the tilting may not be distinguished. Nevertheless, when this effect is observed, it provides valuable additional evidence by identifying geminal pairs.

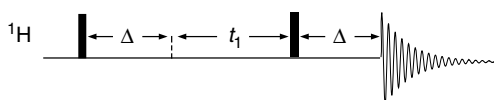
The choice between $\beta = 45^\circ$ and 60° is something of personal taste. Setting $\beta = 45^\circ$ causes the greatest intensity ratio between direct and remote transitions (a factor of about 6 for 45° versus 3 for 60°) and thus gives the greatest reduction in peak complexity, whilst setting $\beta = 60^\circ$ provides slightly higher sensitivity. Since the primary goal of this variant is the reduction of the diagonal and crosspeak footprint, the COSY-45 experiment is probably the one to choose. Due to the more compact peak structures and simplicity of processing, the absolute-value COSY-45 experiment still finds widespread use in routine analyses and automated procedures in the chemistry laboratory.

5.6.4. Delayed COSY: detecting small couplings

The COSY sequences encountered thus far are surprisingly effective at revealing small couplings between protons. However, such crosspeaks are typically rather weak, and their identification as genuine correlations may be difficult. The delayed COSY experiment (Fig. 5.55) enhances the intensity of correlations due to couplings that are smaller than natural linewidths, and since these are often encountered for coupling pathways over many bonds, the experiment is also referred to as *long-range COSY*. Correlations over four or five bonds in rigid or unsaturated systems, such as those from *w*-, allylic or homoallylic couplings, can be observed readily with this experiment even though the coupling may not be resolved in the 1D spectrum (Fig. 5.56).

Previous discussions have stressed the need for an anti-phase disposition between multiplet vectors for coherence transfer to occur. In COSY, this disposition arises from the evolution of spin–spin couplings during t_1 and develops at a slower rate the smaller the coupling. For the case of very small coupling constants, there is insufficient time for this to occur under the typical conditions employed for COSY acquisitions, and hence the crosspeaks from these couplings are weak or undetectable. It is possible to enhance the intensity of these peaks by adjusting the t_1 and t_2 domains such that they are optimised for detection of the coherence transfer signal from these smaller couplings. Although this could in principle be achieved by extending both time periods and hence increasing the digital resolution in both dimensions, this generates enormous data sets and calls for protracted experiments. The approach used in the delayed COSY sequence is to insert a *fixed* delay after each pulse to increase the *apparent* evolution times yet retain the same level of digitisation as in a standard COSY experiment. The use of the additional delays precludes the use of phase-sensitive presentations because of the large phase distortions that arise; so absolute-value processing is necessary.

Figure 5.55. The delayed (or long-range) COSY sequence. Additional fixed delays are inserted into the basic COSY sequence to enhance the appearance of correlations from small couplings (see text).



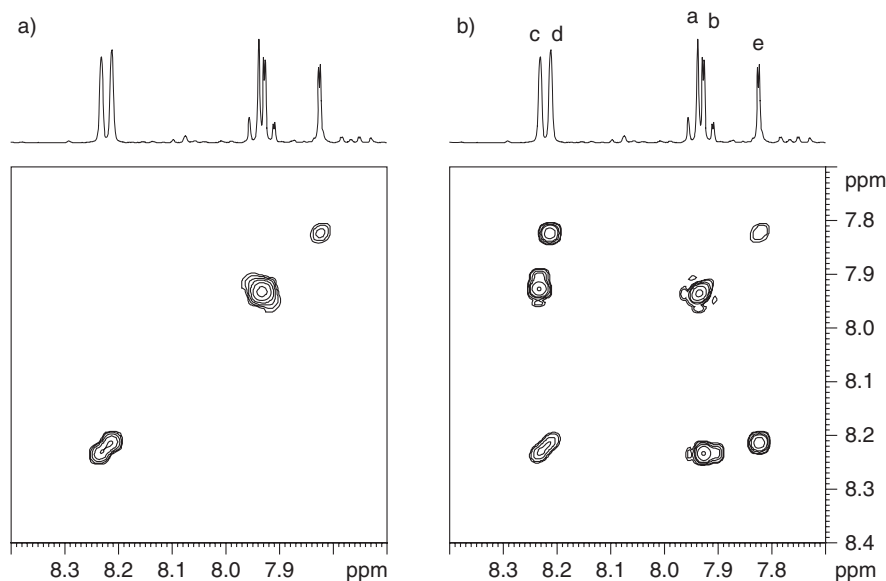
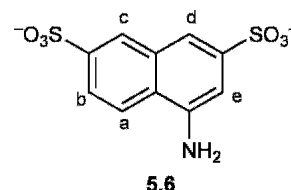


Figure 5.56. (a) The conventional COSY-90 spectrum and (b) the delayed COSY spectrum of **5.6**. Additional Δ delays of 200 ms were used in (b) whilst all other parameters were as for (a). The small (1 Hz), long-range couplings are not apparent in the conventional COSY experiment but the correlations in (b) unambiguously provide proton assignments.



For optimum detection of small couplings, the maximum of the coherence transfer signals should occur at the *midpoint* of each time domain. It may be shown that this maximum arises at a time t where

$$t = \frac{\tan^{-1}(\pi J T_2)}{\pi J} \quad (5.10)$$

When J is small such that $J T_2 \ll 1$, the approximation $t = T_2$ holds, and to ensure that the midpoint of each time domain coincides with this maximum, the additional delay Δ is therefore

$$\Delta = T_2 - 0.5 A Q \quad (5.11)$$

where AQ is the total t_1 or t_2 acquisition time. For typical COSY acquisition parameters and typical proton T_2 values, Δ falls in the range of 50–500 ms. Since the actual values of T_2 are not usually known in advance, and there will in any case exist a spread within the molecule, a compromise setting of around 200 ms should suffice in most cases. The crosspeaks arising from couplings of greater magnitude can be severely attenuated in this experiment, so it is invariably also necessary to record the standard COSY to establish the usual coupling relationships.

5.6.5. Relayed COSY

The relayed COSY experiment shall be considered only very briefly because it has essentially been superseded by the far superior TOCSY experiment described below, although one may still encounter references to this experiment in older literature. The relayed COSY experiment (Fig. 5.57) attempts to overcome problems caused by coincidental overlap of crosspeaks in COSY, which can lead to a breakdown in the stepwise tracing of coupling networks within a molecule. It incorporates an additional coherence transfer step in which that transferred from a spin, A, to its partner, M, as in the standard COSY, is subsequently relayed onto the next coupled spin X in the sequence. This produces a crosspeak in the spectrum between spins A and X *even though there exists no direct coupling between them*, by virtue of them sharing the common coupling partner M. Ambiguities from coincidental overlap of crosspeaks associated with spin M are therefore removed by providing direct evidence for A and X existing within the same spin system.

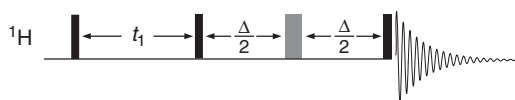


Figure 5.57. The relayed COSY sequence in which an additional transfer step has been appended to the basic COSY experiment.

The relayed coherence transfer [49] is achieved by inserting a fixed time period, Δ , after the second pulse, again in the form of a spin-echo to remove chemical shift effects. During this the M–X coupling evolves to produce anti-phase multiplet vectors (the MX coupling was passive in the initial A–M transfer, so these vectors are in-phase after the second 90° pulse). The third 90° pulse then elicits the M–X coherence transfer prior to detection. The fixed delay Δ should be set optimally to $1/2J$ to maximise this transfer, so a compromise setting of $J = 7$ Hz gives $\Delta = 70$ ms. The direct responses observed in the usual COSY experiment may also be present, but with varying intensities.

5.7. TOTAL CORRELATION SPECTROSCOPY (TOCSY)

The principal feature of all the COSY experiments described above is the direct correlation of homonuclear spins that share a scalar coupling. In proton spectroscopy, this typically provides the chemist with evidence for geminal and vicinal relationships between protons within a molecule. TOCSY (Total Correlation Spectroscopy) [50], not only yields homonuclear proton correlation spectra based on scalar couplings but also is able to establish correlations between protons that sit within the *same spin system*, regardless of whether they are themselves coupled to one another. In other words, provided there is a continuous chain of spin–spin coupled protons, for example, A–B–C–D–... , the TOCSY sequence transfers magnetisation of spin A onto spins B, C, D, etc. by relaying coherence from one proton to the next along the chain. In principle, this can correlate all protons within a spin system, and this feature earns the title TOCSY. The ability to relay magnetisation in this manner provides an enormously powerful means of mapping correlations by making even greater use of the additional dispersion found in two dimensions. This is particularly advantageous in cases of severe resonance overlap, for which COSY spectra can often leave ambiguities. The TOCSY spectrum for **5.1** is shown in Fig. 5.58 and has been recorded so as to provide multistep transfers around the carbohydrate ring. This should be compared with the COSY spectrum of the same material in Fig. 5.12. The additional relayed crosspeaks seen in TOCSY provide correlations between *all* spins within the ring system, and this ability to fully map spin systems is particularly advantageous when discrete units exist within a molecule, as illustrated shortly. A second significant feature of TOCSY that contrasts with COSY is that it utilises the net transfer of *in-phase* magnetisation; so it does not suffer from cancellation of anti-phase peaks under conditions of low digital resolution or large linewidths. In these instances, this feature makes TOCSY the more sensitive of these two methods.

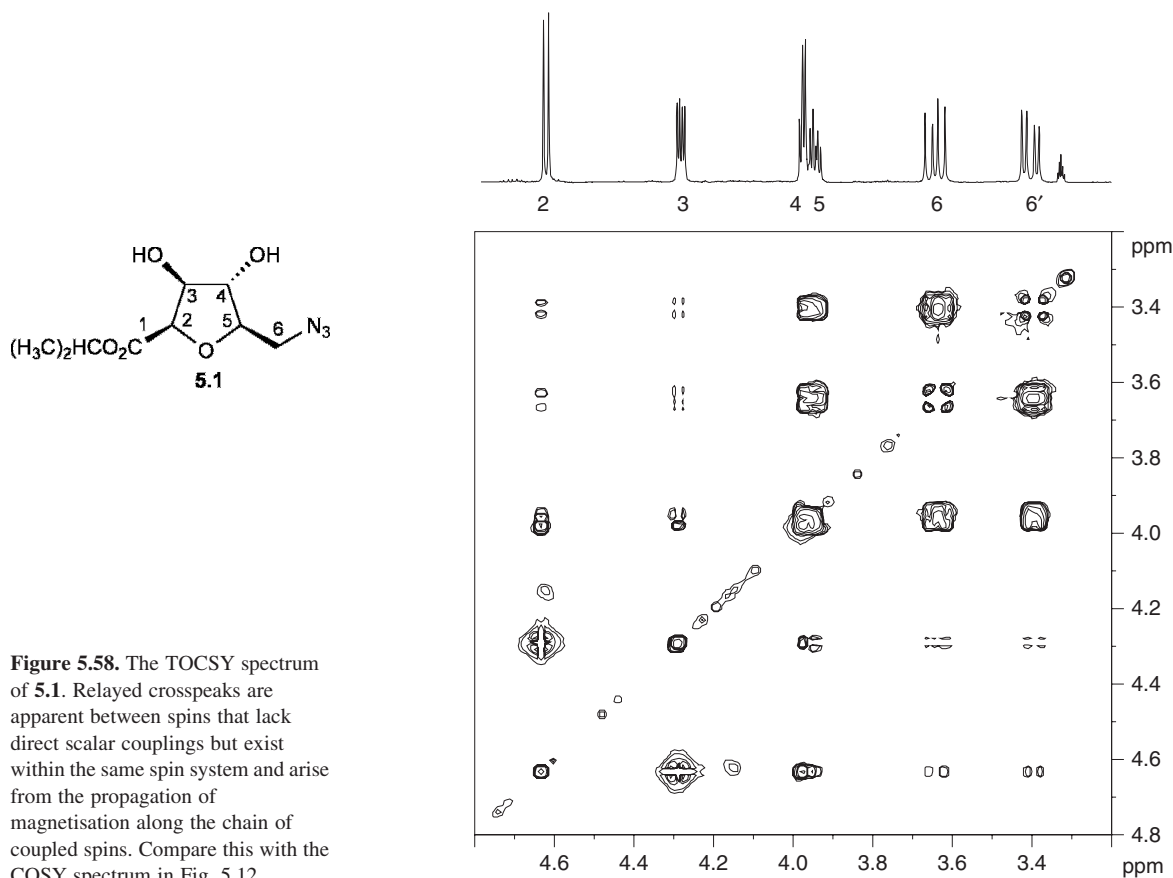


Figure 5.58. The TOCSY spectrum of **5.1**. Relayed crosspeaks are apparent between spins that lack direct scalar couplings but exist within the same spin system and arise from the propagation of magnetisation along the chain of coupled spins. Compare this with the COSY spectrum in Fig. 5.12.

An essentially identical experiment has also been referred to as homonuclear Hartmann–Hahn spectroscopy [51, 52] or HOHAHA (the two differ only in some technical details in the originally published sequences). This name arises from its similarity with methods used in solid-state NMR spectroscopy for the transfer of polarisation from proton to carbon nuclei (so-called cross-polarisation), which are based on the Hartmann–Hahn match described below. For the same reason, the transfer of magnetisation during the TOCSY sequence is sometimes referred to as *homonuclear cross-polarisation*. Throughout this text, the original TOCSY terminology is used, although TOCSY and HOHAHA have been used synonymously in the chemical literature.

5.7.1. The TOCSY sequence

The TOCSY sequence, represented in its most general form in Fig. 5.59, is rather similar to the COSY sequence already described, the only difference being the use of a mixing *sequence* in place of a single mixing *pulse*. This is known as a *spin-lock* (SL) or *isotropic mixing* sequence (this terminology is explained below), and its purpose is to execute the relayed magnetisation transfer mentioned above. Details such as quadrature detection and axial-peak suppression parallel those for COSY; so to understand the operation of the TOCSY experiment, it becomes necessary to appreciate the influence of the spin-lock.

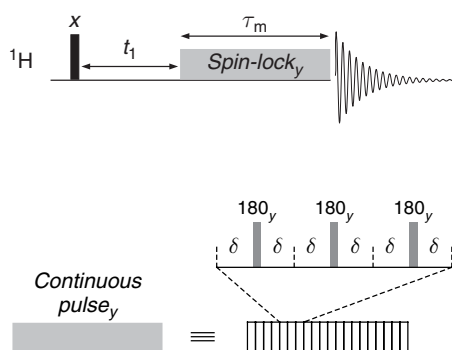


Figure 5.59. The TOCSY sequence. The spin-lock mixing time, τ_m , replaces the single mixing pulse of the basic COSY experiment.

Figure 5.60. The spin-lock in its simplest form is a single, long, low-power pulse. This can be viewed as a continuous sequence of closely spaced 180° pulses bracketed by infinitely small periods, δ .

The spin-lock and coherence transfer

To understand the action of the spin-lock period in the TOCSY experiment, consider the sequence of events during the first transient of the 2D experiment (i.e. that with $t_1 = 0$). The experiment begins by exciting the spins with a 90°_x pulse such that they all lie along the $+y$ axis in the rotating frame. At this point, the spin-lock rf field is applied, this time along the $+y$ axis, parallel to the nuclear vectors. In its simplest form, the spin-lock is a continuous low-power pulse of constant phase applied for a period of typically tens of milliseconds. It is convenient to imagine this to be composed of a continuous sequence of closely spaced 180°_y pulses bracketed by infinitely small periods δ (Fig. 5.60). Each δ – 180° – δ period constitutes nothing more than the homonuclear spin-echo described in Chapter 2, and to picture the evolution of chemical shifts and homonuclear spin–spin couplings during the spin-lock, it is sufficient to consider events within a discrete δ – 180° – δ period. It has already been shown in Section 2.2.4 that a 180° pulse refocuses the evolution of chemical shifts, so after each δ – 180° – δ period, no shift evolution has accrued and all spin vectors remain along the $+y$ axis. Extending this argument for the whole of the mixing period, it is apparent that no net chemical shift evolution occurs during τ_m , and hence the nuclear vectors are said to have been *spin-locked* in the rotating frame along, in this case, the y axis (Fig. 5.61). During the spin-lock mixing, all protons have experienced the same effective field and hence the same *chemical shift offset* (i.e. zero) in the rotating frame. Section 2.2.4 has also shown that, in contrast, *homonuclear* spin–spin couplings *continue* to evolve following a 180° pulse, so that throughout the spin-lock J -couplings behave as they would for a period of free precession. In summary, during the spin-lock mixing, all chemical shift differences in the rotating frame are removed yet spin–spin couplings remain active (Fig. 5.62).

Nuclei that share a coupling and have very similar (or coincident) chemical shifts relative to their coupling constant are said to be *strongly coupled*, and under such conditions, they lose their unique identity and ultimately become indistinguishable. Thus, for a system containing a coupled AB pair, it is not possible to consider the interactions of spin A independently from those of its strongly coupled neighbour B and *vice versa*. Such a strong coupling condition is *forced* upon all spin systems by the application of the spin-lock field since the protons remain coupled yet all possess the same effective chemical shift. The

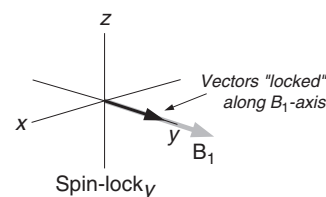
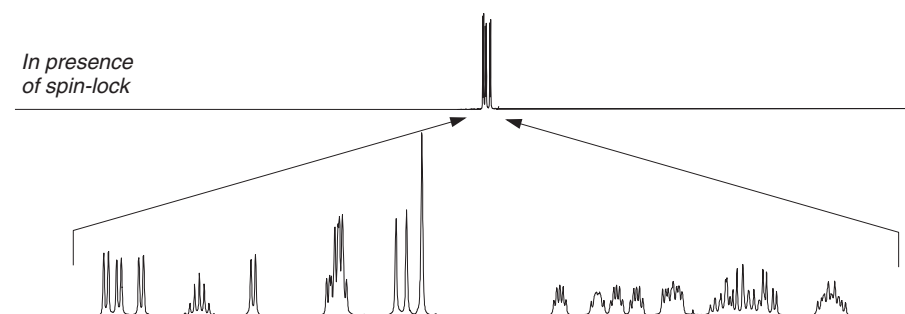


Figure 5.61. Vectors are held along the B_1 axis by the long rf pulse and are said to be *spin-locked* in the rotating frame.

Figure 5.62. A schematic illustration of events during spin-lock mixing. All chemical shift differences between spins are eliminated yet all spin-spin couplings between them remain. This forces the *strong coupling* condition on all spins (see text).



protons therefore lose their 'unique' identity during the mixing period, and this provides the mechanism by which coherence may be shared over all spins within the same spin system. Under these conditions, there exists an oscillatory exchange of coherence between protons during the spin-lock, which, for an AX system, results in complete transfer from A to X after a period of $1/2J_{AX}$ s [50] and a return to A after $1/J$ s (Fig. 5.63). Although events become more complex for larger systems, the general idea of an oscillatory exchange of coherence between spins still holds, leading to a propagation of magnetisation along the chain of coupled spins [53] (Fig. 5.64). For short mixing times of around 20 ms in proton experiments, only single-step transfers have significant intensity and the correlations seen are equivalent to those seen in COSY. Longer mixing periods enable magnetisation to propagate further along the coupled chain and relay peaks arising from multi-step transfers appear, as seen in Fig. 5.58. Small couplings lead to poor transfer efficiencies and can lead to a breakdown in the transfer and to a loss of relay peaks. Since magnetisation may travel in either direction along a spin chain, 2D TOCSY spectra are again symmetrical about the diagonal.

The requirement for nuclei to experience identical local fields during the mixing time for transfer to occur between them is also referred to as the *Hartmann-Hahn match*. More formally and more generally, this may be expressed as follows:

$$\gamma_A B_{1A} = \gamma_X B_{1X} \quad (5.12)$$

where B_{1A} and B_{1X} are the B_1 fields experienced by spins A and X and γ_A and γ_X are their magnetogyric ratios, respectively. The inclusion of the γ s in this means the nuclides involved in the exchange need not be the same, so, for example, transfer can be initiated between proton and carbon if simultaneous rf fields that satisfy Eq. (5.12) can be applied to each nuclide. This, by analogy, leads to hetero-TOCSY or, more amusingly, heteronuclear Hartmann-Hahn spectroscopy (HEHAHA) as a means of establishing heteronuclear correlations [54, 55].

Figure 5.63. The oscillatory transfer of magnetisation between a coupled proton pair under the influence of a spin-lock. The transfer was simulated using an MLEV-17 spin-lock (see text) of increasing duration in each experiment. In each case, this was applied immediately after the selective excitation of the high-frequency doublet with a pure-phase EBURP-2-selective 90° pulse (Chapter 10).

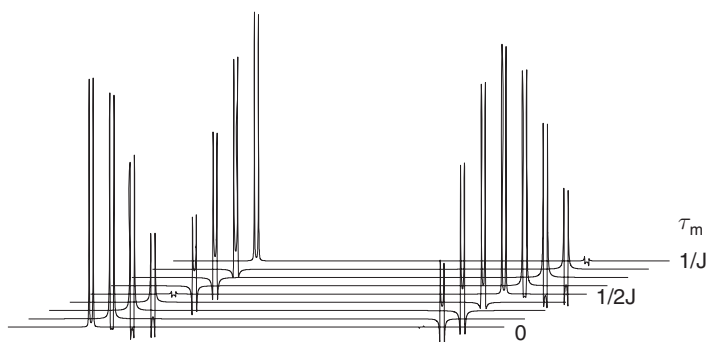
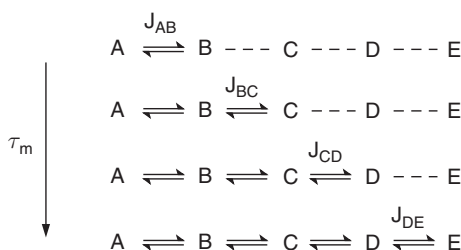


Figure 5.64. Schematic illustration of the propagation of magnetisation along a chain of coupled spins as a function of the spin-lock mixing time. The arrows indicate the cyclic exchange of coherence between nuclei.



5.7.2. Applying TOCSY

The ability of TOCSY to propagate magnetisation along a spin system means that it has become a popular tool for studying molecules that are composed of discrete and often well-defined units. Obvious examples of this are proteins, peptides and oligosaccharides, for which it is often possible to trace the whole amino acid or sugar ring system from a single resolved proton. This is illustrated in the TOCSY spectrum of gramicidin-S, **5.7**, a cyclic decapeptide (Fig. 5.65). Only the aliphatic region is shown, in

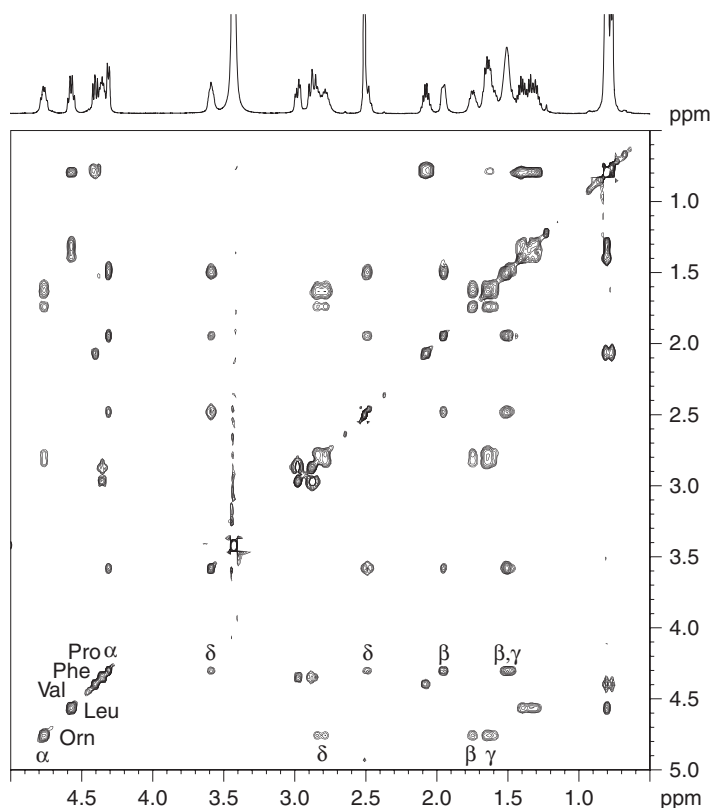
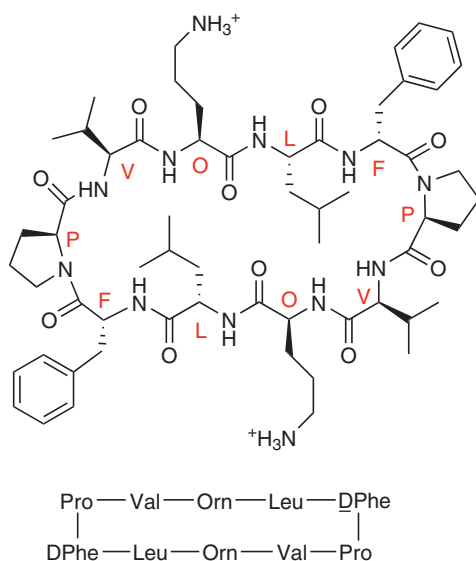


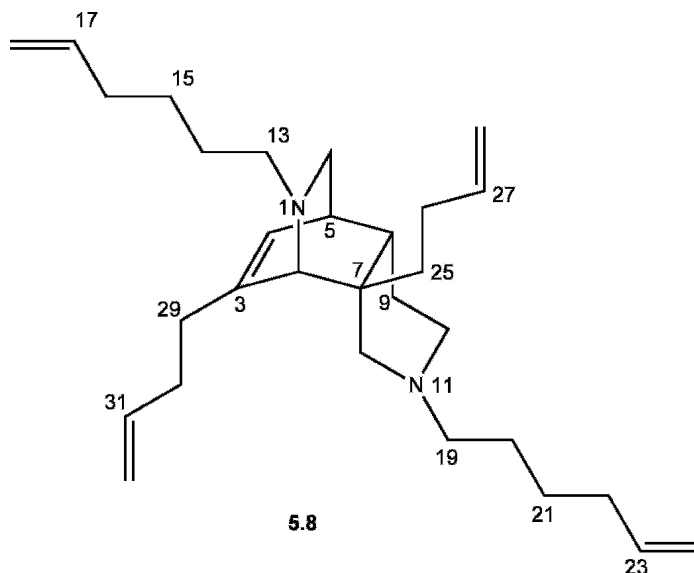
Figure 5.65. The aliphatic region of the TOCSY spectrum of the cyclic decapeptide gramicidin-S, **5.7**. An 80-ms MLEV-17 spin-lock was used, providing complete transfer along the aliphatic side chains of each residue. Correlations from the α -protons of proline and ornithine are labelled, with those out to the δ -protons corresponding to three-step transfers.



5.7

which all side chain protons of each residue can be traced from the associated α -proton (with the exception of the phenylalanine aromatic ring), as illustrated for the proline and ornithine residues. The relaying of magnetisation along a coupled chain of spins can often overcome problems due to close or coincident crosspeaks in COSY by effectively moving

related correlations into regions of the spectrum that would otherwise be devoid of cross-peaks, thereby utilising redundant 'space' in the 2D spectrum. This approach was used in the side chain assignment of **5.8**, an intermediate in the biomimetic synthesis of



members of the manzamine and keramaphidine alkaloids [56, 57], found naturally in marine sponges. Due to extensive crowding in some areas of the aliphatic region, side chain assignments were more readily derived from relayed correlations involving the resolved alkene protons such as H23 (Fig. 5.66). Similarly, the numerous correlations from H4 provided confirmation of assignments derived from a DQF-COSY spectrum.

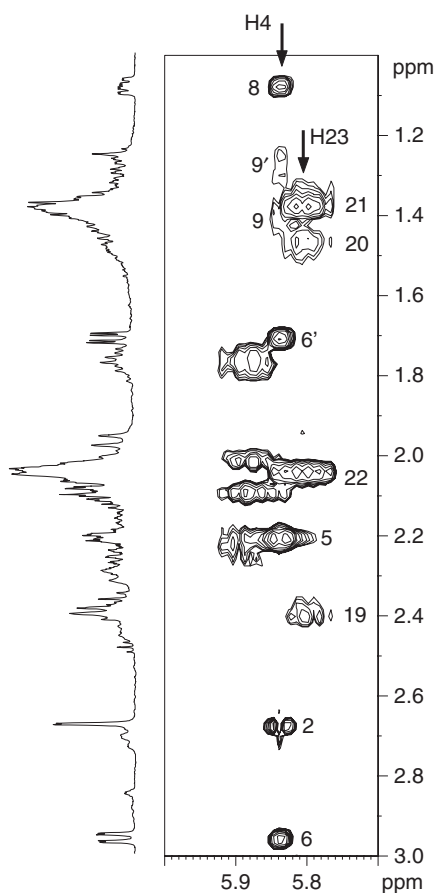


Figure 5.66. A section of the TOCSY spectrum ($\tau_m = 80$ ms) of the biomimetic intermediate **5.8** alongside the conventional 1D spectrum. Correlations from protons H4 and H23 are labelled. Despite extensive overlap in some regions of the 1D spectrum, the numerous relayed correlations in TOCSY occur in an otherwise clear region of the 2D spectrum and provide ready identification of proton shifts.

The somewhat indiscriminate propagation of magnetisation found with TOCSY can also be disadvantageous, however, since it is generally not possible to explicitly state the number of relay steps involved in the production of any given correlation. A time dependence of crosspeak build-up from a number of TOCSY spectra recorded with increasing τ_m (in, e.g., 20 ms steps; Fig. 5.67) can provide an approximate indication of whether the peak arises from 1, 2 or more steps, although it is often not possible to be more precise than this. When using TOCSY on molecules that are not composed of well-characterised monomer units, it is often beneficial to use this in addition to COSY spectra from which more immediate coupling neighbours can be identified. The use of very long spin-lock periods for promoting multistep transfer also leads to sensitivity losses due to relaxation (in the rotating frame) of the spin-locked magnetisation.

It has also been mentioned that TOCSY results in the net transfer of *in-phase* magnetisation, meaning the cancellation effects from *anti-phase* multiplet fine structure associated with COSY are not a feature of TOCSY. Such cancellation can be problematic for molecules that possess large natural linewidths, for example, (bio)-polymers, but it may also prevent the observation of COSY peaks in the spectra of small molecules that have complex multiplet structures, which may cancel under conditions of poor digital resolution. In these cases, the TOCSY experiment may be viewed as the more sensitive option because of the greater crosspeak intensities. The lack of anti-phase structure also means that spectra may be recorded very rapidly under conditions of rather low digital resolution without a loss of peak intensity. Finally, since both diagonal and crosspeaks possess the same phase, both produce double-absorption lineshapes, meaning high-resolution phase-sensitive spectra can be obtained.

One side effect of the spin-lock is that it also enables *incoherent* magnetisation transfer in the rotating frame. In other words, through-space effects known as rotating-frame nuclear Overhauser effects (ROEs) may, in principle, also be observed between spins. These tend to be far weaker than the TOCSY peaks particularly for small molecules and short mixing times, so they are rarely problematic. They may be more of a problem for very large molecules in which they are stronger and faster to build-up. For such molecules, so-called clean TOCSY sequences have been developed [58–60], which eliminate ROE peaks by cancelling them with equal but opposite NOE peaks (Chapter 8); such methods are applicable only to very large molecules where this difference in sign exists. A far greater problem is the appearance of unwelcome TOCSY peaks in ROESY spectra, a topic also addressed in Chapter 8.

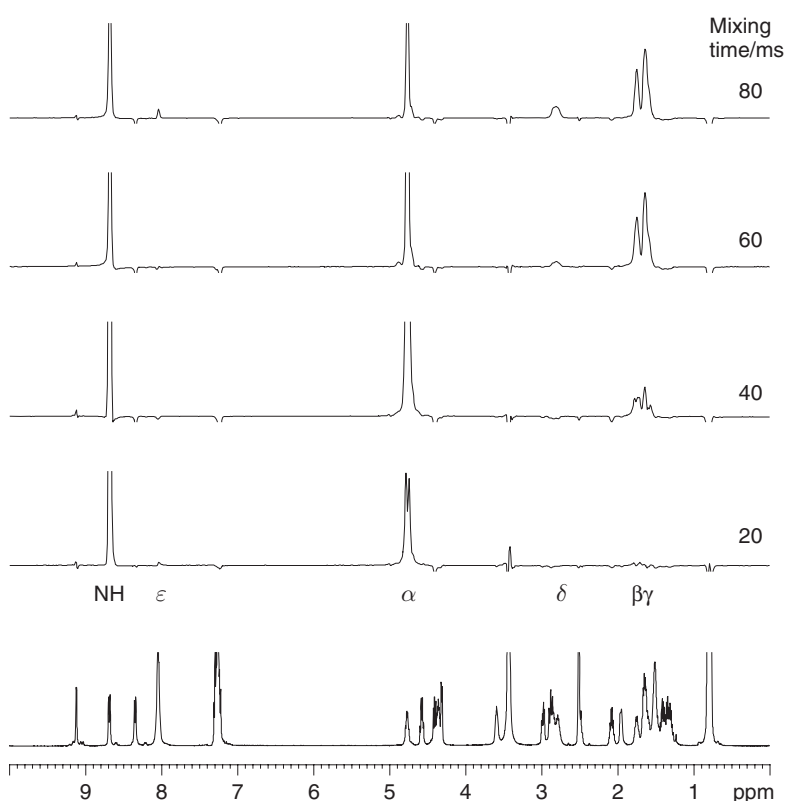


Figure 5.67. The time-dependent propagation of magnetisation along a proton spin system as illustrated for the ornithine residue of gramicidin-S. Traces are extracted from 2D MLEV-17 TOCSY spectra at the amide NH proton shift and show progressive transfer along the side chain as the mixing time increases.

5.7.3. Implementing TOCSY

Although in principle the simple scheme presented in Fig. 5.59 should provide TOCSY spectra, its suitability for practical use is limited by the effective bandwidth of the continuous-wave spin-lock. Spins that are off-resonance from the applied low-power pulse experience a reduced rf field causing the Hartmann–Hahn match to breakdown and transfer to cease. This is analogous to the poor performance of an off-resonance 180° pulse (Section 3.2.1). The solution to these problems is to replace the continuous-wave spin-lock with an extended sequence of ‘composite’ 180° pulses that extend the effective bandwidth without excessive power requirements. Composite pulses themselves are described in Chapter 10 alongside the common mixing schemes employed in TOCSY, so shall not be discussed here. Suffice it to say at this point that these composite pulses act as more efficient broadband 180° pulses within the general scheme of Fig. 5.60.

There are essentially two approaches based on composite-pulse methods in widespread use for the practical implementation of the TOCSY experiment (Fig. 5.68). The first of these [52] (Fig. 5.68a) is based on the so-called MLEV-17 spin-lock, in which an even number of cycles through the MLEV-17 sequence are used to produce the desired total mixing period. To ensure the collection of absorption-mode data, only magnetisation along a single axis should be retained, so it is necessary to eliminate magnetisation not parallel to this before or after the transfer sequence. In this implementation, this is achieved by the use of ‘trim pulses’ applied for 2–3 ms along the chosen axis. These are periods in which a single, low-level pulse is applied, during which magnetisation not parallel to this axis will dephase due to inhomogeneity of the rf field (just as transverse magnetisation rapidly dephases in an inhomogeneous static field). This process eliminates dispersive contributions to the spectrum.

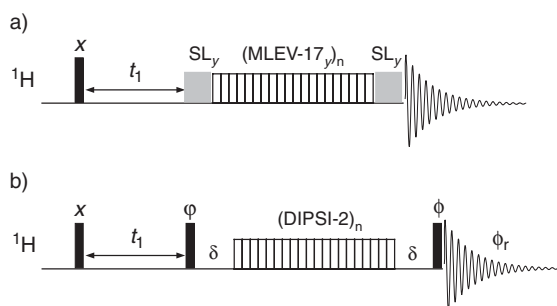
The alternative approach is to regenerate longitudinal magnetisation following the evolution period and to use a suitable *isotropic mixing* scheme to transfer this *z*-magnetisation between spins [61, 62] (isotropic here means that magnetisation transfer is equally effective along the *x*-, *y*- or *z*-axes, so the overall sequence has a similar effect to that described above). The *z*-magnetisation is then reconverted to pure-phase transverse magnetisation following the mixing, enabling the collection of absorption-mode data. The idea of generating and subsequently selecting *z*-magnetisation with the view to collecting pure-phase spectra has been termed ‘*z*-filtration’ [63]. This also has the advantage for older instruments that the proton ‘decoupler’ may be used for generating the low-power mixing scheme [61] if the main proton transmitter is incapable of suitable power level control; this restriction does not arise on modern instruments. A number of isotropic mixing schemes may be used in this approach, including MLEV-16, WALTZ-16 [64, 65] and DIPSI-2 [66,67], the last of these being particularly well suited for transfer in ^1H – ^1H TOCSY.

In either of these two general schemes, a greater effective bandwidth is achieved with the composite-pulse mixing, and significantly lower powers are required for this than for the 90° preparation pulse. For MLEV, the rf field strength need be about twice the desired bandwidth, whilst for the more efficient DIPSI-2 it may be about equal to it. Thus, a 10-ppm window at 400 MHz requires $\gamma B_1 \approx 8$ kHz ($90^\circ \approx 30 \mu\text{s}$) or 4 kHz ($90^\circ \approx 60 \mu\text{s}$) respectively, either of which can be achieved comfortably.

Gradient-selected TOCSY

For samples of sufficient strength to require only a single scan per increment, gradient versions of TOCSY may be attractive alternatives for the rapid recording of spectra. By analogy with the previous COSY discussions, the absolute-value TOCSY sequence simply requires equal gradients to be placed on either side of the spin-lock sequence (Fig. 5.69a) to

Figure 5.68. Two practical schemes for implementing TOCSY based on (a) the MLEV-17 mixing scheme and (b) the DIPSI-2 isotropic mixing scheme. The MLEV sequence is bracketed by short, continuous-wave, spin-lock trim pulses (SL) to provide pure-phase data. In scheme (b), this can be achieved by phase-cycling the 90° *z*-filter pulses that surround the mixing scheme. This demands the independent inversion of each bracketing 90° pulse with coincident receiver inversion, thus $\varphi = x, -x, x, -x$; $\phi = x, x, -x, -x$ and $\phi_r = x, -x, -x, x$. The δ periods allow for the necessary power switching.



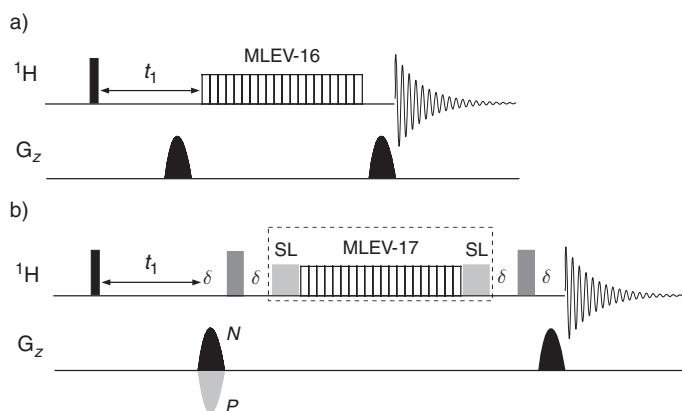


Figure 5.69. Gradient-selected TOCSY. Sequence (a) is suitable for absolute-value presentations with a 1:1 gradient combination selecting the N-type spectrum. Sequence (b) provides phase-sensitive data sets via the echo-antiecho method for which separate P- and N-type data are collected through inversion of the first gradient. The boxed section may contain any suitable mixing element including the 90°-DIPS12-90° scheme described above.

selectively refocus the N-type pathway to provide f_1 frequency discrimination. Trim pulses are not required since pure-phase spectra are not produced by this method. As for absolute-value COSY, this experiment may be well suited to automated acquisition schemes because of the simplicity of processing, although in general the phase-sensitive version is preferred for reasons of resolution. This may be performed by placing the gradients within spin-echoes to refocus shift evolution. Quadrature detection is then afforded by collecting P- and N-type data separately for each t_1 increment via gradient inversion and processing these according to the echo-antiecho procedure of Section 5.5.2 (Fig. 5.69b).

5.7.4. 1D TOCSY

As an alternative to the collection of a full 2D data set, the 1D analogue can prove advantageous in some circumstances. In general, most 2D sequences can be adapted to produce the 1D equivalent [68, 69], meaning the experiments can be quicker to acquire and can be recorded under conditions of higher digital resolution. These may prove particularly attractive when only specific information is required, as is often the case for small- to medium-sized molecules, or when small sample quantities demand many scans for adequate signal-to-noise and so preclude the collection of 2D data sets. All 1D analogues of multi-dimensional experiments begin by selectively exciting one resonance in the spectrum and using this as the source for all subsequent magnetisation transfer. The resulting spectra may therefore be viewed as being equivalent to a high-resolution row through the related 2D spectrum at the shift of the excited spin. Selective excitation methods are described in Chapter 10, so they are not addressed here. Numerous methods for generating 1D TOCSY spectra without [70–73] or with [74–77] gradient assistance have been presented, a generalised scheme using as an example the MLEV-17 sequence being shown in Fig. 5.70. Following the excitation of the chosen spins, magnetisation transfer is initiated immediately with the mixing scheme since now there is no need for the evolution period. The only resonances appearing in the resulting spectra are those that have ‘received’ magnetisation from the source spin.

The experiment produces 1D subspectra for discrete spin systems within the molecule, potentially revealing multiplet structures that were otherwise overlapped or buried. Furthermore, the higher digital resolution afforded by the 1D analogue may resolve ambiguities arising from crosspeak overlap in 2D spectra and may also provide a more rapid method for following magnetisation transfer along a chain of spins as a function of mixing time. Figure 5.71 shows 1D TOCSY spectra for the carbopeptide **5.9** in which transfer is from the

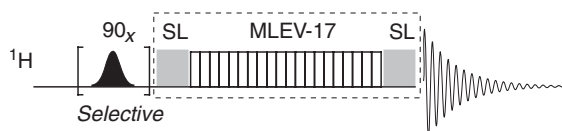
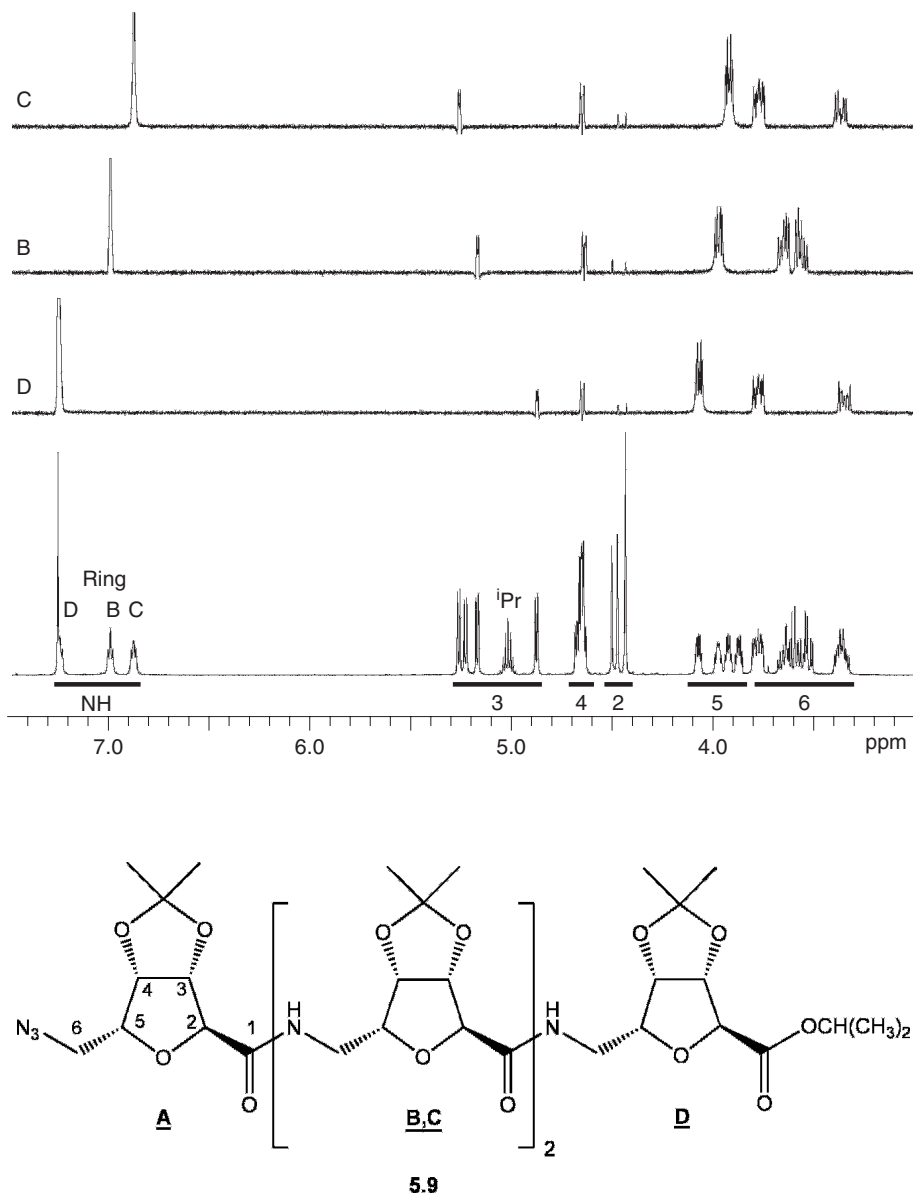


Figure 5.70. The general sequence for 1D selective TOCSY. Any suitable selective 90° pulse scheme (see Chapter 10) can be used to selectively excite the target resonance from which transfer is initiated. The boxed section can contain any suitable mixing element.

Figure 5.71. One-dimensional TOCSY spectra of the tetrameric carbopeptoid **5.9** in CDCl_3 . Each amide proton was selectively excited and used as the starting point for coherence transfer. Selective excitation was achieved with the excitation sculpting method of Chapter 10 and mixing used a 97-ms MLEV-17 spin-lock.



selected amide protons with the individual ring systems resolved in each case. This approach is often applicable to the study of oligosaccharides where the anomeric protons can provide a convenient starting point from which to establish intraresidue assignments.

Eliminating zero-quantum artefacts

The ability to reveal otherwise hidden multiplet structures with the 1D TOCSY experiment can prove to be extremely beneficial to spectrum analysis, but a faithful representation of multiplet structures can be hampered by undesirable artefacts arising from ZQCs (a form of transverse magnetisation; Section 5.4) generated prior to and/or during the isotropic mixing scheme employed. Although unobservable, these coherences can be transferred into observable signals by subsequent rf pulses and produce responses in spectra that have an anti-phase coupling structure (akin to those in DQF-COSY described in Section 5.6.2) and are orthogonal to the desired TOCSY peaks, meaning these also appear as dispersive contributions when the desired peaks are phased to have the usual absorption lineshape. The zero-quantum contributions lead to lineshape distortions of TOCSY peaks in both 2D and 1D spectra, and whilst undesirable in both, these can present a greater nuisance in the 1D spectra where the high-resolution analysis of multiplet structure is most useful (Fig. 5.72b). Thus, some effort has gone into designing methods to eliminate these contributions, and two methods are presented here in the context of the TOCSY experiment. The first does not require pulsed field gradients whilst the second, the most recent and successful approach, does. The elimination of these responses is a complex task, given that ZQCs are insensitive to both phase-cycling routines and pulsed field gradients. The available approaches are, however, quite general and can be applied to any

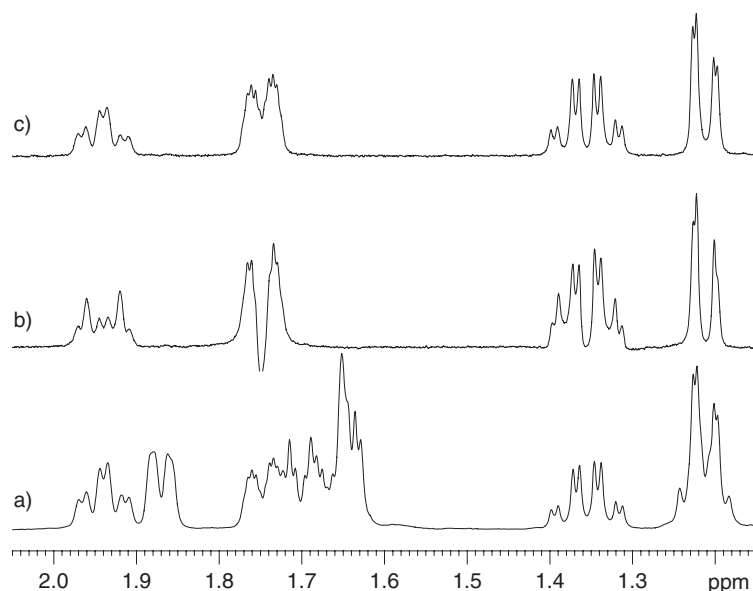


Figure 5.72. Zero-quantum interference in selective 1D TOCSY spectra. (a) Partial ^1H spectrum and the corresponding regions of 1D TOCSY recorded with 80-ms DIPSI-2 (b) without zero-quantum suppression and (c) with the zero-quantum dephasing scheme shown in Fig. 5.74 employing adiabatic smoothed CHIRP pulses with 40 kHz sweep widths and durations of 15 and 10 ms.

sequence in which these coherences are troublesome, so the techniques themselves are described more fully in Chapter 10.

The simplest approach to zero-quantum suppression employs a z -filter scheme in which the desired TOCSY magnetisation is placed along the z axis for a defined period τ_{zf} before being returned to the transverse plane for detection (Fig. 5.73). Repetition of the experiment with a random variation of the τ_{zf} period leads to differential evolution of the ZQCs such that the summation of many experiments leads to a destructive addition of the zero-quantum responses, as described in Section 10.6. The desired magnetisation, being longitudinal during the z -filter, is not influenced by this variation and so adds constructively. Typically, the τ_{zf} delay varies over a 2–20 ms range and requires the addition of approximately 10 experiments for acceptable suppression. The need for this repetition is clearly a major limitation of this technique, meaning it may be applicable to selective 1D TOCSY experiments but essentially impractical for 2D experiments. Even then, it may demand extended acquisition periods beyond what is required to satisfy signal-to-noise requirements.

A more sophisticated approach achieves suppression *in a single scan* and thus has wider applicability, being equally useful for 1D and 2D experiments [78]. This incorporates a method for zero-quantum dephasing based on the simultaneous application of a pulsed field gradient and a swept-frequency 180° inversion pulse during a z -filter delay, as described in Section 10.6. Implementation with the DIPSI-2 isotropic mixing scheme is illustrated in Fig. 5.74, again for the 1D version. The dephasing element is applied on either side of the

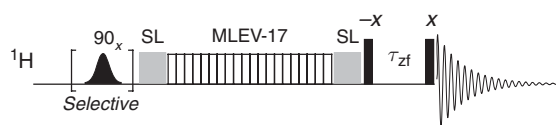


Figure 5.73. The z -filter scheme for suppressing zero-quantum contributions in 1D TOCSY. The delay τ_{zf} is randomly varied between experiments and the resulting spectra co-added. When using DIPSI-2, the two δ periods on either side of the isotropic mixing scheme can act directly as independent τ_{zf} periods and may be randomised in a similar manner.

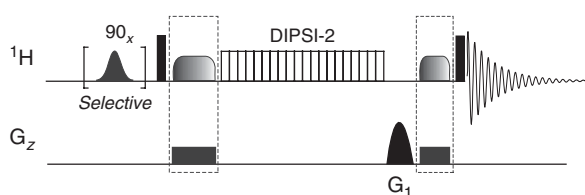


Figure 5.74. The zero-quantum dephasing scheme applied to TOCSY. The boxed regions contain the dephasing elements in which the gradients are applied during the swept inversion pulse; the two elements are of different durations to avoid accidental refocusing. G_1 represents a purge gradient.

isotropic mixing scheme with different total durations such that the dephasing generated by the first is not accidentally refocused by the second. The effectiveness of the scheme is illustrated in Fig. 5.72 where appreciable lineshape distortions are seen without the use of the dephasing element whereas clean multiplet structures matching those observed for the 1D spectrum are revealed with this included.

5.8. CORRELATING DILUTE SPINS WITH INADEQUATE

One of the major goals in determining the structure of an organic molecule is the unambiguous identification of the carbon skeleton of the compound. Carbon–carbon connectivities are, however, rarely determined directly, rather we imply their presence from the correlations observed between protons, such as in COSY-type experiments, or between proton and carbon nuclei, such as from the long-range heteronuclear correlation experiments described in the following chapter. The need for one to take this indirect approach lies in the relatively poor sensitivity and low natural abundance (1.1%) of the carbon-13 isotope; hence the reference to the spin as being dilute. The abundance of molecules containing two (adjacent) ^{13}C spins is only 0.01%, so in attempting to directly identify ^{13}C – ^{13}C linkages, we are forced to observe only one in every $\sim 10,000$ molecules in our sample (in the absence of isotopic enrichment). In other words, one must detect the ^{13}C satellites in the ^{13}C spectrum itself, and clearly larger sample quantities and extended acquisition periods are required for this. Despite these limitations, and because the ability to directly trace the carbon skeleton of a molecule is the ultimate approach to organic structure elucidation, considerable effort has gone into developing methods to identify C–C connectivities directly, all generally based on the humorously entitled INADEQUATE sequence (Incredible Natural Abundance Double Quantum Transfer Experiment) [79, 80]. These techniques are also suitable for correlating nuclides other than carbon that exist with relatively low natural abundance levels of around 1–20%, including ^{29}Si , ^{119}Sn and ^{183}W (4.7, 8.6 and 14.4%, respectively); see reference [80] for examples. More recent developments have incorporated proton detection to enhance sensitivity, principally within the realm of organic structure elucidation, and these methods will also be described below.

Despite the power of these techniques for structural elucidation, they are certainly not the first to turn to when considering a structural problem because of the large sample quantities typically required, but in favourable cases, they may prove effective, and with molecules that possess very few or even no protons, one may have few other options. In addition, the more widespread availability of cryogenic probes is likely to extend the range of applications for these techniques for both the ^{13}C and ^1H detected approaches.

5.8.1. 2D INADEQUATE

The principal complication when attempting to correlate low abundance spins (commonly referred to as *dilute* or *rare* spins) is the interference caused by the dominant parent resonances that lack homonuclear couplings and therefore cannot provide connectivity data. The more popular correlation techniques such as COSY, whilst suitable in principle, are not well suited in these cases, and the INADEQUATE sequence is more appropriate. The basis of INADEQUATE is double-quantum filtration to suppress the uninformative parent resonances [81], this filtration being analogous to that which we have already encountered in the DQF-COSY experiment (Section 5.6.1). Natural-abundance carbon-13 spins are very well suited to such filtration because they are restricted to AX or AB two-spin systems only (because a molecule containing more than two ^{13}C centres will exist at such low abundance as to be non-existent) and because $^1J_{\text{CC}}$ couplings cover a limited range, meaning the sequence can be reasonably well optimised for the generation of double-quantum coherence. Furthermore, one-bond C–C coupling constants are typically an order of magnitude greater than those operating over two or three bonds, meaning the experiment can also be optimised to detect only the short-range couplings indicative of direct carbon–carbon connectivity.

The two-dimensional correlation spectrum [82] is generated by allowing the double-quantum coherences associated with coupled spins to evolve during a variable t_1 period, following which they must be reconverted to single-quantum carbon magnetisation for observation. The complete INADEQUATE sequence is given in Fig. 5.75.

The generation of double-quantum coherence requires an anti-phase disposition of coupling vectors, which here develop during a period $\Delta = 1/2J_{\text{CC}}$. This is provided in the form of a homonuclear spin-echo to make the excitation independent of chemical shifts. The t_1 period represents a genuine double-quantum evolution period in which these

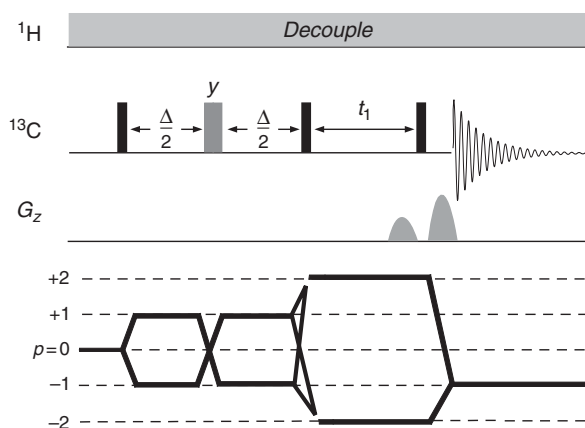


Figure 5.75. The INADEQUATE sequence and the corresponding coherence transfer pathway. The experiment selects double-quantum coherence during the evolution period (analogous to the selection in the DQF-COSY experiment, Section 5.6.1) with a suitable phase cycle or optionally via gradient selection (shown greyed). In doing so, it rejects all contributions from all uncoupled spins.

coherences evolve at the *sums* of the rotating-frame frequencies of the two coupled spins, that is, at the sums of their *offsets* from the transmitter frequency. Thus, for two coupled spins A and X that have offsets of ν_A and ν_X , the f_1 crosspeak frequency will be $(\nu_A + \nu_X)$. Following the reconversion of double-quantum into SQC, crosspeaks in f_2 will be observed with the conventional chemical shift frequencies of A and X. Throughout the sequence, broadband proton decoupling can be applied to prevent evolution of heteronuclear couplings and to generate the ^1H to ^{13}C nuclear Overhauser enhancement to aid sensitivity. Pulsed field gradients may also be incorporated for more complete suppression of the parent ^{12}C resonances for which a 1:2 ratio provides double-quantum filtration.

The resulting spectrum, here illustrated for *n*-butanol **5.10** (Fig. 5.76), differs considerably in appearance from the other correlation spectra encountered in this chapter, although again the f_2 dimension represents the chemical shifts of each participating spin. Since two coupled spins share the same DQ frequency in f_1 , correlations are made by following horizontal traces parallel to f_2 . Carbon connectivity is therefore established by a sequence of vertical and horizontal steps. If the DQ excitation sequence is optimised for one-bond C–C couplings, then each step identifies adjacent carbon centres in the molecule. The spectra display no diagonal peaks, although the midpoints of correlated spin pairs appear along a ‘pseudo’ diagonal (the DQ diagonal) of slope 2, that is, where $f_1 = 2f_2$. This characteristic symmetry can be useful in differentiating genuine responses from artefacts. Each crosspeak is composed of a doublet with a splitting of J_{CC} arising from the AX (or AB) fine structure.

An example of the power of this method in structural elucidation is shown for the sesquiterpene lactone acetylismontanolid (Fig. 5.77). The complete carbon skeleton of the molecule can be traced directly in the spectrum, with breakdown occurring only when heteroatom linkages arise. Unfortunately, it is rare indeed to have sufficient sample quantities at hand to consider employing this technique, and still it remains of limited application in its original form. Nevertheless, in very proton-sparse systems, it may present the only option as in the identification of the nitration product of **5.11a**. The product was to

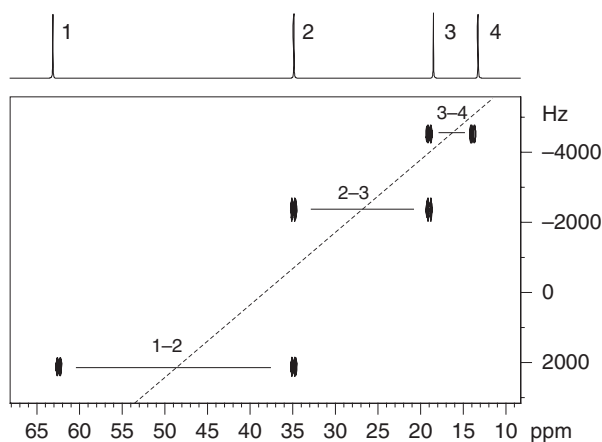
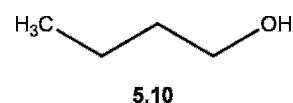
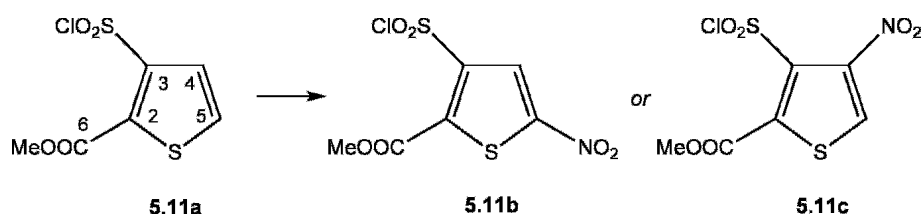
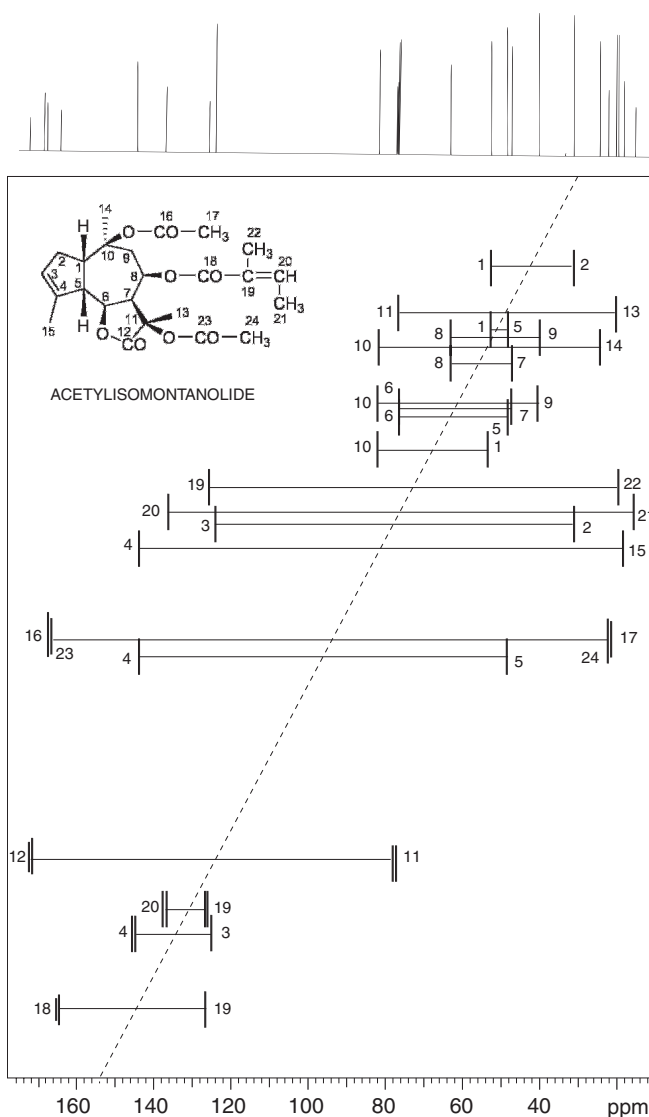


Figure 5.76. The 2D INADEQUATE (magnitude mode) and conventional carbon spectrum of *n*-butanol **5.10**. The dotted line indicates the double-quantum ‘pseudo’ diagonal.

Figure 5.77. The 2D ^{13}C INADEQUATE and conventional carbon spectrum of the sesquiterpene lactone acetylismontanolid in CDCl_3 (reproduced with permission from [83], Copyright 1995, Elsevier).



undergo reduction of NO_2 to NH_2 followed by further derivatisation of NH_2 and SO_2 as a template in the parallel synthesis of potential antibiotics. As such, knowledge of the core template structure was critical prior to further elaboration, but carbon shifts alone were not considered definitive enough to differentiate 4- from 5-nitro products, and methods based on long-range proton–carbon correlations (HMBC; see Chapter 6) also would not differentiate these. The ability to trace carbon connectivities directly in 2D INADEQUATE (Fig. 5.78) provided evidence for the CH at the 4 position and hence unambiguous proof of the nitration site at the 5 position as in **5.11b**.

5.8.2. 1D INADEQUATE

The one-dimensional INADEQUATE experiment was in fact the original version and was proposed as a means of measuring carbon–carbon coupling constants [81]. The use of double-quantum filtration removes the parent resonances that dominate the spectrum and mask the desired splittings, especially when smaller two- and three-bond

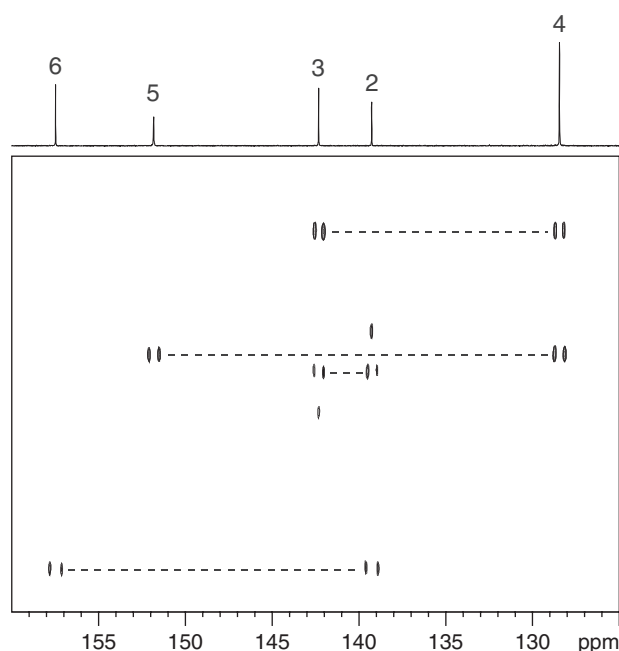


Figure 5.78. The 2D ^{13}C INADEQUATE spectrum of the thiophene **5.11b** in CDCl_3 . Data were collected on ~ 150 mg of sample using a ^{13}C -optimised cryogenic probe with the experiment optimised for $^1J_{\text{CC}} = 70$ Hz.

couplings are to be measured. This can also suppress resonances from low-level impurities that may otherwise interfere. Replacing the t_1 evolution period by a short, fixed delay (sufficient only to allow spectrometer phase changes or the application of gradient pulses) results in a 1D DQF experiment in which carbon–carbon couplings appear as anti-phase doublets (Fig. 5.79). From these patterns, the values of J_{CC} can be determined directly, if sufficiently resolved and have value in studies of stereochemistry and conformation [84–86]. An alternative application that has been used extensively is in the tracking of doubly ^{13}C -labelled precursors in the study of metabolic or biosynthetic pathways [87]. The DQ filtration is used to suppress background signals of those species that do not contain the original ^{13}C – ^{13}C fragment (to the limit of natural abundance), whilst retaining only those that have survived the pathway intact and is nowadays achieved most effectively with pulsed field gradients.

5.8.3. Implementing INADEQUATE

The basic components of the INADEQUATE phase cycle comprise double-quantum filtration and f_1 quadrature detection. The filtration may be achieved as for the DQF-COSY experiment described previously, that is, all pulses involved in the DQ excitation (those prior to t_1 in this case) are stepped x , y , $-x$, $-y$ with receiver inversion on each step (an equivalent scheme found in spectrometer pulse sequences is to step the *final* 90° pulse x , y , $-x$, $-y$ as the receiver steps in the opposite sense x , $-y$, $-x$, y ; other possibilities also exist). This simple scheme may not be sufficient to fully suppress singlet contributions, which appear along $f_1 = 0$ as axial peaks and are distinct from genuine C–C correlations. Extension with the EXORCYCLE sequence (Section 7.2.2) on the 180° pulse together with CYCLOPS (Section 3.2.5) may improve this. Cleaner suppression could also be achieved

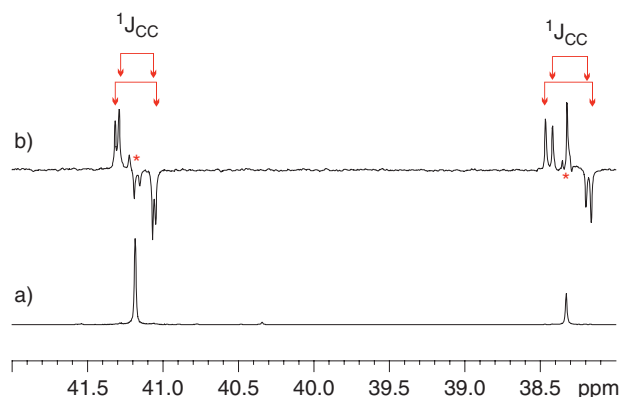


Figure 5.79. The 1D INADEQUATE experiment (b) selects for the carbon-13 satellites in the conventional carbon-13 spectrum (a) and provides a means of measuring carbon–carbon coupling constants (here marked as anti-phase doublets). *Some residual parent resonances from lone ^{13}C centres remain in (b).

by the use of pulsed field gradients in a 1:2 ratio, which for sensitivity reasons benefits from gradient probe optimised for ^{13}C observation and even more so from a cryogenically cooled probe head.

Quadrature detection requires the observed signals to exhibit 90° phase increments on each t_1 step. Since the desired signals pass through double-quantum coherence, they are *twice* as sensitive to rf phase shifts, meaning the excitation pulses prior to t_1 must themselves be incremented in 45° steps only. Such phase increments are readily achieved on modern spectrometers, so the usual States or TPPI approaches to phase-sensitive data collection can be applied. For a pure-phase display, the implementation of field gradient pulses as in Fig. 5.75 requires that each of these are applied within ^{13}C spin-echoes to provide chemical shift refocusing. During the development of the INADEQUATE sequences, the available hardware was incapable of producing small phase shifts, and a number of alternative schemes that avoided 45° rf phase shifts altogether were developed. One popular approach used to generate absolute-value spectra was to use pulse width dependence to enhance the intensity of the N-type signals relative to the P-type [88]. Increasing the final pulse angle to 135° produces a 6:1 intensity ratio of N:P, so the P-type signal essentially disappears and frequency discrimination results, albeit with mixed-mode lineshapes that demand magnitude calculation.

The success of INADEQUATE may be compromised by deleterious off-resonance effects that are particularly troublesome for nuclei such as carbon-13, which have a relatively large chemical shift range. Variations employing composite pulses to counteract offset effects have therefore been developed to minimise losses [89, 90]. An alternative approach is to replace the ^{13}C 180° pulses with composite adiabatic refocusing pulses (see Chapter 10).

Experimental set-up

There is no doubt that the deciding factor when considering the use of ^{13}C INADEQUATE is sensitivity, and far greater sample quantities are required for this experiment than for other 2D sequences. As a rule of thumb, one should be able to obtain a conventional 1D ^{13}C spectrum *in a single scan* following a 90° excitation pulse, and this should display a signal-to-noise ratio of *at least* 20:1. For realistic sample quantities encountered in the chemical laboratory, this is rarely achievable, and even in favourable cases, at least overnight acquisitions are typically required. The post-processing algorithm CCBond [91, 92] (available as a feature in some software) has proved extremely successful at automatically analysing INADEQUATE spectra even when the signal-to-noise was too poor for the human eye to recognise connectivity patterns. Using such an approach, spectra with an order of magnitude poorer signal-to-noise than is conventionally required may be subject to reliable analysis [93, 94], clearly enhancing the utility of this method (for other experimental approaches to improving sensitivity see the following section).

Maximum sensitivity requires optimum generation of DQC. For weakly coupled systems, Δ should be equal to $1/2J_{\text{CC}}$, whilst the presence of strong coupling ($\Delta/J_{\text{CC}} < 3$) requires optimum periods of $3/2J_{\text{CC}}$ [95]. As ever, the choice of Δ represents some compromise over anticipated J_{CC} values. One-bond couplings range typically from 35 to 70 Hz [79, 85, 96, 97] (Table 5.7) but are more often less than 50 Hz in the absence of C–C unsaturation, so Δ of around 10–14 ms is a reasonable choice. Couplings over multiple bonds are considerably smaller, often ≤ 5 Hz for $^{23}J_{\text{CC}}$ (Table 5.8), and correspondingly longer delays are required if these correlations are being sought. Since the optimum repetition rate is 1.3 times the ^{13}C T_1 s, the use of relaxation agents to speed data acquisition should also be considered, particularly when quaternary centres may be present. For organic solutions, chromium acetylacetonate is widely used for this, and even dissolved oxygen has been shown to be advantageous [98]. Gadolinium triethylenetetraaminehexaacetate (Gd(TTHA)) [99] has been recommended for aqueous solutions, and experience suggests

Table 5.7. Typical ranges for one-bond C–C coupling constants

C–C group	$^1J_{\text{CC}}$ (Hz)
C–C	35–40
C–C–OH(R)	40–60
C=C (alkene)	70–80
C=C (aromatic)	55–70
C $\equiv$ C	170–220

The principal factor in determining the magnitudes of these is carbon hybridisation.

Table 5.8. Typical values for long-range C–C coupling constants

J coupling	C–C group	$^nJ_{CC}$ (Hz)
$^2J_{CC}/^3J_{CC}$	C...C (aliphatic)	≤ 5
$^3J_{CC}$	C=C=C–C (trans)	~ 7
$^3J_{CC}$	C=C=C–C (cis)	~ 3
$^2J_{CC}$	C(CO)–C	~ 15
$^2J_{CC}$	C=C–C	3–8
$^4J_{CC}$	Any	< 1

that concentrations up to 10 mM produce acceptable results without significantly affecting the carbon resonances, although this can substantially broaden the *proton* spectrum of the sample.

Due to the large spectral widths involved, spectra are collected with low resolution in both dimensions: around 10–20 Hz/pt in f_2 and 50–100 Hz/pt in f_1 . Processing then requires window functions that minimise distortions arising from the truncated FIDs, such as the shifted sine-bell or squared-sine-bell. To minimise the f_1 spectral width, the transmitter frequency should be placed in the centre of the 1D ^{13}C spectrum so that DQ frequencies fall on either side of this, that is, both positive and negative DQ frequencies appear in f_1 . It is also possible to further reduce the f_1 spectral width by deliberately folding peaks in the f_1 dimension by making this equal to that in f_2 . This causes no ambiguity in interpretation because both participating crosspeaks fold in together and connectivities can still be identified in horizontal traces.

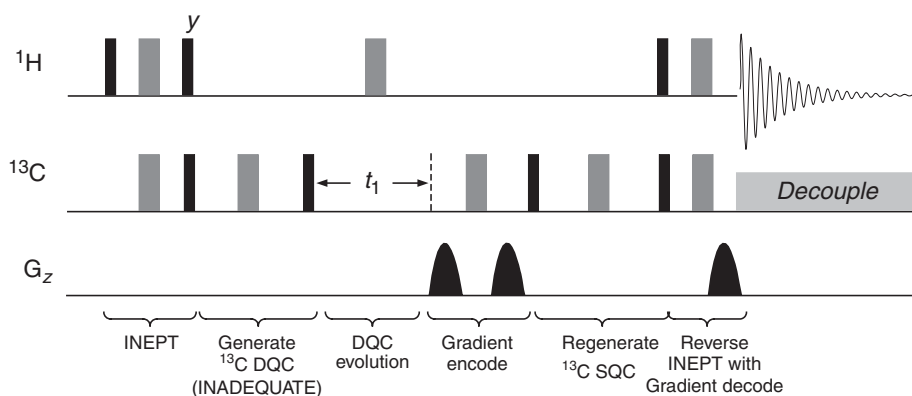
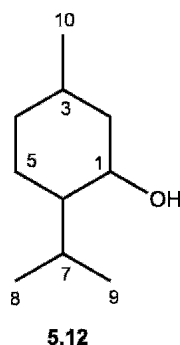
5.9. CORRELATING DILUTE SPINS WITH ADEQUATE

A number of variations on the basic INADEQUATE sequence have been presented over the years, particularly with reference to ^{13}C experiments, all primarily aimed at improving the appallingly low sensitivity of the original technique. Proton–carbon polarisation transfer prior to the INADEQUATE sequence via INEPT [100] or DEPT [101] may be used as a means of enhancing ^{13}C population differences and of allowing more rapid repetition rates since these would be dictated by faster proton spin relaxation. However, these preclude the use of broadband proton decoupling and hence gain no enhancement from the NOE, so only marginal improvements may be obtained. Conversely, magnetisation may be transferred onto protons directly attached to ^{13}C sites by a reverse-INEPT step *after* the INADEQUATE sequence. This variant, referred to as INSIPID [102], benefits from the higher sensitivity of proton detection. Alas, it also suffers severely from the need to suppress the signals of protons attached to lone ^{13}C centres as well as those attached to ^{12}C , which possess intensities that are, respectively, 100 and $\sim 10,000$ times greater than those of the desired ^1H – ^{13}C – ^{13}C moieties! More recent experiments, referred to originally as proton-detected INEPT-INADEQUATE [34] or more boldly in one development as ADEQUATE [103], tackle the sensitivity problem by utilising both polarisation transfer and proton observation and overcome the proton suppression problems by exploiting pulsed field gradients for signal selection. In principle, this leads to sensitivity gains by a factor of 32 $((\gamma_{\text{H}}/\gamma_{\text{C}})^{5/2})$, although a factor of around 8–16 seems more realistic when allowing for experimental losses [34]. The resulting 2D spectrum presents a stepwise correlation of protons that sit on neighbouring ^{13}C centres and so maps direct carbon connectivities within the molecular framework. This powerful technique should prove valuable in the structural elucidation of unknown materials, although the obvious limitation with this approach is the need for proton-bearing carbons in the molecule, which may limit its utility for proton-sparse systems. Furthermore, these techniques are likely to be employed only after more sensitive methods, such as those based on proton–proton and proton–carbon correlations, have failed to solve the problem at hand.

5.9.1. 2D ADEQUATE

The operation of the proton-enhanced sequences is best understood by reference to the original INEPT-INADEQUATE sequence shown in simplified form in Fig. 5.80 in which the individual component parts have been identified and may be followed in a stepwise manner. Thus, initial transfer from proton to carbon is via the INEPT sequence introduced in Chapter 4 followed by the generation of ^{13}C DQC as for the INADEQUATE above. This again evolves to encode t_1 after which gradient selection begins. The ^{13}C DQ then returns to single quantum, and the ^{13}C – ^{13}C -coupling is refocused by the spin-echo. The final step is INEPT in reverse to transfer from carbon back onto proton, followed by

Figure 5.80. The gradient-selected INEPT-INADEQUATE sequence for identifying carbon–carbon connectivities through proton observation (time delays in the sequence have been removed for clarity). The sequence selects for ^1H – ^{13}C – ^{13}C fragments. Despite the complexity of the sequence, its operation may be understood by recognising discrete, simpler segments within it that have defined roles to play, which in this case are INEPT and INADEQUATE steps.



gradient refocusing of the desired responses and then observation in the presence of ^{13}C decoupling. The sequence can thus be viewed as a variation of the HSQC technique of Chapter 6 in which DQ ^{13}C coherence evolves during t_1 instead of the single-quantum ^{13}C coherence utilised in HSQC. The resulting spectrum has carbon DQ frequencies along f_1 with proton shifts along f_2 in which correlated protons are indicative of H–C–C–H fragments. As an illustration, the spectrum in Fig. 5.81 was recorded on a 50 mg sample of unlabelled menthol **5.12** in 16 h at 400 MHz, although ‘adequate’ results would have been achieved in less than half this time.

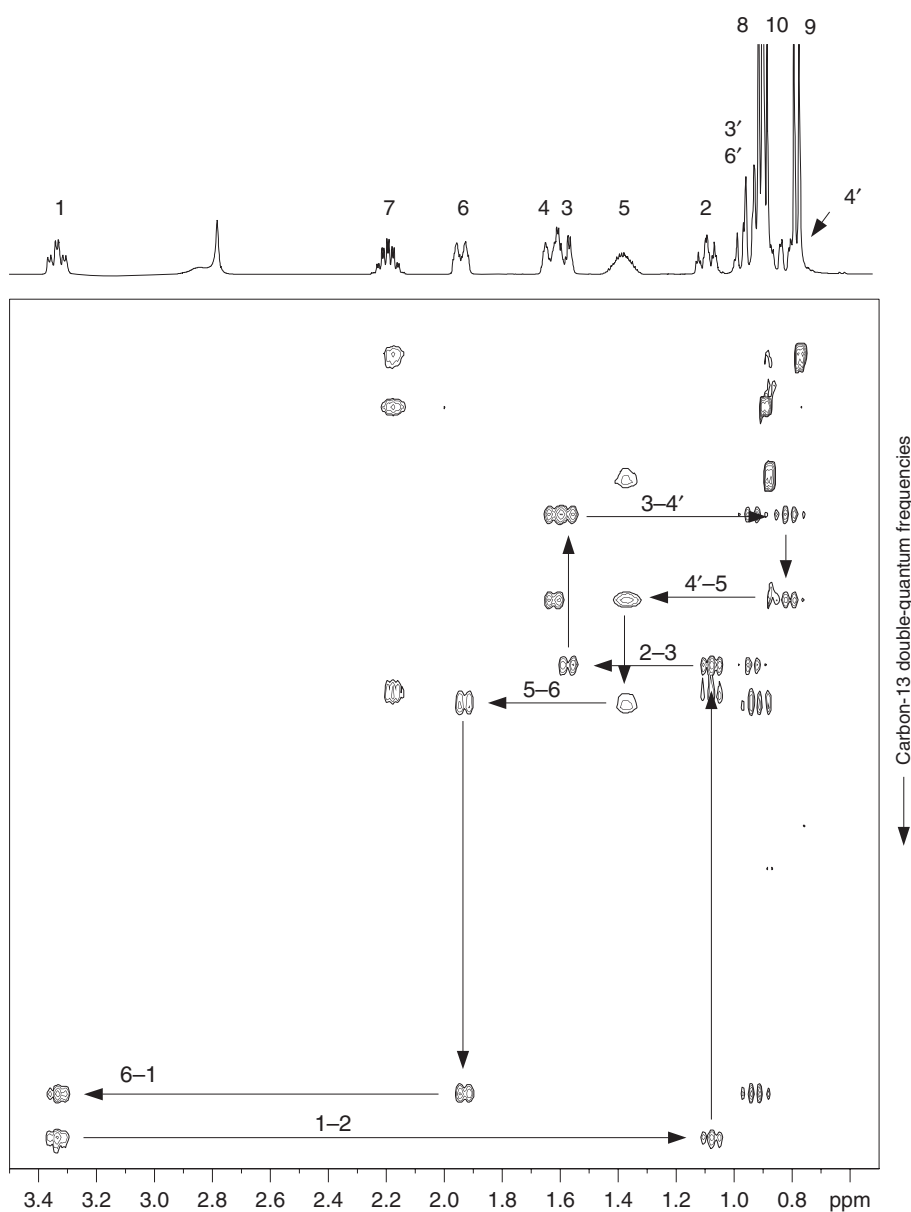


Figure 5.81. The proton-detected 1,1-ADEQUATE spectrum of menthol **5.12** (50 mg in CDCl_3) recorded at 400 MHz. The stepwise tracing of the carbon skeleton of the cyclohexane ring is illustrated in which carbon connectivities are identified in horizontal traces (alternative ‘routes’ involving the diastereotopic 3, 4 and 6 partners also exist but, for clarity, are not shown).

Table 5.9. The scalar couplings exploited in the ADEQUATE family of sequences

ADEQUATE version	Couplings exploited	Bonds correlated
1,1-	$^1J_{\text{CH}}, ^1J_{\text{CC}}$	2
1, <i>n</i> -	$^1J_{\text{CH}}, ^nJ_{\text{CC}}$	4
<i>n</i> ,1-	$^nJ_{\text{CH}}, ^1J_{\text{CC}}$	4
<i>m</i> , <i>n</i> -	$^mJ_{\text{CH}}, ^nJ_{\text{CC}}$	6

The bond correlations indicate the *maximum* number of bonds over which correlations may be identified.

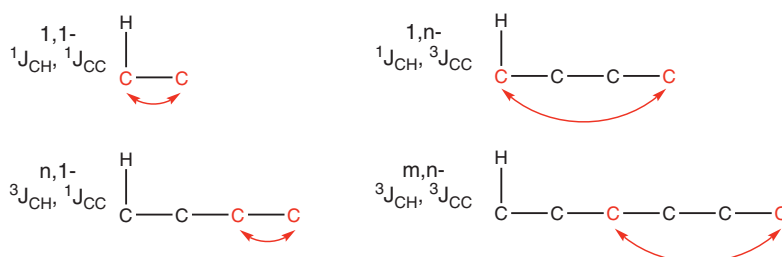


Figure 5.82. Schematic illustration of the correlations explored with the ADEQUATE sequences. The maximum number of bonds are indicated for typical J_{CH} and J_{CC} values but shorter-range (two-bond) correlations and those crossing heteroatoms will also be detected. The linked carbon centres coloured red are those that generate the double-quantum coherence (DQC) and are thus ultimately correlated.

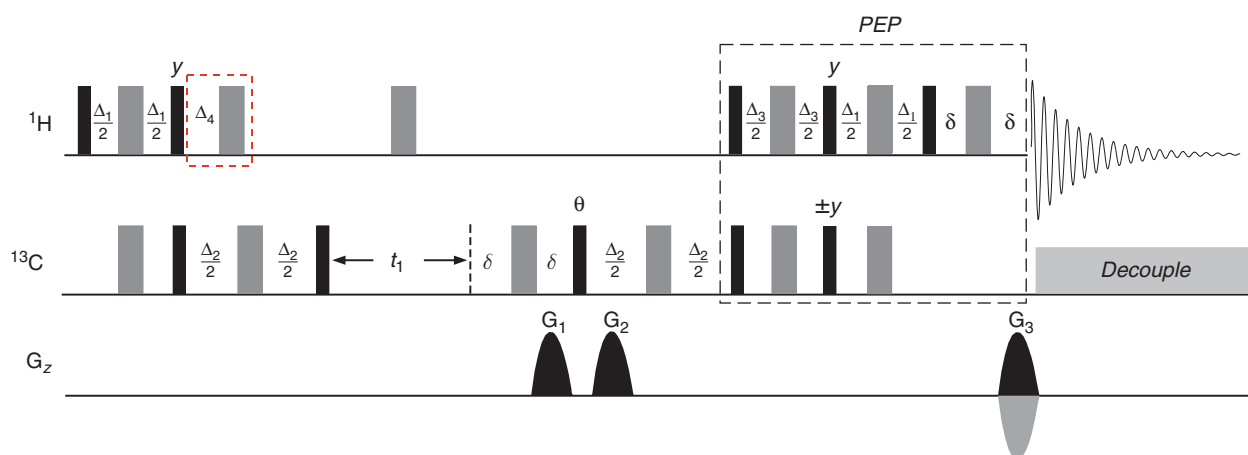
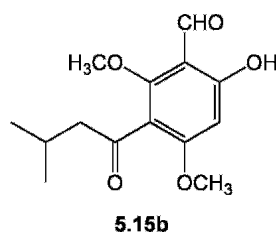
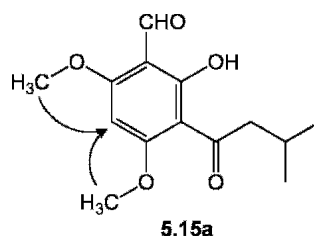
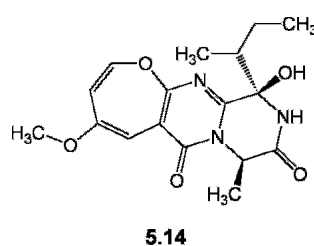
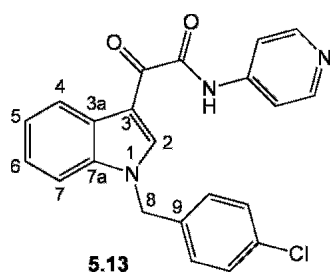


Figure 5.83. The 1,1-ADEQUATE sequence. Delays are $\Delta_1 = 1/(2^1J_{\text{CH}})$, $\Delta_2 = 1/(2^1J_{\text{CC}})$, $\Delta_3 = 1/(6^1J_{\text{CH}})$, δ = gradient encoding and ^{13}C pulse angle $\theta = 60^\circ$. In the 1,*n*- experiment, the Δ_2 J_{CC} evolution period is optimised for long-range carbon–carbon couplings. Gradient pulses together with alternation of the final carbon 90° pulse select echo/anti-echo signals for f_1 quadrature detection. For the multiplicity-edited version, the additional red boxed element is employed with $\Delta_4 = 1/(2^1J_{\text{CH}})$. For details of the PEP element see Section 6.3.3.

Since the original sequence, a more refined family of experiments (known collectively as the ADEQUATE techniques) have been presented [103] offering the ability to correlate over single and multiple bonds by employing both one-bond and long-range J_{CH} and J_{CC} couplings, as summarised in Table 5.9. The types of molecular fragments interrogated by these sequences are summarised in Fig. 5.82, correlating, in principle, nuclei up to six bonds apart and potentially providing a wealth of structural data. The sequence for the 1,1- and 1,*n*-ADEQUATE experiments is illustrated in Fig. 5.83 and features a number of enhancements including the use of a 60° ^{13}C pulse for optimum transfer of double-quantum to single-quantum ^{13}C magnetisation and the inclusion of the preservation of equivalent pathways (PEP) cluster for optimum signal transfer from carbon back to proton (see Section 6.3.3). The 1,*n*- version correlates remotely coupled carbons by optimising the Δ_2 coupling evolution delays for the considerably smaller long-range J_{CC} coupling constants (Table 5.8) rather than $^1J_{\text{CC}}$ values. The *n*,1- and *m*,*n*-ADEQUATE techniques employ long-range J_{CH} couplings for initial transfer from proton to carbon and may thus be viewed as the HMBC analogues of the above; see reference [103] for sequence details. The use of long-range couplings in this manner will further compromise sensitivity, so their application is likely to be further restricted despite their potentially rich information content.

5.9.2. Enhancements to ADEQUATE

In recent years, there have been further developments that have sought to simplify the appearance, improve performance or enhance the content of the ADEQUATE techniques.



As described in the original publication [103], one modification of the 1,1- and 1,*n*-sequence includes an additional element that is executed when ^{13}C SQC has been regenerated to refocus the chemical shift of the carbon to which the observed proton is attached. This results in the f_1 dimension displaying the conventional ^{13}C chemical shift axis (rather than double-quantum frequencies) such that the spectrum appearance matches that of ^1H -detected heteronuclear correlation experiments such as HSQC or HMBC. The observed correlations then relate the observed proton to the *remote* carbon centres, which, in the case of the 1,1- experiment, equates to those carbon atoms that are two bonds away. This correlation is equivalent to that which would be observed for $^2J_{\text{CH}}$ correlations in the HMBC long-range correlation experiments (Chapter 6) even though the couplings exploited differ in these two techniques (i.e. $^1J_{\text{CH}} + ^1J_{\text{CC}}$ versus $^2J_{\text{CH}}$). Indeed the refocused 1,1-ADEQUATE has been suggested as one means of differentiating $^2J_{\text{CH}}$ from $^{3/4}J_{\text{CH}}$ correlations observed in HMBC experiments [104]; other potentially more sensitive methods also exist, see Section 6.4.3. For example, the 1,1- experiment was used to support HMBC data in the identification of C–C connectivity and assignment of $^2J_{\text{CH}}$ correlations in the proof of structure of the synthetic indol-3-yl-2-oxo acetamide anti-tumour agent known as D-24851 **5.13** [105]. The appearance of the H8–C9 and the lack of H8–C3 correlation provided proof of substitution position. Similarly, the 1,1- and 1,*n*-experiments were used in support of ^1H – ^{13}C and ^1H – ^{15}N HMBC data in the identification of oxepinamide A **5.14** isolated from marine fungi [106]. The 1,*n*-experiment has also been applied to the assignment of proton-sparse, highly substituted aromatic compounds for which HMBC affords ambiguous results [107]. Thus, the ‘pseudo- $^4J_{\text{CH}}$ ’ correlations observed in this experiment were able to distinguish between the two possible synthetic regioisomers **5.15a** and **b** and identify the product as the former by virtue of the four-bond correlations from the two O-methyl groups to the aromatic methine carbon.

The performance of ADEQUATE experiments can be significantly compromised by deleterious off-resonance effects associated with the many ^{13}C 180° pulses employed, and the use of frequency-swept adiabatic pulses (Section 10.2) has been shown to greatly improve spectral quality [108]. Both the first and the last ^{13}C 180° pulses of Fig. 5.83 may be replaced with smoothed CHIRP pulses, where carbon inversion is required, and the remaining four may be substituted for composite-smoothed CHIRP pulses to effect the desired carbon chemical shift refocusing. For situations in which the molecules of interest are likely to contain a wide range of $^1J_{\text{CC}}$ values, notably when both aliphatic and olefinic or aromatic moieties exist, an ACCORDION-optimised version of ADEQUATE that seeks to detect correlations over a wider range of J_{CC} coupling constants has been presented [109], in an analogous manner to the approach employed in HMBC experiments for the detection of $^nJ_{\text{CH}}$ couplings (Section 6.4.3). In a separate development, a simple modification of the experiment, illustrated by the boxed area in Fig. 5.83, provides for multiplicity editing of the spectrum whereby the observed correlations arising from protons belonging to CH_2 groups are inverted relative to those from CH or CH_3 (as in the multiplicity-edited HSQC variants of Chapter 6), thus further enhancing information content. Finally we note that further variants of the ADEQUATE experiment have been proposed for the measurement of one-bond and long-range carbon–carbon coupling constants [110–112], which, although rarely used to date, have potential application in stereochemical and conformational analysis [84–86, 113].

Finally, we finish with a small digression. The original sequence for identifying ^1H – ^{13}C – ^{13}C connectivities, shown in Fig. 5.81, serves to illustrate a feature common to many modern pulse sequences. Although at first sight it may appear to be frighteningly complex and is undoubtedly one of the most elaborate sequences in this book, it can be broken down into more readily digestible fragments, as illustrated. Although the details of this sequence may appear somewhat esoteric, an appreciation of its operation is rather more accessible when viewed in this stepwise manner as it becomes apparent that this is built-up from simpler sequences or elements already encountered (the aforementioned INEPT and INADEQUATE). As already mentioned, the sequence can then simply be viewed as a variation on the HSQC technique in which carbon double-quantum rather than single-quantum coherence is employed. When faced with trying to follow a new or unfamiliar pulse sequence, a good approach is to try to recognise smaller fragments within it that are derived from shorter, more familiar or recognisable sequences and to piece these elements together to follow the flow of magnetisation through the experiment. We shall encounter further examples of this in the following chapter.

REFERENCES

- [1] J. Jeener, *Ampère International Summer School*, Basko Polje, (former) Yugoslavia, 1971.
- [2] W. P. Aue, E. Bartholdi and R. R. Ernst, *J. Chem. Phys.*, 1976, **64**, 2229–2246.
- [3] J. N. S. Evans, *Biomolecular NMR Spectroscopy*, Oxford University Press, Oxford, 1995.
- [4] J. Cavanagh, W. J. Fairbrother, A. G. Palmer, N. J. Skelton and M. Rance, *Protein NMR Spectroscopy: Principles and Practice*, 2nd edition Academic Press (Elsevier), San Diego, 2006.
- [5] J. Hahn, Higher-order ^{31}P NMR spectroscopy of polyphosphorus compounds In: *Phosphorus-31 NMR Spectroscopy in Stereochemical Analysis* (Eds.: J.G. Verkade and L.D. Quin) VCH, Florida, 1987, pp. 331–364.
- [6] D. J. States, R. A. Haberkorn and D. J. Ruben, *J. Magn. Reson.*, 1982, **48**, 286–292.
- [7] M. Ohuchi, M. Hosono, K. Furihata and H. Seto, *J. Magn. Reson.*, 1987, **72**, 279–297.
- [8] D. Marion and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 1983, **113**, 967–974.
- [9] J. Keeler and D. Neuhaus, *J. Magn. Reson.*, 1985, **63**, 454–472.
- [10] D. Marion, M. Ikura, R. Tschudin and A. Bax, *J. Magn. Reson.*, 1989, **85**, 393–399.
- [11] A. L. Davis, J. Keeler, E. D. Laue and D. Moskau, *J. Magn. Reson.*, 1992, **98**, 207–216.
- [12] C. J. Turner and H. D. W. Hill, *J. Magn. Reson.*, 1986, **66**, 410–421.
- [13] A. Bax, R. Freeman and G. A. Morris, *J. Magn. Reson.*, 1981, **43**, 333–338.
- [14] A. F. Mehlkopf, D. Korb, T. A. Tiggelman and R. Freeman, *J. Magn. Reson.*, 1984, **58**, 315–323.
- [15] G. A. Morris, *J. Magn. Reson.*, 1992, **100**, 316–328.
- [16] R. Baumann, G. Wider, R. R. Ernst and K. Wüthrich, *J. Magn. Reson.*, 1981, **44**, 402–406.
- [17] G. A. Morris, *J. Magn. Reson.*, 1988, **80**, 547–552.
- [18] G. A. Morris and D. Cowburn, *Magn. Reson. Chem.*, 1989, **27**, 1085–1089.
- [19] G. A. Morris, H. Barjat and T. J. Horne, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1997, **31**, 197–257.
- [20] K. R. Metz, M. M. Lam and A. G. Webb, *Concepts Magn. Reson.*, 2000, **12**, 21–42.
- [21] T. J. Horne and G. A. Morris, *J. Magn. Reson. Ser A*, 1996, **123**, 246–252.
- [22] A. Gibbs, G. A. Morris, A. G. Swanson and D. Cowburn, *J. Magn. Reson. Ser A*, 1993, **101**, 351–356.
- [23] A. D. Bain, *J. Magn. Reson.*, 1984, **56**, 418–427.
- [24] G. Bodenhausen, H. Kogler and R. R. Ernst, *J. Magn. Reson.*, 1984, **58**, 370–388.
- [25] H. Kessler, M. Gehrke and C. Griesinger, *Angew. Chem. Int. Ed.*, 1988, **27**, 490–536.
- [26] J. Keeler, R. T. Clowes, A. L. Davis and E. D. Laue, *Methods Enzymol.*, 1994, **239**, 145–207.
- [27] S. Berger, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1997, **30**, 137–156.
- [28] T. Parella, *Magn. Reson. Chem.*, 1998, **36**, 467–495.
- [29] P. C. Lauterbur, *Nature*, 1973, **242**, 190–191.
- [30] A. Bax, P. G. d'Long, A. F. Mehlkopf and J. Smidt, *Chem. Phys. Lett.*, 1980, **69**, 567–570.
- [31] P. Barker and R. Freeman, *J. Magn. Reson.*, 1985, **64**, 334–338.
- [32] R. E. Hurd, *J. Magn. Reson.*, 1990, **87**, 422–428.
- [33] J. Jeener, B. H. Meier, P. Bachmann and R. R. Ernst, *J. Chem. Phys.*, 1979, **71**, 4546–4553.
- [34] J. Weigelt and G. Otting, *J. Magn. Reson. Ser A*, 1995, **113**, 128–130.
- [35] V. Sklenář, M. Piotto, R. Leppik and V. Saudek, *J. Magn. Reson. Ser A*, 1993, **102**, 241–245.
- [36] T. L. Hwang and A. J. Shaka, *J. Magn. Reson. Ser A*, 1995, **112**, 275–279.
- [37] G. Kontaxis, J. Stonehouse, E. D. Laue and J. Keeler, *J. Magn. Reson. Ser A*, 1994, **111**, 70–76.
- [38] M. Rance, O. W. Sørensen, G. Bodenhausen, G. Wagner, R. R. Ernst and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 1983, **117**, 479–485.
- [39] U. Piantini, O. W. Sørensen and R. R. Ernst, *J. Am. Chem. Soc.*, 1982, **104**, 6800–6801.
- [40] A. L. Davis, E. D. Laue, J. Keeler, D. Moskau and J. Lohman, *J. Magn. Reson.*, 1991, **94**, 637–644.
- [41] M. Eberstadt, G. Gemmecker, D. F. Mierke and H. Kessler, *Angew. Chem. Int. Ed.*, 1995, **34**, 1671–1695.
- [42] D. Neuhaus, G. Wagner, M. Vasák, J. H. R. Kágy and K. Wüthrich, *Eur. J. Biochem.*, 1985, **151**, 257–273.
- [43] H. Kessler and H. Oschkinat, *Angew. Chem. Int. Ed.*, 1985, **24**, 690–692.
- [44] H. Kessler, A. Müller and H. Oschkinat, *Magn. Reson. Chem.*, 1985, **23**, 844–852.
- [45] H. Oschkinat and R. Freeman, *J. Magn. Reson.*, 1984, **60**, 164–169.
- [46] A. J. Shaka and R. Freeman, *J. Magn. Reson.*, 1983, **51**, 169–173.
- [47] N. Müller, R. R. Ernst and K. Wüthrich, *J. Am. Chem. Soc.*, 1986, **108**, 6482–6492.
- [48] J. Boyd and C. Redfield, *J. Magn. Reson.*, 1986, **68**, 67–84.
- [49] G. Eich, G. Bodenhausen and R. R. Ernst, *J. Am. Chem. Soc.*, 1982, **104**, 3731–3732.
- [50] L. Braunschweiler and R. R. Ernst, *J. Magn. Reson.*, 1983, **53**, 521–528.
- [51] D. G. Davis and A. Bax, *J. Am. Chem. Soc.*, 1985, **107**, 2820–2821.
- [52] A. Bax and D. G. Davis, *J. Magn. Reson.*, 1985, **65**, 355–360.
- [53] J. Cavanagh, W. J. Chazin and M. Rance, *J. Magn. Reson.*, 1990, **87**, 110–131.
- [54] G. A. Morris and A. Gibbs, *J. Magn. Reson.*, 1991, **91**, 444–449.
- [55] A. Gibbs and G. A. Morris, *Magn. Reson. Chem.*, 1992, **30**, 662–665.
- [56] J. E. Baldwin, L. Bischoff, T. D. W. Claridge, F. A. Heupel, D. R. Spring and R. C. Whitehead, *Tetrahedron*, 1997, **53**, 2271–2290.
- [57] J. E. Baldwin, T. D. W. Claridge, A. J. Culshaw, F. A. Heupel, V. Lee, D. R. Spring, R. C. Whitehead, R. J. Boughtflower, I. M. Mutton and R. J. Upton, *Angew. Chem. Int. Ed.*, 1998, **37**, 2661–2663.
- [58] C. Griesinger, G. Otting, K. Wüthrich and R. R. Ernst, *J. Am. Chem. Soc.*, 1988, **110**, 7870–7872.
- [59] J. Briand and R. R. Ernst, *Chem. Phys. Lett.*, 1991, **185**, 276–285.
- [60] J. Cavanagh and M. Rance, *J. Magn. Reson.*, 1992, **96**, 670–678.
- [61] M. Rance, *J. Magn. Reson.*, 1987, **74**, 557–564.
- [62] R. Bazzo and I. D. Campbell, *J. Magn. Reson.*, 1988, **76**, 358–361.
- [63] O. W. Sørensen, M. Rance and R. R. Ernst, *J. Magn. Reson.*, 1984, **56**, 527–534.
- [64] A. J. Shaka, J. Keeler and R. Freeman, *J. Magn. Reson.*, 1983, **53**, 313–340.

- [65] A. J. Shaka, J. Keeler, T. Frenkiel and R. Freeman, *J. Magn. Reson.*, 1983, **52**, 335–338.
- [66] S. P. Rucker and A. J. Shaka, *Mol. Phys.*, 1989, **68**, 509–517.
- [67] A. J. Shaka, C. J. Lee and A. Pines, *J. Magn. Reson.*, 1988, **77**, 274–293.
- [68] H. Kessler, S. Mronga and G. Gemmecker, *Magn. Reson. Chem.*, 1991, **29**, 527–557.
- [69] T. Parella, *Magn. Reson. Chem.*, 1996, **34**, 329–347.
- [70] D. G. Davis and A. Bax, *J. Am. Chem. Soc.*, 1985, **107**, 7197–7198.
- [71] H. Kessler, U. Anders, G. Gemmecker and S. Steuernagel, *J. Magn. Reson.*, 1989, **85**, 1–14.
- [72] H. Kessler, H. Oschkinat, C. Griesinger and W. Bermel, *J. Magn. Reson.*, 1986, **70**, 106–133.
- [73] S. Subramanian and A. Bax, *J. Magn. Reson.*, 1987, **71**, 325–330.
- [74] P. Adell, T. Parella, F. Sánchez-Ferrando and A. Virgili, *J. Magn. Reson. Ser B*, 1995, **108**, 77–80.
- [75] T. Fäcke and S. Berger, *J. Magn. Reson. Ser A*, 1995, **113**, 257–259.
- [76] C. Dalvit and G. Bovermann, *Magn. Reson. Chem.*, 1995, **33**, 156–159.
- [77] G. Z. Xu and J. S. Evans, *J. Magn. Reson. Ser B*, 1996, **111**, 183–185.
- [78] M. J. Thrippleton and J. Keeler, *Angew. Chem. Int. Ed.*, 2003, **42**, 3938–3941.
- [79] J. Buddrus and H. Bauer, *Angew. Chem. Int. Ed.*, 1987, **26**, 625–642.
- [80] J. Buddrus and J. Lambert, *Magn. Reson. Chem.*, 2002, **40**, 3–23.
- [81] A. Bax, R. Freeman and S. P. Kempell, *J. Am. Chem. Soc.*, 1980, **102**, 4849–4851.
- [82] A. Bax, R. Freeman and T. H. Frenkiel, *J. Am. Chem. Soc.*, 1981, **103**, 2102–2104.
- [83] M. Budesinsky and D. Saman, *Annu. Rep. NMR Spectrosc.*, 1995, **30**, 231–475.
- [84] J. L. Marshall, *Carbon-carbon and carbon-proton NMR couplings: Applications to organic stereochemistry and conformational analysis*, VCH, Florida, 1983.
- [85] L. B. Krivdin and G. A. Kalabin, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1989, **21**, 293–448.
- [86] L. B. Krivdin and S. V. Zinchenko, *Curr. Org. Chem.*, 1998, **2**, 173–193.
- [87] J. C. Vederas, *Nat. Prod. Rep.*, 1987, **4**, 277–337.
- [88] T. H. Mareci and R. Freeman, *J. Magn. Reson.*, 1982, **48**, 158–163.
- [89] J. Lambert, H. J. Kuhn and J. Buddrus, *Angew. Chem. Int. Ed.*, 1989, **28**, 738–740.
- [90] A. M. Torres, T. T. Nakashima, R. E. D. McClung and D. R. Muhandiram, *J. Magn. Reson.*, 1992, **99**, 99–117.
- [91] R. Dunkel, C. L. Mayne, J. Curtis, R. J. Pugmire and D. M. Grant, *J. Magn. Reson.*, 1990, **90**, 290–302.
- [92] R. Dunkel, C. L. Mayne, R. J. Pugmire and D. M. Grant, *Anal. Chem.*, 1992, **64**, 3133–3149.
- [93] R. Dunkel, C. L. Mayne, M. P. Foster, C. M. Ireland, D. Li, N. L. Owen, R. J. Pugmire and D. M. Grant, *Anal. Chem.*, 1992, **64**, 3150–3160.
- [94] J. K. Harper, R. Dunkel, S. G. Wood, N. L. Owen, D. Li, R. G. Cates and D. M. Grant, *J. Chem. Soc., Perkin Trans. 2*, 1996, 191–200.
- [95] A. Bax and R. Freeman, *J. Magn. Reson.*, 1980, **41**, 507–511.
- [96] E. Breitmaier and W. Voelter, *Carbon-13 NMR Spectroscopy*, 3rd edition, VCH, Weinheim, 1987.
- [97] K. Kamienska-Trela, *Annu. Rep. NMR Spectrosc.*, 1995, **30**, 131–230.
- [98] D. L. Mattiello and R. Freeman, *J. Magn. Reson.*, 1998, **135**, 514–521.
- [99] J. Lettvin and A. D. Sherry, *J. Magn. Reson.*, 1977, **28**, 459–461.
- [100] O. W. Sørensen, R. Freeman, T. A. Frenkiel, T. H. Mareci and R. Schuck, *J. Magn. Reson.*, 1982, **46**, 180–184.
- [101] S. W. Sparks and P. D. Ellis, *J. Magn. Reson.*, 1985, **62**, 1–11.
- [102] P. J. Keller and K. E. Vogele, *J. Magn. Reson.*, 1986, **68**, 389–392.
- [103] B. Reif, M. Kock, R. Kerssebaum, H. Kang, W. Fenical and C. Griesinger, *J. Magn. Reson. Ser A*, 1996, **118**, 282–285.
- [104] M. Kock, B. Reif, W. Fenical and C. Griesinger, *Tetrahedron Lett.*, 1996, **37**, 363–366.
- [105] M. Knaack, P. Emig, J. W. Bats, M. Kiesel, A. Müller and E. Günther, *Eur. J. Org. Chem.*, 2001, 3843–3847.
- [106] G. N. Belofsky, M. Anguera, P. R. Jensen, W. Fenical and M. Köck, *Chem. Eur. J.*, 2000, **6**, 1355–1360.
- [107] M. Kock, B. Reif, M. Gerlach and M. Reggelin, *Molecules*, 1996, **1**, 41–45.
- [108] M. Kock, R. Kerssebaum and W. Bermel, *Magn. Reson. Chem.*, 2003, **41**, 65–69.
- [109] R. T. Williamson, B. L. Marquez, W. H. Gerwick and F. E. Koehn, *Magn. Reson. Chem.*, 2001, **39**, 544–548.
- [110] B. Reif, M. Kock, R. Kerssebaum, J. Schleucher and C. Griesinger, *J. Magn. Reson. Ser B*, 1996, **112**, 295–301.
- [111] K. E. Kövér and P. Forgó, *J. Magn. Reson.*, 2004, **166**, 47–52.
- [112] T. N. Pham, K. E. Kövér, L. Jin and D. Uhrin, *J. Magn. Reson.*, 2005, **176**, 199–206.
- [113] B. Bose, S. Zhao, R. Stenutz, F. Cloran, P. B. Bondo, G. Bondo, B. Hertz, I. Carmichael and A. S. Serianni, *J. Am. Chem. Soc.*, 1998, **120**, 11158–11173.

Chapter 6

Correlations through the chemical bond II: Heteronuclear shift correlation

6.1. INTRODUCTION

This chapter on establishing correlations through the chemical bond concentrates on techniques that correlate different nuclides, the so-called *heteronuclear* shift correlations. For an organic chemist, this means, in the vast majority of cases, establishing connectivities between proton and carbon nuclei, and as such the techniques encountered in the sections that follow are primarily concerned with these. That is, not to say that the techniques are not suitable for correlating other nuclides, for example, ^1H with ^{15}N or ^{31}P , or even ^{31}P with ^{13}C and so on. Indeed many of the modern techniques used routinely in the chemical laboratory were originally implemented as methods for ^1H – ^{15}N correlations in proteins and peptides. The principal techniques described in the sections that follow are summarised in Table 6.1.

Various methods that allow the editing of the 1D spectrum of the heteronuclear spin have already been described in Chapter 4, for example, those based on spin-echoes or polarisation transfer, thus providing valuable information on the numbers of attached protons. They do not, however, provide any *direct* evidence for which protons are attached to which heteronucleus (X spin) in the molecule and for this heteronuclear 2D correlations are widely used to transfer previously established proton assignments onto the directly bonded heteronucleus or, on occasions, *vice versa*. This may then provide further evidence to support or reject the proposed structure as being correct or may provide assignments that can be used as the basis of further investigations. In addition, the experiment may be used as a means of spreading the resonances of a complex proton spectrum according to the chemical shift of the directly attached nucleus, utilising the typical greater dispersion of the X spin chemical shifts to assist with proton interpretation. The high sensitivity of modern correlation techniques often provides a fast method for indirectly determining chemical shifts of the X-nucleus and avoids the need for its direct observation altogether, offering considerable time savings. Thus, the ^1H – ^1H COSY and the ^1H – ^{13}C correlation experiments (as the HMQC or HSQC sequences) represent the primary 2D techniques in structural organic chemistry.

For more complex problems, there exist methods that combine the features of two, otherwise separate, techniques, which is referred to here as ‘hybrid’ experiments. Although a wide range of combinations have been devised, essentially limited by the imagination of the spectroscopist, two principal features are of considerable utility. The first is editing of the correlation spectrum such that it contains both shift and multiplicity data, by analogy with the 1D editing methods presented in Chapter 4. The second feature is the relaying of correlations to enhance the information content of the spectrum by providing additional neighbouring group information. Common implementations of these schemes are found in the form of the edited HSQC and the HSQC-TOCSY experiment, both described in this chapter.

Beyond these methods that utilise one-bond heteronuclear couplings, correlations with a heteronucleus over more than one bond, so-called long-range or multiple-bond correlations, can provide a wealth of connectivity information on how molecular fragments are linked together. These methods have become increasingly important in recent years and the techniques for establishing such correlations have been embellished in various ways to enhance information content, providing the chemist with a range of complimentary techniques with which to define structure. The final class of techniques that will be considered seeks correlations between, or involving, two heteronuclei, these generally being referred to as ‘X–Y correlations’, which may be employed when ^1H –X correlations provide insufficient information or when these simply do not exist. Those methods that also utilise coupling to protons (^1H –X–Y correlations) may be derived conceptually from those developed for establishing ^1H – ^{13}C – ^{15}N correlations in biological macromolecules and mostly find application in the realm of organometallic chemistry where suitable NMR-active X/Y spin pairs exist.

Table 6.1. The principal applications of the main techniques described in this chapter

Technique	Principal applications
HMQC	Correlating coupled heteronuclear spins across a single bond and hence identifying directly connected nuclei, most often ^1H – ^{13}C . Employs detection of high-sensitivity nuclide, e.g. ^1H , ^{19}F , ^{31}P (an ‘inverse technique’). Experimentally robust sequence, well suited to routine structural characterisation
HSQC	Correlating coupled heteronuclear spins across a single bond and hence identifying directly connected nuclei. Employs detection of high-sensitivity nuclide, e.g. ^1H , ^{19}F , ^{31}P (an ‘inverse technique’). Provides improved resolution over HMQC, so it is better suited for crowded spectra but can be more sensitive to experimental imperfections
HMBC	Correlating coupled spins across multiple bonds. Employs detection of high-sensitivity nuclide, e.g. ^1H , ^{19}F , ^{31}P (an ‘inverse technique’). Essentially HMQC tuned for the detection of small couplings. Most valuable in correlating ^1H – ^{13}C over two or three bonds. Powerful tool for linking together structural fragments
HETCOR	Correlating coupled heteronuclear spins across a single bond. Employs detection of the lower- γ nuclide, typically ^{13}C , so has significantly lower sensitivity than inverse techniques. Benefits from high resolution in ^{13}C dimension, so may find use when this is critical, otherwise superseded by above methods
H–X–Y	Triple-resonance methods for correlating protons and a heteratom (Y) whilst using a second heteroatom (X) to either relay the correlations or edit the correlation spectrum

6.2. SENSITIVITY

The original methods for determining heteronuclear shift correlations were based on the observation of the low- γ X-nucleus, with the proton being indirectly detected and consequently appearing along the f_1 dimension of the 2D experiment. This approach was adopted because, as we shall see, the original 2D sequences were derived from early polarisation transfer experiments, such as INEPT (Section 4.4), which were themselves designed to enhance the sensitivity of low- γ observations, and because early pulsed NMR instruments were designed with this mode of operation in mind. During the last decade or so, the approach to data collection has fundamentally changed to one in which the high- γ nucleus, most frequently the proton, is observed, with the heteronucleus now detected indirectly. This switch has given rise to a body of experiments frequently referred to as ‘inverse’ shift correlations, the motivation for change being improved sensitivity.

The dependence of the strength of an NMR signal on magnetogyric ratio has been discussed previously in Section 4.4 where the concept of polarisation transfer was introduced as a means of sensitivity enhancement. From the qualitative arguments presented in that section, a more formal expression for the signal-to-noise ratio of a 1D experiment involving spin-1/2 nuclei was given as:

$$\frac{S}{N} \propto NAT^{-1} B_0^{3/2} \gamma_{\text{exc}} \gamma_{\text{obs}}^{3/2} T_2^* (\text{NS})^{1/2} \quad (6.1)$$

where N is the number of molecules in the observed sample volume, A is a term that represents the abundance of the NMR-active spins involved in the experiment, T is the temperature, B_0 is the static magnetic field, γ_{exc} and γ_{obs} represent the magnetogyric ratios of the initially excited and observed spins, respectively, T_2^* is the effective transverse relaxation time and NS is the total number of accumulated scans. When choosing how to perform a heteronuclear shift correlation experiment, four general schemes may be devised, as represented in Fig. 6.1, according to which spin is used as the initial magnetisation ‘source’ and which is observed (represented in the γ factors in Eq. (6.1)). Alongside each schematic is summarised the relative theoretical sensitivities expected for an H–X pair based on the participating γ values. Figure 6.1b represents the approach adopted in the traditional shift correlation experiments, in which initial proton magnetisation is frequency-labelled in t_1 and then transferred onto the X spin for detection. Figure 6.1d is the modern ‘inverse’ approach in which the proton nucleus is used both as the source and the observed spin. Clearly the predicted sensitivity is significantly greater than that of the traditional method, and the dependence on the magnetogyric ratio of the low- γ spin has been completely removed, although the natural abundance of this spin is still an important factor for all approaches (term A in the above equation). The lower the γ of the X spin, the greater the gains arising from proton detection; compare the figures for ^{31}P and ^{15}N in Fig. 6.1, for example. Additional theoretical gains by a factor of 2 or 3 can be anticipated for XH_2 or XH_3 groups, respectively, with the proton-detected experiments.

The impressive gains illustrated in Fig. 6.1 for inverse versus traditional methods may not be met in practice when the details of a particular sequence are considered. Significant factors may include the different relaxation behaviour of participating spins or the presence of multiplet splittings that spread a resonance and so reduce the signal-to-noise ratio.

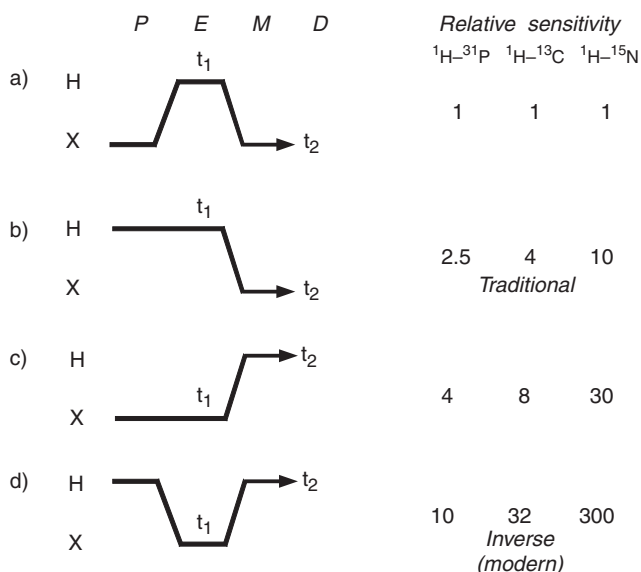


Figure 6.1. The four general schemes to produce 2D heteronuclear shift correlation spectra. (a) X-H-X, (b) H-H-X, (c) X-X-H and (d) H-X-H. The relative sensitivities of these approaches are compared for proton correlation experiments with phosphorus-31, carbon-13 and nitrogen-15.

However, even a realistic gain of a factor of 4 in $^1\text{H}-^{13}\text{C}$ correlations corresponds to a time saving of a factor of 16, meaning experiments that once required an overnight acquisition with ^{13}C detection can be collected in about an hour, whilst those traditionally requiring a couple of hours can be completed within a matter of minutes. Furthermore, studies on very dilute samples that would have been considered intractable become viable targets with proton detection. Such considerations have led to the universal adoption of proton-detected inverse-correlation methods whenever possible in both chemical and biological spectroscopy, and it is these techniques that are focussed upon below, along with only a relatively brief consideration of the more traditional X-detected methods that may still have utility in specific circumstances.

The adoption of the inverse approach also has implications for the design of the NMR instrument. Conventional probes were constructed so as to optimise the sensitivity for observation of the low- γ X-nucleus, which entails placing the X-nucleus coil closest to the sample and positioning the proton coil outside this. Inverse probes have this configuration switched such that the proton coil sits closest to the sample for optimum sensitivity, thus providing a greater *filling factor*. However, even with conventional probes, the proton-detected experiments can still be performed, albeit with less than optimum sensitivity, and may still provide a faster approach than the former X-observe experiments.

6.3. HETERONUCLEAR SINGLE-BOND CORRELATION SPECTROSCOPY

There are two techniques in widespread use that provide single-bond heteronuclear shift correlations, known colloquially as HMQC and HSQC. The correlation data provided by these two methods are essentially equivalent, the methods differing only in finer details, which, for routine spectroscopy, are often of little consequence. As such, both methods are ubiquitous throughout the chemical literature and hence will be described in the sections that follow. Historically, the HMQC experiment has been favoured by the chemical community and HSQC by biological spectroscopists. This is, at least in part, due to the manner in which the early experiments were presented, *viz* HMQC for $^1\text{H}-^{13}\text{C}$ correlations in small molecules and HSQC for $^1\text{H}-^{15}\text{N}$ correlations in proteins. At a practical level, the HMQC experiment tends to be more robust with respect to experimental imperfections or miscalibrations, whilst the HSQC experiment has more favourable characteristics for very high-resolution work and is more flexible with regard to modification and extension of the sequence, so it is now widely used in the chemical laboratory also; these points are pursued in the following sections. Both techniques employ the optimum approach for establishing heteronuclear connectivity utilising proton detection and following the general scheme of Fig. 6.1d. However, this approach poses technical difficulties in the suppression of the parent resonance arising from protons bound to nuclides with $I \neq 1/2$, for example $^1\text{H}-^{12}\text{C}$ and $^1\text{H}-^{14}\text{N}$. This is the dominant line observed in 1D proton spectra but is merely a source of interference in heteronuclear correlations since it is only the low-intensity *satellites* that can give rise to the desired correlations, that is, the $^1\text{H}-^{13}\text{C}$ or $^1\text{H}-^{15}\text{N}$ protons in the above examples. The necessary suppression is nowadays most effectively executed by the application of pulsed field gradients (PFGs) which have had an enormous impact on heteronuclear correlation spectroscopy in particular.

6.3.1. Heteronuclear multiple-quantum correlation (HMQC)

The HMQC (Heteronuclear Multiple-Quantum Correlation) experiment is one of the two commonly employed proton-detected single-bond correlation experiments. Although this was suggested many years ago [1, 2], the experiment lacked widespread use until a scheme that was able to overcome the technical difficulties associated with proton observation described above was presented [3]. Since then, and particularly since the advent of PFGs, the technique has come to dominate organic NMR spectroscopy.

The 2D spectrum provides a simple map of connectivities in which a crosspeak correlates two attached nuclei, as seen in the ^1H - ^{13}C HMQC spectrum of menthol **6.1** (Fig. 6.2). This illustrates three of the most significant features of the experiment when applied to routine structural problems. The first is the ability to transfer known proton assignments, determined, for example, with the methods described in Chapter 5, onto the spectrum of the heteronucleus, thus extending characterisation of the molecule. The second is the dispersion of the proton resonances according to the heteronuclear shift, which itself can aid the initial interpretation of the proton spectrum. For example, the region between 0.7 and 1.2 ppm of Fig. 6.2 contains a number of overlapped proton resonances that defy direct analysis even at 500 MHz. A clearer picture of events in this region emerges when these are dispersed along the ^{13}C dimension, revealing the presence of seven distinct groups. This property becomes increasingly valuable as the complexity and overlap within proton spectra increases. The final feature that can prove surprisingly useful in structural assignment is the ability to identify *diastereotopic* geminal pairs. These are not always readily identifiable in COSY spectra owing to the lack of differentiation of geminal and vicinal couplings and can lead to ambiguity in proton assignment. Only for these geminal pairs will two correlations to a single carbon resonance be observed, as seen for the correlations of C6, C4 and C3 in Fig. 6.2. In contrast to previous *homonuclear* 2D spectra encountered in this book, *heteronuclear* shift correlation spectra lack a diagonal and are not symmetrical about $f_1=f_2$, a simple consequence of there being different nuclides represented in the two dimensions.

The HMQC sequence

Despite the slightly foreboding title, the basic HMQC sequence is rather simple, comprising only four rf pulses (Fig. 6.3), the operation of which is considered here for a simple ^1H - ^{13}C spin pair. The sequence starts with proton excitation followed by evolution of proton magnetisation under the influence of the one-bond carbon-proton coupling.

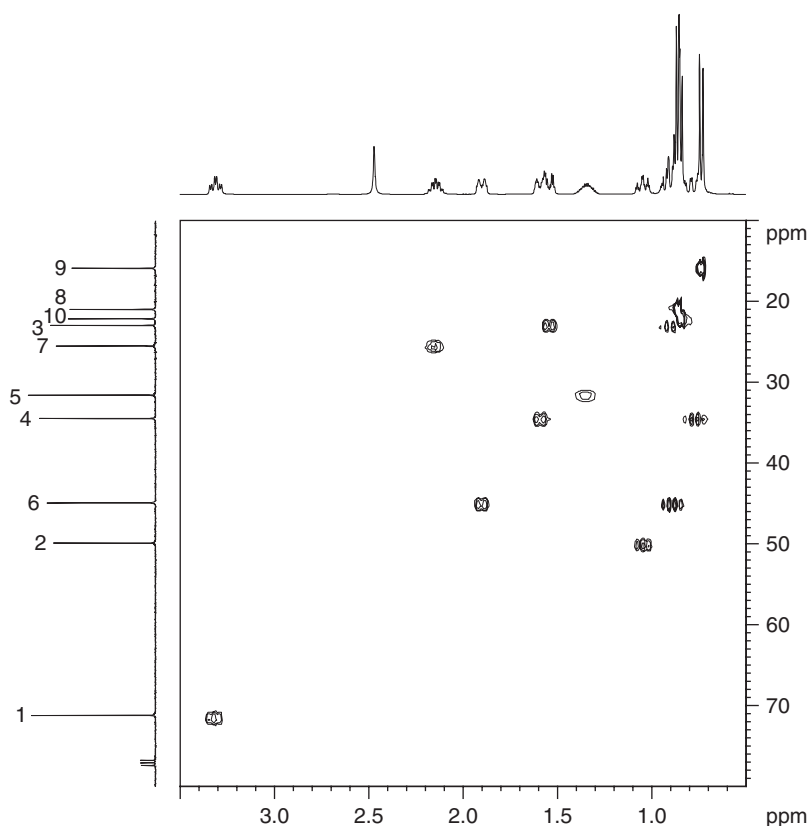
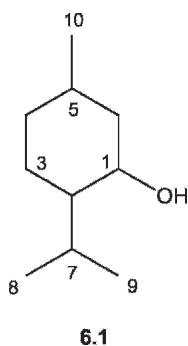


Figure 6.2. The 500-MHz HMQC single-bond correlation spectrum of menthol **6.1**. The conventional 1D proton and carbon spectra are also shown for reference.

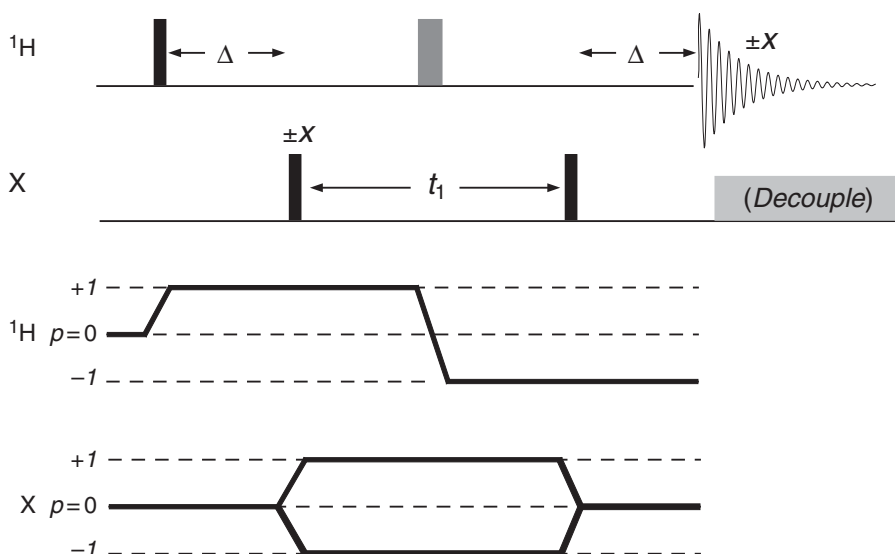


Figure 6.3. The HMQC sequence and associated coherence transfer pathway. The Δ periods are set to $1/2J_{\text{CH}}$ to enable defocusing and subsequent refocusing of the one-bond heteronuclear coupling.

During a period Δ , anti-phase proton magnetisation develops with respect to $^1J_{\text{CH}}$ and to maximise this, Δ is set to $1/2J_{\text{CH}}$ (it will be shown shortly that proton chemical shift evolution during Δ is later refocused, so need not be considered). Accepting a compromise value of around 150 Hz for $^1J_{\text{CH}}$ (Table 6.2), Δ is therefore typically set to around 3.3 ms. As in the case of INEPT or COSY, this anti-phase magnetisation may be transferred to the coupled partner by the action of a subsequent rf pulse, and the role of the first carbon pulse in HMQC is to generate proton–carbon multiple-quantum coherence (hence the title of the experiment). Multiple-quantum coherence was described in Section 5.4 as a pooling of the transverse magnetisation of coupled spins, in this case a proton and its directly bound carbon, that evolves coherently but that cannot be directly observed. If one were to start data collection of the proton signal directly after this carbon pulse, there would be nothing to detect, provided the Δ delay was set precisely to $1/2J_{\text{CH}}$.¹

This coherence is, in fact, a combination of both heteronuclear double- and zero-quantum coherence, as represented on the coherence transfer pathway of Fig. 6.3. Thus, for example, $^1H_p = +1$ and $^{13}C_p = +1$ corresponds to double-quantum coherence ($\Sigma p = 2$) whilst $^1H_p = +1$ and $^{13}C_p = -1$ corresponds to zero-quantum coherence ($\Sigma p = 0$). The salient point at this stage is how such coherences evolve during the subsequent t_1 period. Since they contain terms for both transverse proton and carbon magnetisation, they will evolve under the influence of *both proton and carbon chemical shifts* (although a feature of multiple-quantum coherences is that they will *not* evolve under the ‘active’ coupling, so J_{CH} need not be considered at this point). What is ultimately required, however, is frequency labelling according to *carbon* shifts only, since this is what one wishes to characterise in the indirectly detected f_1 dimension. To remove the effect of proton shifts during t_1 , a spin-echo is incorporated by placing a proton 180° pulse at the midpoint of t_1 so that by the end of the evolution period, these shifts have refocused and thus have no influence in f_1 . Evolution of the carbon shifts is unaffected by the proton pulse, so these remain to produce the desired frequency labelling. The final carbon pulse then reconverts

Table 6.2. Typical ranges for one-bond carbon–proton coupling constants

Proton environment	Typical $^1J_{\text{CH}}$ range (Hz)
Aliphatic, CH_n-	125–135
Aliphatic, CH_nX ($\text{X} = \text{N}, \text{O}, \text{S}$)	135–155
Alkene	155–170
Alkyne	240–250
Aromatic	155–165

¹ Note that this is exactly the procedure described in Section 3.5.1 for the calibration of pulses on the indirectly observed spin and, allowing for the proton 180° pulse, is also closely related to the start of the DEPT sequence (Section 4.4.3), which similarly relies on the generation of multiple-quantum coherence.

the multiple-quantum coherence back to observable single-quantum proton magnetisation, which is once again anti-phase with respect to $^1J_{CH}$. When collecting the proton FID, it is generally desirable to apply broadband decoupling of the *carbon* spins to remove the J_{CH} doublet fine structure, thus doubling the signal-to-noise ratio. To avoid cancellation of the anti-phase proton satellites, a second Δ period is inserted to refocus the proton–carbon coupling, after which the proton magnetisation is detected. Conventional quadrature detection in f_1 is implemented by incrementing the phase of the carbon pulse prior to t_1 according to either the States or TPPI procedures to yield a phase-sensitive display. The result is a 2D spectrum with ^1H shifts represented in f_2 , ^{13}C shifts in f_1 and crosspeaks indicating one-bond connectivities (Fig. 6.4a and b). The doublet structure for each correlation seen in the absence of carbon decoupling reflects the $^1J_{CH}$ coupling constant and provides a direct measure of this parameter.

One subtle point to be aware of is that although this sequence (and HSQC below) nominally detects single-bond correlations, in exceptional circumstances, these may be missing and longer-range correlations may appear. This occurs when the actual coupling constant involved is far from the value assumed when calculating Δ , a situation most likely to occur for alkynes where the one-bond and two-bond ($H-C\equiv C$) couplings are unusually large. A large two-bond coupling (>50 Hz) can be sufficient to produce a crosspeak and care should be taken not to confuse this with a single-bond correlations when dealing with these systems.

The influence of homonuclear proton couplings

It was noted above that proton magnetisation will also evolve according to its chemical shift during Δ after initial excitation. However, this is exactly refocused during the second Δ period because of the presence of the proton spin-echo, so it does not give rise to phase errors in the proton dimension. Of greater concern is the evolution of proton magnetisation in the two Δ periods and of heteronuclear multiple-quantum coherence during t_1 , under the influence of homonuclear proton–proton couplings. Since these homonuclear couplings are *not* refocused by a spin-echo, they will evolve in both Δ periods and potentially contribute to unwanted phase errors in the proton dimension. However, Δ is set according to $^1J_{CH}$, which is typically at least an order of magnitude greater than J_{HH} , so that in practice the degree of evolution due to proton–proton coupling is rather small. In other words, Δ is too short for significant evolution to occur, and the small phase errors that may arise are rarely troublesome (as described below, this is not the case when seeking correlations through long-range heteronuclear couplings that are comparable in size to homonuclear proton couplings). In contrast, multiple-quantum coherence evolves during t_1 under *passive* J_{HH} couplings without being refocused and thus the final carbon resonances are spread by proton–proton couplings along f_1 ! This may seem a little odd, but it is a consequence of the fact that during t_1 both proton and carbon coherences evolve; we simply choose to remove proton chemical shifts with the spin-echo. In fact, because of the rather low digital

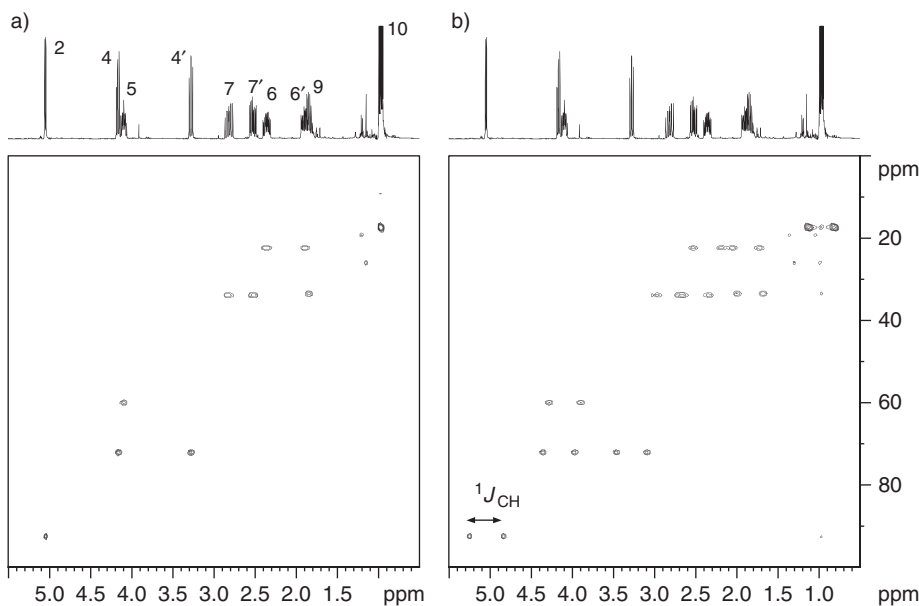
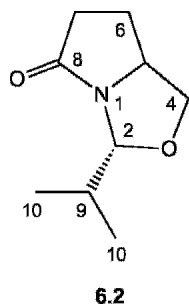


Figure 6.4. The 400-MHz HMQC spectra of **6.2** recorded (a) with and (b) without carbon decoupling during data collection. In the absence of decoupling, each crosspeak appears with doublet structure along f_2 arising from $^1J_{CH}$. These doublets are merely the usual ^{13}C satellites observed in the 1D proton spectrum. 1 K data points were collected for $256t_1$ increments of two transients each. Data were processed with $\pi/2$ -shifted sine-bells in both dimensions and presented in phase-sensitive mode. Zero filling once in t_1 resulted in digital resolutions of 4 and 40 Hz/pt in f_2 and f_1 , respectively.

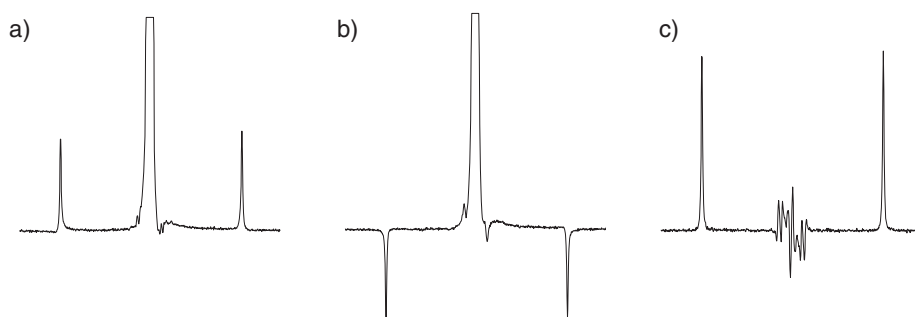


Figure 6.5. Selection of satellite resonances in the HMQC experiment through phase cycling. The phase of the carbon-13 satellites can be inverted by inverting the phase of the first 90° carbon pulse (a vs b). Subtraction of these two data sets by inverting the receiver phase also cancels the parent ^1H - ^{12}C resonance but reinforces the satellites (c).

resolution used in the carbon-13 dimension, as described below, these proton couplings are rarely resolved but do contribute to undesirable *broadening* of the resonance along f_1 . These homonuclear couplings *do not* appear in the f_1 dimension of HSQC, and this feature is the principal difference between the two spectra (see Section 6.3.2 and Fig. 6.7).

Interference from parent ^1H - ^{12}C / ^1H - ^{14}N resonances

The HMQC sequence aims at detecting only those protons that are bound to a spin- $1/2$ heteronucleus, or in other words, only the satellites of the conventional proton spectrum. In the case of ^{13}C , this means that only 1 in every 100 proton spins contribute to the 2D spectrum (the other 99 being attached to NMR-inactive ^{12}C), whilst for ^{15}N with a natural abundance of a mere 0.37%, only 1 in ~ 300 contribute. When the HMQC FID is recorded, *all protons* will induce a signal in the receiver *on each scan* and the unwanted resonances, which clearly represent the vast majority, must be removed with a suitable phase cycle if the correlation peaks are to be revealed (the notable exception to this is when pulsed field gradients are employed for signal selection, see section 6.3.3). By inverting the first ^{13}C pulse on alternate scans, the phase of the ^{13}C satellites is inverted whereas the ^{12}C -bound protons remain unaffected (Fig. 6.5). Simultaneous inversion of the receiver will thus lead to cancellation of the unwanted resonances with corresponding addition of the desired satellites. This two-step procedure is the fundamental phase cycle of the HMQC experiment, as indicated in Fig. 6.3.

The problem with this scheme is that clean suppression of the unwanted resonances is unlikely to be achieved by phase cycling alone, with residual signals contributing to undesirable bands of t_1 noise in the resulting spectra, which may mask the genuine correlations. Extensive time averaging can help to progressively reduce the intensity of these artefacts but defeats the time-saving advantages otherwise made possible by this higher-sensitivity approach, and the use of a ‘double-difference’ phase cycle [4] has been shown to improve the suppression. The detection of all proton resonances on each scan also imposes dynamic range limitations, in that a substantial part of the digitiser collects data that are ultimately discarded. A practically useful approach requires attenuation of the parent proton resonance *before* data collection begins, and Section 6.3.3 describes two methods that are widely employed to meet this goal.

6.3.2. Heteronuclear single-quantum correlation (HSQC)

The HSQC (Heteronuclear Single-Quantum Correlation) experiment [5] also follows the general scheme of Fig. 6.1d but differs from HMQC in that only transverse (single-quantum) magnetisation of the heteronuclear spin evolves during the t_1 period ($\Sigma p = \pm 1$), rather than ^1H -X multiple-quantum coherence; (Fig. 6.6). The transverse heteronuclear magnetisation is generated by polarisation transfer from the attached protons via the INEPT sequence, exactly as described for the 1D experiment in Section 4.4.2 (compare the first part of Fig. 6.6 with that of Fig. 4.23a). The X-nucleus magnetisation evolves during t_1 with the proton 180° pulse at its midpoint refocusing ^1H -X coupling evolution, thus decoupling the ^1H -X interaction, so only heteronuclear chemical shifts remain in f_1 . Following t_1 , the heteronuclear magnetisation is transferred back onto the proton by an INEPT step in reverse to produce, once again, in-phase proton magnetisation for detection in the presence of X spin decoupling. The basic two-step phase cycle is analogous to that of HMQC requiring inversion of the first 90° X pulse with associated inversion of the receiver. The overall transfer process may be summarised using a shorthand notation indicating these key transfer and encoding stages:

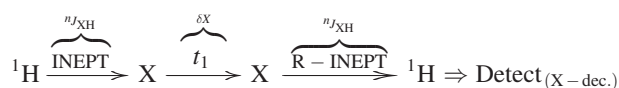


Figure 6.6. The HSQC experiment and associated coherence transfer pathway. The experiment uses the INEPT sequence to generate transverse X magnetisation that evolves and is then transferred back to the proton by an INEPT step in reverse. Note that, in contrast to HMQC, only single-quantum X coherence evolves during t_1 (see text).

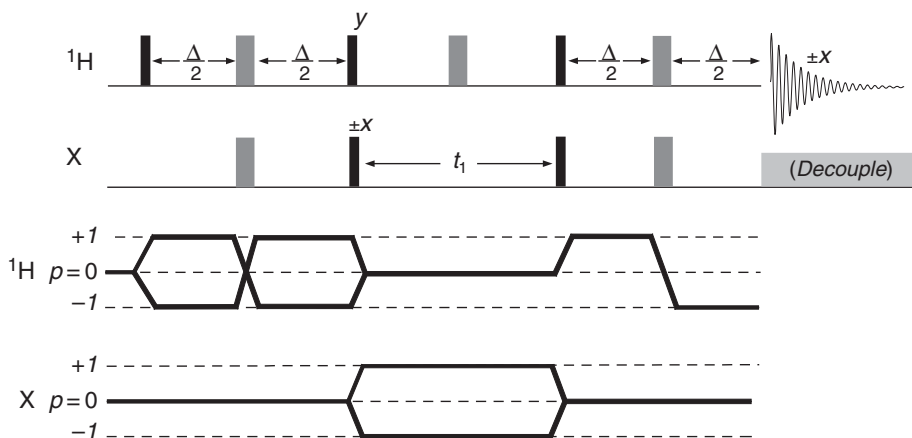
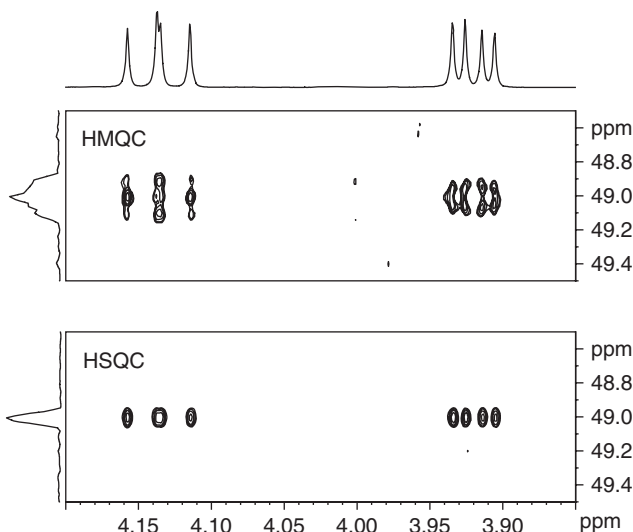


Figure 6.7. A comparison of experimental crosspeaks taken from HMQC and HSQC spectra acquired under identical conditions of high f_1 resolution (2.5 Hz/pt). The upper 1D trace is taken from the conventional 1D proton spectrum, and the vertical traces are f_1 projections from the 2D spectra. The additional broadening in the HMQC spectrum arises from unresolved homonuclear proton couplings in f_1 .



Although this ultimately produces correlations similar to those of the HMQC experiment, the crosspeaks *do not* contain homonuclear ^1H – ^1H couplings along f_1 . This is because only X-nucleus magnetisation evolves during t_1 , which is not, therefore, influenced by homonuclear proton couplings. This results in improved resolution in this dimension, the principal advantage of HSQC over HMQC for small molecules [6] (Fig. 6.7). The potentially greater f_1 resolution of HSQC makes this approach superior when the heteronuclear spectrum is poorly dispersed, although for many routine small-molecule assignments, ^{13}C dispersion is not limiting and either approach suffices. The most notable disadvantage of HSQC over HMQC is the greater number of pulses it utilises, especially 180° pulses on the heteronucleus, promoting intensity losses from rf inhomogeneity, pulse miscalibration or off-resonance excitation. This has greater significance for ^{13}C than ^{15}N owing to the greater frequency spread. Such losses can be minimised by careful probe tuning and, if necessary, the use of composite 180° pulses or still better frequency-swept adiabatic pulses (see discussions in Chapter 10). As with HMQC, suppression of the dominant parent resonance is required if the experiment is to be of general utility, as described below.

6.3.3. Practical implementations

To make the HMQC/HSQC sequences generally applicable, it is desirable, if not essential, to suppress the resonances of those protons that cannot give rise to correlations in a more effective manner than can be achieved with phase cycling alone. There are essentially two schemes for achieving this in widespread use. The more recent and certainly most effective approach is through the incorporation of PFGs into the sequences. The older yet still very effective scheme aims at selectively saturating the parent resonances so that they have negligible intensity when detected. The effectiveness of the principal methods for suppressing the parent signal is compared with the 1D ^1H – ^{13}C HMQC spectra of Fig. 6.8 and discussed further below.

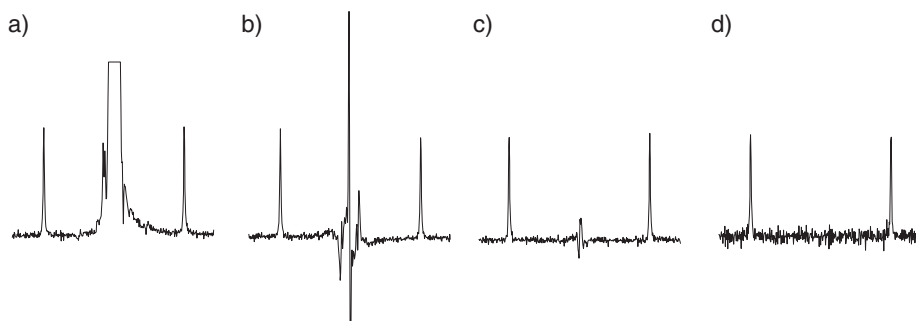


Figure 6.8. A comparison of signal suppression methods used in proton-detected heteronuclear correlation experiments (see descriptions in text). Spectrum (a) is taken from a conventional 1D proton spectrum without suppression of the parent resonance and displays the required ^{13}C satellites. Other spectra are recorded with (b) phase cycling, (c) optimised BIRD presaturation, and (d) pulsed field gradients to remove the parent line. All spectra were recorded under otherwise identical acquisition conditions and result from two transients. Complete suppression can be achieved with gradient selection, but at some cost in sensitivity in this case (see text).

Gradient selection [7]

Pulsed field gradients undoubtedly represent the ultimate approach to signal suppression (Fig. 6.8d). Attenuation ratios in excess of 1000:1 are readily achieved in a single scan, meaning the suppression of the parent ^1H - ^{12}C or ^1H - ^{14}N signals is complete and conveniently implemented. Phase cycling is also not essential because signal selection is achieved solely by gradient refocusing, and in situations where sample quantities are not limiting, these experiments may be performed within a matter of minutes.

The gradient-selected sequence operates by employing a suitable combination of gradients that refocus only those responses that have followed the desired transfer pathway. The scheme of Fig. 6.9a is suitable for the collection of absolute-value data as this selects only one ^{13}C transfer pathway during t_1 (preferably the echo or N-type) and therefore provides f_1 quadrature detection directly.² To understand the signal selection process, consider the action of each gradient in turn, paying due attention to the coherence orders, p (as represented in the coherence transfer pathway) and magnetogyric ratios of the participating spins. The coherence transfer pathway represents only the pathway we wish to preserve, others are not shown since they will not be selected. Assuming that the gradients have the same profile and are of the same duration but differ only in their strengths, G_n , it is straightforward to summarise the phase induced by the gradients using the shorthand notation of Section 5.5. Thus, the first gradient of Fig. 6.9a acts when both proton and carbon have coherence order $p = +1$ (heteronuclear double-quantum coherence), so the effect of the gradient is written as $G_1(\gamma_{\text{H}} + \gamma_{\text{C}})$. For the second gradient, this becomes $G_2(-\gamma_{\text{H}} + \gamma_{\text{C}})$ and for the third $G_3(-\gamma_{\text{H}})$. To preserve this pathway, the overall phase induced by the gradients must be zero, that is

$$G_1(\gamma_{\text{H}} + \gamma_{\text{C}}) + G_2(-\gamma_{\text{H}} + \gamma_{\text{C}}) + G_3(-\gamma_{\text{H}}) = 0 \quad (6.2)$$

Note that only the *ratios* of the γ values become important in heteronuclear experiments, and since $\gamma_{\text{H}}/\gamma_{\text{C}} = 4$, the above expression is simplified to the following form:

$$G_1(5) + G_2(-3) + G_3(-4) = 0 \quad (6.3)$$

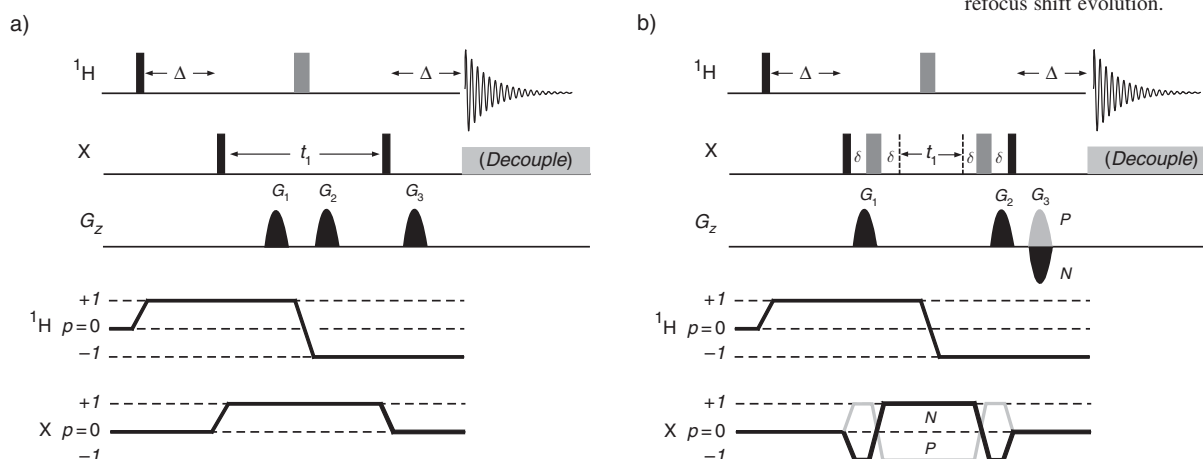


Figure 6.9. Sequences for the gradient-selected HMQC experiment. Sequence (a) is suitable for the collection of absolute-value data. Sequence (b) provides phase-sensitive data *via* the echo-antiecho procedure. The N- and P-type pathways are selected with the last gradient whilst the first two gradients are placed within spin-echoes to refocus shift evolution.

² This retention of only one of the two possible transfer pathways with gradient selection is the reason for the reduced signal-to-noise ratio in Fig. 6.8d (which was acquired with sequence 6.9a with $t_1 = 0$) when compared to the other spectra. The precise details of how gradients are incorporated into pulse sequences will dictate whether such signal losses occur [7]. From a users' point of view, it is sufficient to be aware that such losses can arise with gradient selection and, when sensitivity is critical, to choose those techniques designed to avoid or compensate for these such as the PEP-enhanced sequences.

There are a number of gradient ratios that will satisfy this expression and lead to the desired signal selection, for example, 2:2:1, 5:3:4 or 3:5:0. The optimum choice, in the presence of various experimental imperfections, has been considered in some detail [8], and the authors recommend the use of gradients in the ratio 3:5:0 (i.e. using only two gradient pulses). With the appropriate gradient ratio, it remains to ensure that the gradients are of sufficient strength to achieve the desired suppression. This absolute-value approach demands a magnitude calculation, so it is most suited to the fully automated production of routine spectra when resolution and sensitivity are less critical.

To achieve a phase-sensitive presentation, it is necessary to retain both N and P ^{13}C pathways during t_1 . This, however, is fundamentally impossible in a single scan if gradients are applied during the t_1 period since no gradient combination is able to *simultaneously* refocus signals from both +1 and -1 pathways. For example, the net effect of the 2:2:1 gradient combination on the ^{13}C P-type pathway (coherence order -1 during t_1) will be

$$G_1(\gamma_{\text{H}} - \gamma_{\text{C}}) + G_2(-\gamma_{\text{H}} - \gamma_{\text{C}}) + G_3(-\gamma_{\text{H}}) = (2 \times 3) + (2 \times -5) + (1 \times -4) = -8 \quad (6.4)$$

and the signal will remain dephased. There are two basic approaches to overcoming this limitation; either to avoid the use of gradients during t_1 altogether [9, 10], thus allowing the use of the conventional States or TPPI methods of quadrature detection, or to collect the N- and P-type signals on alternate scans and combine them via the echo-antiecho method of processing [11–13]. Figure 6.9b shows the scheme based on the echo-antiecho method. To obtain pure-phase spectra, it is necessary to refocus carbon-13 chemical shift evolution that occurs during the gradient pulses, so additional spin-echoes bracket t_1 , otherwise the sequence resembles that of Fig. 6.9a. The two different pathways are collected by inverting the sign of the last gradient so, for example, the N-type is refocused with 2:2:-1 and the P-type with 2:2:1 (note that this selection is opposite to that of Fig. 6.9a because of the use of additional 180° ^{13}C pulses).

A logic similar to that above applies to gradient selection in the HSQC experiment, for which various different approaches are also possible [7]. A suitable sequence employing the echo-antiecho approach is illustrated in Fig. 6.10 and requires only two gradients in proportion to the magnetogyric ratios of the X and ^1H spins since each acts on single-quantum X and ^1H magnetisation. Thus, for a ^1H - ^{13}C correlation experiment, ratios of 4:1 and 4:-1 will select the N- and P-type coherences on alternate scans. Once again, the first gradient is applied within a spin-echo to refocus X spin chemical shift evolution during the gradient pulse, whereas the second is applied during the usual INEPT refocusing period. The experiment can be summarised using the following reduced notation:

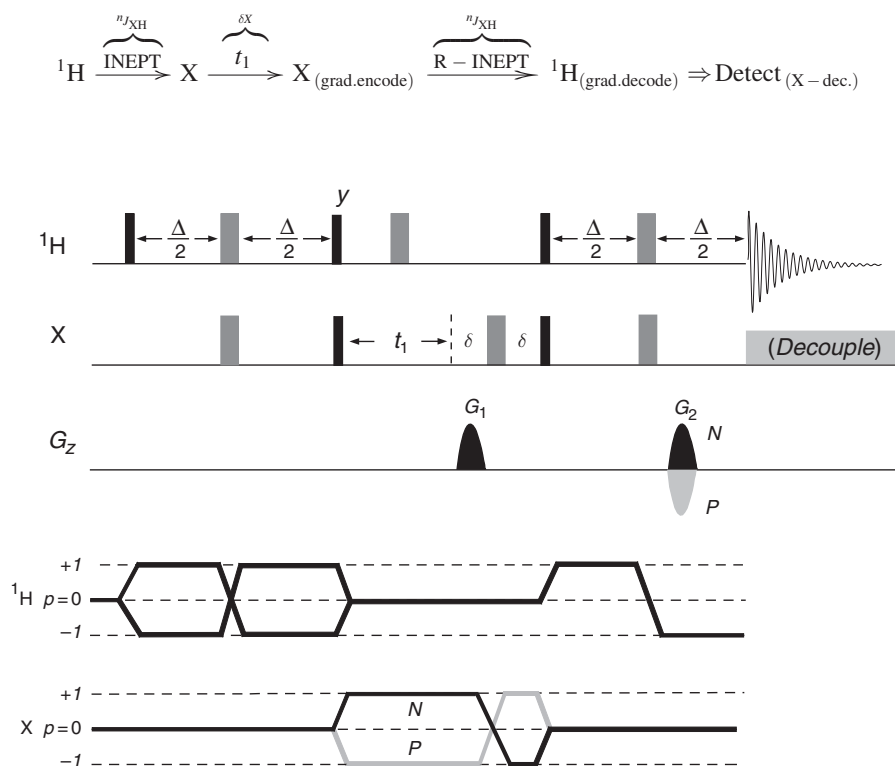


Figure 6.10. A gradient-selected, phase-sensitive HSQC sequence using the echo-antiecho approach. The N- and P-type pathways are selected by the last gradient.

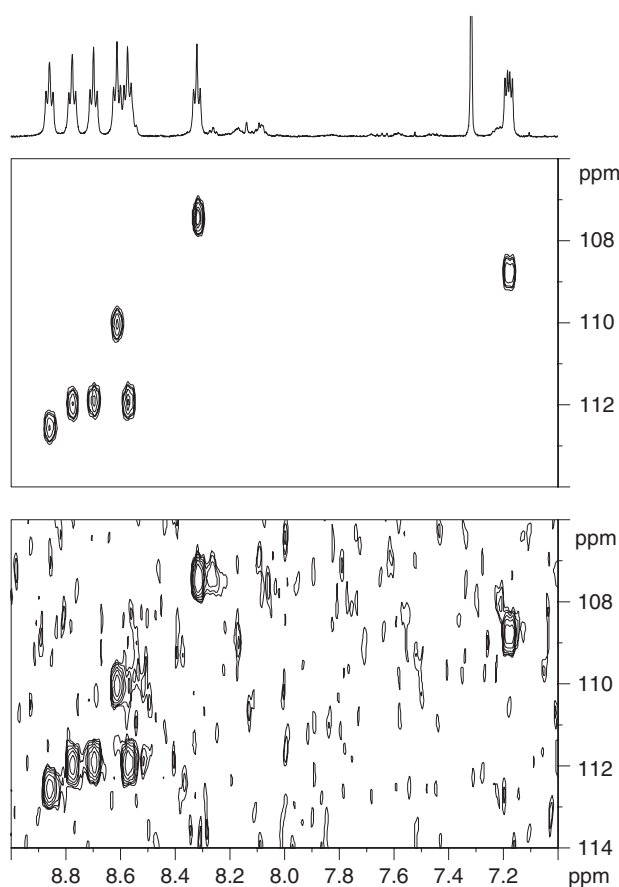
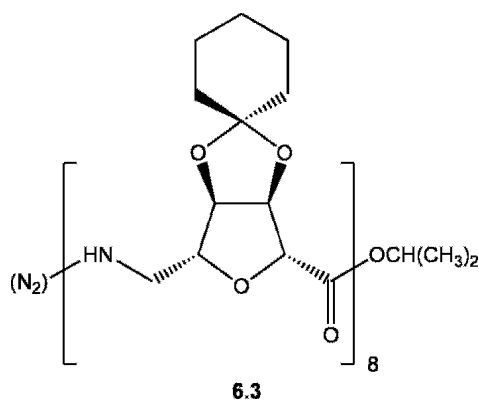


Figure 6.11. A gradient-selected HSQC spectrum of the carbopeptoid **6.3** at natural ^{15}N abundance plotted at high and at low contour levels to show the thermal noise floor. No t_1 noise artefacts remain from the parent ^1H – ^{14}N resonances (^{15}N is referenced to external liquid ammonia).

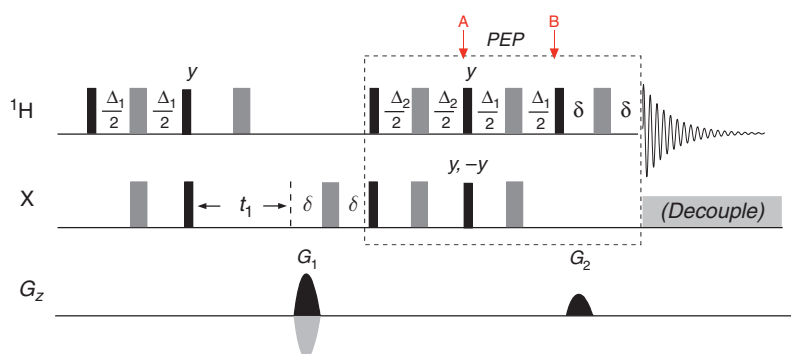
The ability to completely suppress the parent ^1H – ^{12}C or ^1H – ^{14}N resonances produces spectra that are largely devoid of the bands of t_1 noise that may otherwise plague the experiment. This is illustrated in Fig. 6.11, which shows a section of the gradient-selected ^1H – ^{15}N HSQC spectrum of the carbopeptoid **6.3** recorded with ^{15}N decoupling, plotted conventionally and with the contour levels reduced to show the baseline thermal noise.



Sensitivity improvement: PEP

The signal losses associated with gradient selection that arise from the selective refocusing of a single N or P pathway can be ameliorated to some extent by the use of the sensitivity improvement scheme now known as the PEP (Preservation of Equivalent Pathways) [14–16]. This can yield sensitivity gains up to a factor of 2 relative to the standard sequences by recovering one of the magnetisation components that is usually lost in conventional HSQC methods and is applicable to both non-gradient and gradient-selected experiments. In the HSQC experiment, heteronuclear single-quantum magnetisation that exists at the end of the t_1 period may be decomposed into two orthogonal components that lie along the x and y axes. The subsequent $90^\circ(\text{H}, \text{X})$ pulses are able to transfer only one of these back into single-quantum proton magnetisation, whilst the other gives rise to ^1H – X multiple-quantum coherence that remains unobservable, meaning only one-half of the

Figure 6.12. The PEP-HSQC sequence for sensitivity improvement; see text for discussions.



initial proton magnetisation contributes to the observed signal. The PEP method extends the HSQC sequence (Fig. 6.12) so as to reconvert the unobservable component into observable single-quantum coherence for simultaneous detection with its orthogonal partner. Thus, at point A in the sequence of Fig. 6.12 (the point at which detection would begin in the standard HSQC experiment), the refocused ^1H single-quantum magnetisation produced by the reverse INEPT step is placed along the z axis by the $90^\circ(^1\text{H})$ pulse for storage. Subsequently, the multiple-quantum signals are transferred into single-quantum proton magnetisation by the simultaneous $90^\circ(\text{X})$ pulse, effectively initiating a second reverse INEPT transfer. Within this, the anti-phase magnetisation so generated undergoes refocusing of the heteronuclear $^1J_{\text{XH}}$ coupling (as ultimately required for detection in the presence of X spin decoupling) at which point the component held in storage along the z axis is again placed into the transverse plane by the final $90^\circ(^1\text{H})$ pulse (point B). Subsequent gradient refocusing then allows detection of *both* components of proton magnetisation. Since these two components are again orthogonal, they would produce phase-twisted lineshapes in the resulting spectrum, so it becomes necessary to record two data sets per t_1 increment in which the phase of one of the $90^\circ(\text{X})$ pulses after t_1 is inverted and the data sets stored separately (this may also be combined with gradient inversion according to the echo–antiecho protocol for f_1 quadrature detection). This inverts the phase of one of the two orthogonal components such that appropriate addition and subtraction of the data sets then separates these components and allows for quadrature detection with absorption-mode lineshapes.

The maximum theoretical gain in signal intensity provided by the PEP extension when using the echo–antiecho scheme is a factor of 2 although experimental gains are likely to be less than this due to losses through relaxation and pulse imperfections arising from the PEP extension. Furthermore, the whole process operates to give retention of both pathways most efficiently only in the case of XH pairs but to a lesser extent when the heteroatom is bound to more than one proton. In situations when only XH pairs are to be observed, the INEPT delays should be optimised for $\Delta_1 = \Delta_2 = 1/2J_{\text{XH}}$, whereas for the detection of XH_2 or XH_3 as well, the Δ_2 period should be best reduced to $1/4J_{\text{XH}}$ [16]. In this case, the theoretical enhancement factors become 1.7 (XH), 1.4 (XH_2) and 1.2 (XH_3). Figure 6.13 compares the signal intensities for a disaccharide anomeric CH resonance observed for HSQC with and without the PEP extension and shows a 1.4-fold sensitivity gain using the PEP methodology when selecting for all multiplicities.

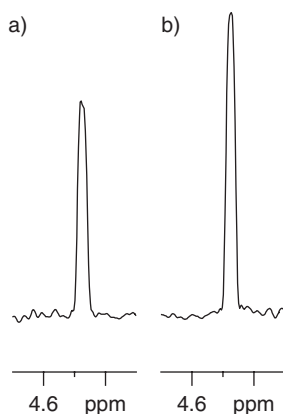


Figure 6.13. Traces through an anomeric CH resonance of a disaccharide taken from (a) HSQC and (b) PEP-HSQC. Both spectra were collected with pulsed field gradients with delays $\Delta_1 = 1/2J_{\text{CH}}$ and $\Delta_2 = 1/4J_{\text{CH}}$ optimised for $^1J_{\text{CH}} = 145 \text{ Hz}$. Trace (b) demonstrates a 40% signal increase.

BIRD-HMQC

Not every NMR spectrometer or probe head is equipped with pulsed field gradients capabilities, yet it may still be possible to execute the basic heteronuclear shift correlation experiments described above. In such cases, it is desirable to employ an alternative scheme to remove the interfering parent resonances and produce spectra devoid of objectionable artefacts. In the bilinear rotation decoupling (BIRD) variant (Fig. 6.14), unwanted ^1H – ^{12}C resonances are suppressed prior to the HMQC sequence by an ingenious presaturation scheme. This commences with the inversion of *only the carbon-12-bound protons* by the so-called ‘BIRD pulse’ (the action of which is described below), leaving the carbon-13-bound protons unaffected. Following the inversion, a recovery period, τ , allows the magnetisation vectors to relax back towards the equilibrium $+z$ axis, until they pass through the x – y plane (Fig. 6.15). At this point, the HMQC sequence itself begins, and because there exists no longitudinal ^1H – ^{12}C magnetisation, no transverse component is ever generated for these spins and hence the desired suppression of the resonances is achieved. During detection and the next inversion recovery period, the ^1H – ^{13}C magnetisation simply relaxes back towards its equilibrium value in readiness for the sequence to begin once more since it is not inverted by the BIRD pulse.

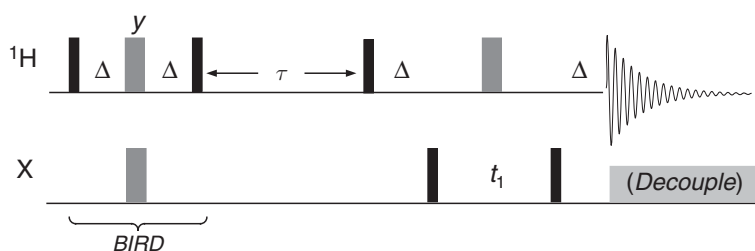


Figure 6.14. The BIRD variant of the HMQC experiment. The conventional HMQC sequence is employed but is preceded by the BIRD inversion element and an inversion recovery delay τ . This procedure ultimately leads to saturation of the unwanted parent resonances.

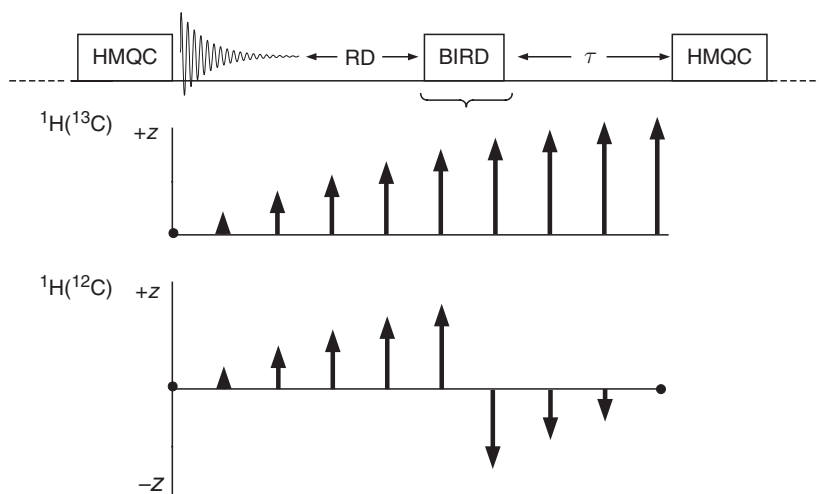


Figure 6.15. Elimination of parent ^1H - ^{12}C proton resonances through the BIRD inversion recovery sequence. At the start of data collection, no longitudinal proton magnetisation exists but this reappears during the acquisition period and subsequent recovery delay (RD) through spin relaxation. The BIRD element selectively inverts only those protons attached to carbon-12, which then continues to relax during the inversion recovery delay τ . With an appropriate choice of τ , the ^1H - ^{12}C magnetisation has no longitudinal component when the HMQC sequence starts, so it does not contribute to the detected FID.

The BIRD pulse [17] is in fact a cluster of pulses (Fig. 6.14) used as a tool in NMR to differentiate spins that possess a heteronuclear coupling from those that do not. The effect of the pulse can vary depending on the phases of the pulses within the cluster, so we concentrate here on the selective inversion described above. For illustrative purposes, proton pulse phases of x, y, x will be considered as this provides a clearer picture with the vector model, although equivalent results are achieved with phases $x, x, -x$, as in the original publication. The scheme (Fig. 6.16) begins with a proton excitation pulse followed by a spin-echo. Since carbon-12-bound protons have no one-bond heteronuclear coupling, only their chemical shifts evolve during Δ . These are subsequently refocused by the $180^\circ(^1\text{H})$ pulse so that at the end of the second Δ period, the second $90^\circ(^1\text{H})$ pulse places the vector along the $-z$ axis and produces the desired inversion of the ^1H - ^{12}C resonances. For those spins that possess a heteronuclear coupling, chemical shifts will refocus as above, so we need to consider only the effect of the coupling itself. If Δ is set to $1/2J_{\text{CH}}$, the two proton vectors will lie along $\pm x$ immediately prior to the $180^\circ(^1\text{H}, ^{13}\text{C})$ pulses, after which the coupling will *continue* to evolve. Thus, after a total period 2Δ , the doublet vectors lie along $-y$ and are then returned to $+z$ by the action of the final proton pulse, and for these spins it is as if the BIRD has never appeared.

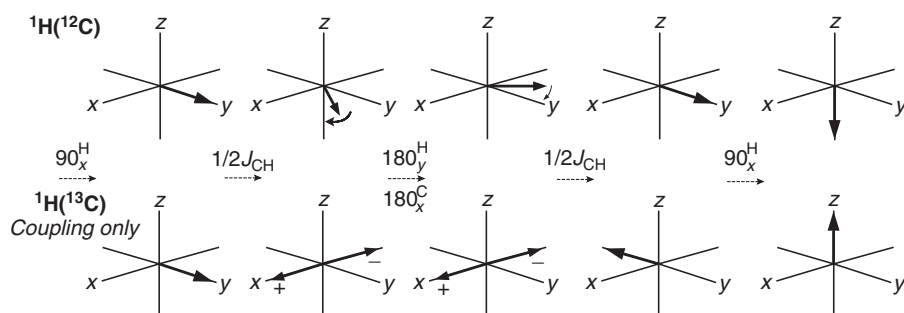


Figure 6.16. Selective inversion of carbon-12-bound protons with the BIRD element. Proton chemical shift evolution is refocused in the sequence but heteronuclear one-bond couplings evolve throughout.

The success of the BIRD suppression scheme depends crucially on the correct setting of recovery delays and repetition rates, according to the proton T_1 values of the molecule. In practice, one is unlikely to know precisely what these are, and in any case there will exist a spread of these within the molecule, so a best-guess compromise must be made. It turns out [3] that optimum sensitivity is achieved by setting timings according to the *shortest* T_1 value in the molecule and once again setting the recycle time of the experiment, that is, the time between the start of data acquisition of one experiment and the beginning of the HMQC sequence of the next ($t_2 + \text{RD} + \tau$ of Fig. 6.15), to around $1.3T_1$. The inversion recovery period, τ , must be set to approximately $0.5T_1$ for efficient suppression and the proton acquisition time, t_2 , set according to the desired digital resolution. The relaxation delay, RD, is then chosen to make up the desired recycle time ($t_2 + \text{RD} \approx 0.8T_1$). For example, assuming that the smallest anticipated T_1 in a molecule to be 600 ms and that t_2 is set to 200 ms, (corresponding to an f_2 digital resolution of 5 Hz/pt), the above guidelines suggests one selects $\tau = 300$ ms and $\text{RD} = 280$ ms.³ Since one is generally forced to make a best guess at the T_1 values, the experimental settings are fine tuned to give optimum suppression of the unwanted resonances. This is most conveniently achieved by running the experiment in an interactive set-up mode that allows real-time adjustment of parameters and altering τ to produce the *minimum* FID.

The BIRD scheme is remarkably efficient at suppressing the troublesome ^1H - ^{12}C resonances and associated t_1 noise and has been widely employed in the study of small molecules. However, this method is not suitable for the study of very large molecules because during the τ period, the *negative* NOE (Chapter 8) generated from the inverted protons causes a *reduction* in the signal intensity of the observed protons and therefore compromises sensitivity. The traditional approach for large molecules in the absence of field gradients [18] has been to apply a strong spin-lock period during the first INEPT sequence of the HSQC experiment. This destroys all magnetisation not aligned with the spin-lock axis and suppresses the unwanted parent signals.

Practical set-up

The high crosspeak dispersion typically associated with heteronuclear shift correlation experiments alongside the lack of any requirement for well-defined crosspeak fine structure means that HMQC or HSQC experiments can be recorded with rather low digital resolution for routine applications, enhancing their time efficiency. An acquired digital resolution of 5 Hz/pt in the proton f_2 dimension and only 50 Hz/pt in the heteronucleus f_1 dimension are generally sufficient to resolve correlations. Improved f_1 resolution can be achieved by linear prediction of the FIDs when sensitivity allows, and/or the digital resolution enhanced by zero filling (usually at least once). Alternatively, the use of linear prediction can lead to significant time savings when large sample quantities are available, by reducing the number of t_1 increments that must be recorded and computationally regenerating those missing t_1 data points to provide adequate f_1 resolution [19, 20]. A more exotic route to high resolution in the f_1 dimension is to employ multiple aliasing of resonances in this dimension through the digitisation of a very narrow f_1 bandwidth. To regain the traditional unaliased display, computational manipulation is necessary, which yields a high-resolution spectrum [21–23]; the software required for this has yet to become widely available, and the careful use of the more widely available linear prediction has been proposed as an effective alternative [20]. It must be remembered, however, that very high resolution in f_1 is not generally desirable for the HMQC experiment since one should avoid resolving multiplet structure arising from the homonuclear proton couplings and thus degrading the signal-to-noise ratio. Beyond this, processing requires only simple apodisation in both dimensions and either shifted squared sine-bells or Gaussian windows function well for phase-sensitive data sets.

Proton-carbon correlation experiments based on the methods described above can be surprisingly fast to acquire for routine organic samples, especially when pulsed field gradients and probes optimised for proton detection are employed. Repetition rates are dictated by the shorter relaxation times of the ^{13}C -bound protons and can therefore be faster than homonuclear COSY spectra, for example, where it is the ^{12}C -bound protons that are monitored. They are usually quicker to obtain and often more informative than the 1D carbon spectrum, often accessible in a matter of minutes, and are now routine experiments in the organic laboratory. Combining these methods with spectrum-editing techniques as described below further extends their utility as structural tools and may increasingly relegate direct X-observe 1D experiments such as APT and DEPT to the periphery of common structural elucidation methods.

³ Such rapid pulsing may seem excessively fast for the resonances one wishes to observe, especially for those protons with longer relaxation times. However, one should note that the proton T_1 s generally measured relate to carbon-12-bound protons, whereas in HMQC, one is interested in carbon-13-bound protons. This nucleus acts as an additional dipolar relaxation source for the directly attached proton leading to shortening of the proton T_1 value, consistent with the rapid repetition.

6.3.4. Hybrid experiments

As was alluded to earlier, shift correlation spectra may be further enhanced by combining them with other pulse techniques to produce spectra with altered characteristics or increased information content. This section is necessarily brief and is intended to provide an oversight of the types of techniques that have been developed and that are likely to play an increasingly prominent role in organic structure elucidation. The first two sections below represent two of the more useful methods for structural elucidation and also illustrate the manner in which different techniques may be concatenated to produce ‘new’ experiments. The third illustrates how the heteronuclear spin may be used in the editing or simplification of 1D proton spectra.

Two-dimensional multiplicity editing

Short of the ability to identify diastereotopic XH_2 groups, the heteronuclear shift correlation experiments so far encountered provide no direct evidence for the multiplicities of the XH_n groups giving rise to each correlation, analogous to the way in which broadband-decoupled heteronuclear spectra provide no multiplicity information. Various methods are now available to provide such editing *within* the 2D heteronuclear correlation experiment itself, thus killing two birds with one stone, if you like. These provide more information on routine analysis and require less time than conventional 1D editing methods. Furthermore, such experiments provide access to heteronuclear multiplicity information when small sample quantities preclude the direct observation of X spin-edited spectra, thus further extending the range of sample quantities one can consider accessible to structural studies.

Incorporating the DEPT sequence of Chapter 4 within HMQC [24] (Fig. 6.17) offers one method for directly editing the correlation spectrum. The role of the DEPT sequence prior to the HMQC is to generate the heteronuclear multiple-quantum coherence that evolves during t_1 . A full analysis [24, 25] shows that different multiple-quantum terms are used in this sequence than in 1D DEPT, the net result being that the crosspeak intensities have a different dependence on the θ proton-editing pulse. The most useful implementation uses $\theta = 180^\circ$ to produce spectra with XH_2 correlations inverted relative to those of XH and XH_3 groups, equivalent to the conventional DEPT-135 1D sequence (a phase-sensitive presentation must be used!). Editing may be readily introduced to the HSQC sequence [10, 26, 27] by the simple addition of a spin-echo ($\Delta - 180^\circ(^1\text{H}, \text{X}) - \Delta$) after t_1 . During this, only the heteronuclear coupling evolves, exactly as in the pulsed 1D J -modulated spin-echo sequence described in Section 4.3.1. Hence, setting $\Delta = 1/2J$ also provides a 2D correlation spectrum in which XH_2 responses are inverted relative to those of XH and XH_3 . In one example of this approach, concatenating this echo with the existing t_1 period produces the modified HSQC scheme of Fig. 6.18 [7, 28]. The edited HSQC spectrum of the substituted disaccharide **6.4** is shown in Fig. 6.19 and clearly differentiates CH from CH_2 correlations.

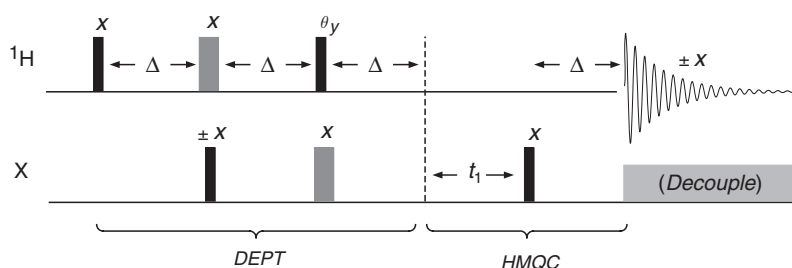


Figure 6.17. The DEPT-HMQC sequence for multiplicity editing within the 2D correlation experiment. The conventional DEPT sequence precedes HMQC and provides editing through the proton θ tip angle. Setting $\theta = 180^\circ$ inverts XH_2 responses relative to those of XH and XH_3 .

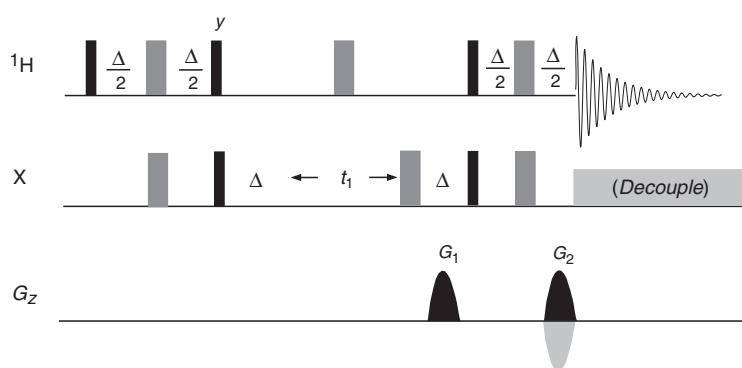


Figure 6.18. The gradient-selected, spin-echo HSQC sequence for multiplicity editing within the 2D correlation experiment. Setting $\Delta = 1/2J$ inverts XH_2 responses relative to those of XH and XH_3 .

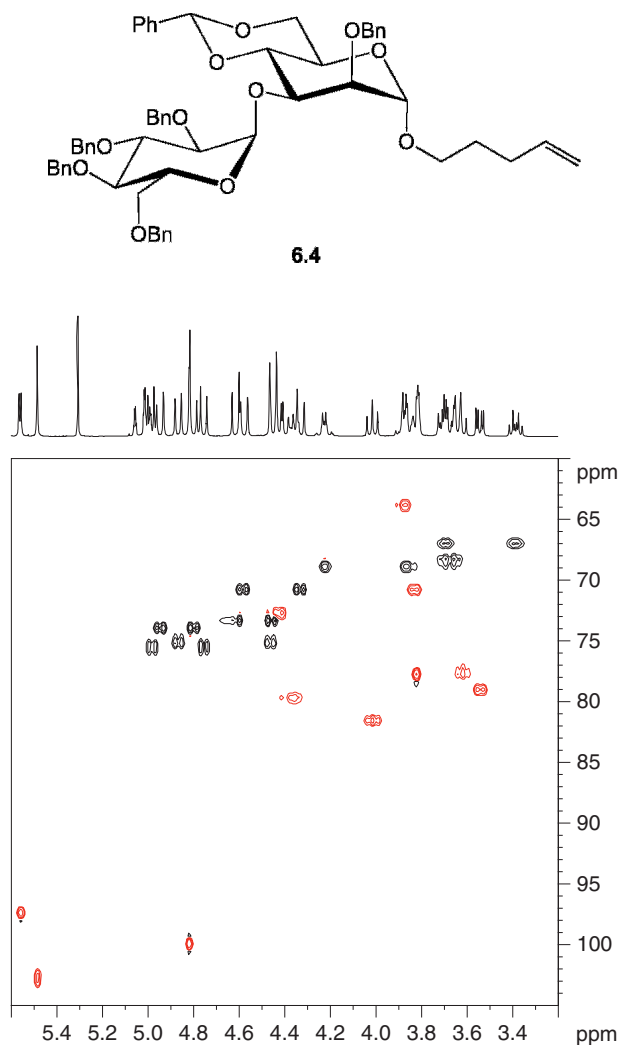
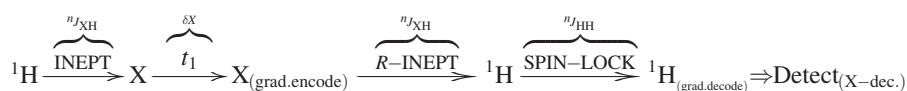


Figure 6.19. The multiplicity-edited HSQC spectrum of the disaccharide **6.4** in which positive CH correlations (red) are distinguished from negative CH₂ correlations (black).

A potential problem with these editing approaches is the cancellation of overlapping correlations with opposite phases in crowded regions of 2D spectra. Alternative sequences based on editing through multiple-quantum filtration produce spectra containing correlations from *only* XH or XH₂ or XH₃ groups, so the problem of signal cancellation is removed. These methods are too numerous to discuss here but include REPAY [29], HYSEL [30, 31], HmQC [25], MAXY [32] and others [10, 33]. Even more exotic modifications include the addition of spin-lock transfer [34, 35] in a manner similar to that described below. When to turn to such levels of sophistication will be dictated by the nature and complexity of the problem in hand, but the efficiency of such editing schemes with the advent of pulsed field gradients makes these worthy of consideration for the investigation of complex structures.

Utilising *X* spin shift dispersion

When analysing molecules that display very crowded proton spectra, the 2D homonuclear shift correlation experiments discussed in Chapter 5 may still provide spectra that are too overlapped to allow a complete interpretation. In such instances, the potentially greater dispersion of the parent heteroatom chemical shifts can be used as an additional means for separating proton–proton correlations. Furthermore, in cases of exactly overlapping *proton* resonances, the one-bond heteronuclear correlation experiments do not provide unambiguous identification of the parent heteroatom, and one approach to overcoming such problems lies in the transfer of the heteronuclear correlation information onto neighbouring protons. Thus, adding a TOCSY spin-lock mixing period after the HMQC/HSQC sequence and immediately prior to data collection [36, 37] transfers magnetisation that has returned to the proton from which it originated onto neighbouring *J*-coupled protons (Fig. 6.20). The process may again be summarised for HSQC-TOCSY in shorthand notation as



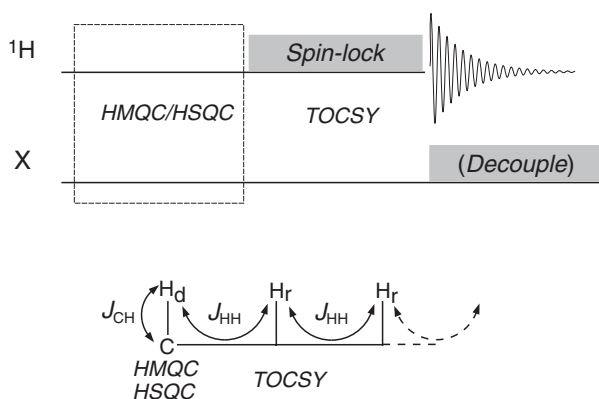


Figure 6.20. The schematic HMQC/HSQC-TOCSY sequence and the coupling pathway it maps. Direct correlations are produced for the proton (H_d) bound to the X-heteroatom as in the basic shift correlation sequence, and further relayed correlations are produced for those protons receiving magnetisation through the TOCSY transfer (H_r).

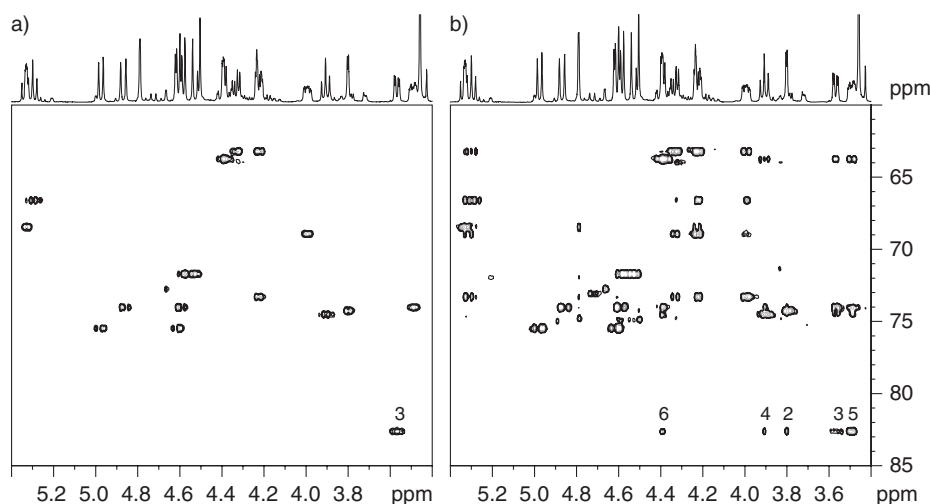
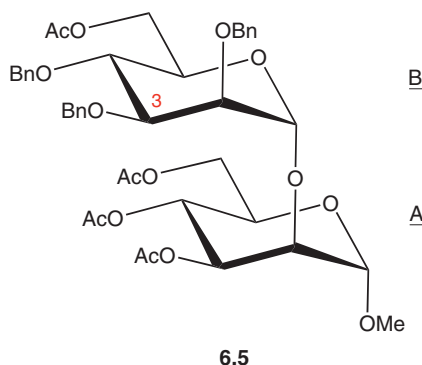


Figure 6.21. Selected regions of the (a) HSQC and (b) HSQC-TOCSY spectra of the disaccharide **6.5**. The proton correlations originating from H_3 of ring B are labelled and provide an almost complete map of protons within this ring (only the correlation to H_1 is missing).

Extended mixing periods can again be used to *relay* magnetisation along the proton network, potentially providing a complete proton sub-spectrum of the molecular fragment to which the heteroatom belongs. The result may be viewed as a X spin-edited 2D TOCSY experiment, the proton correlations now appearing along rows taken at the hetero-spin f_1 chemical shifts. With sufficient shift dispersion in the X spin dimension, overlap present in the 2D TOCSY is removed. The HSQC-TOCSY spectrum of the disaccharide **6.5** (Fig. 6.21b) contains numerous additional correlations over the HSQC spectrum (Fig. 6.21a) and can be used to map the proton coupling pathways. This is most clearly seen at the C3 shift of ring B, from which the H_3 proton produces TOCSY correlations to all other protons in the ring, with the exception of H_1 (which in this case had zero coupling with H_2 , thus causing a breakdown in transfer).



The enormous simplification of crowded spectra in this manner makes this a very powerful technique. However, a point to bear in mind when considering applying these methods is that crosspeaks in the ^{13}C -edited experiment originate only from ^{13}C satellites because of the initial HMQC/HSQC step, and the experiment is therefore of significantly lower sensitivity than homonuclear 2D TOCSY, in which all protons may participate. The experiment will generally find use after initial stages of investigation of complex spectra

using the techniques already presented where ambiguities remain. The use of long-range heteronuclear correlations described shortly should also be considered in such cases.

Further modifications (the addition of a spin-echo) allow the direct and relayed peaks to be differentiated through inversion of the direct correlations [38], and gradient-selected versions of these sequences without [39, 40] and with [10, 41] such editing have also been proposed. Although the addition of TOCSY transfer is probably the most useful extension, any homonuclear mixing scheme can, in principle, be added, including a COSY, NOESY (NOE spectroscopy) or ROESY (ROE spectroscopy) step.

Editing and filtering one-dimensional proton spectra

The idea of using the heteroatom to edit the 2D spectrum as described above is equally applicable to the editing of 1D proton spectra. Generally speaking, there are two reasons as to why one may consider doing this. Firstly, one may wish to *edit* the proton spectrum according to the heteroatom multiplicities to produce subspectra that are the proton analogues of, for example, APT- or DEPT-edited carbon spectra. These analogues provide a faster and more sensitive route to identifying carbon multiplicities within a molecule and allow the analysis of smaller sample quantities by virtue of proton detection. Secondly, one may wish to selectively observe only the protons attached to a heteroatom isotope label. In this way, one uses the label to *filter* the proton spectrum, transferring the selectivity associated with the hetero-label onto the more sensitive proton. Although the natural-abundance proton satellites of unlabelled positions will also pass the filter, they will be present at significantly lower intensity.

The HMQC or HSQC sequences may be transformed into their 1D equivalents by simply removing the incremental t_1 time period (Fig. 6.22) so that the experiment becomes just a heteronuclear filter. Only magnetisation that has passed via the X spin will be observed in the final spectrum and again the suppression of all unwanted signals is greatly improved by the use of pulsed field gradients. The selective observation of ^{13}C -labelled glycine in an aqueous mixture is illustrated in Fig. 6.23.

The classical approach to generating multiplicity-edited proton spectra was to use the INEPT or DEPT sequences in reverse to transfer initial carbon magnetisation onto the proton for detection. These suffered from low sensitivity and poor suppression of ^1H - ^{12}C resonances, so they were never popular. Greater sensitivity can be achieved by using the inverse $^1\text{H} \rightarrow ^{13}\text{C} \rightarrow ^1\text{H}$ approach combined with multiple-quantum filtering [24, 30] as for the 2D editing methods discussed above, with methods employing editing via gradient selection providing far cleaner results [25, 31, 32, 42]. A readily implemented method is the 1D variant of the multiplicity-edited HSQC described above, an example of which is shown in Fig. 6.24, which distinguishes methine from methylene groups and also eliminates hydroxyl protons. Relative to the standard 1D proton spectrum, there is a price to pay in

Figure 6.22. The 1D HMQC sequence. This can be used for editing spectra by selecting only those protons bound to NMR-active heteroatoms. Improved suppression of unwanted resonances can be achieved through the incorporation of pulsed field gradients, as for the 2D experiment.

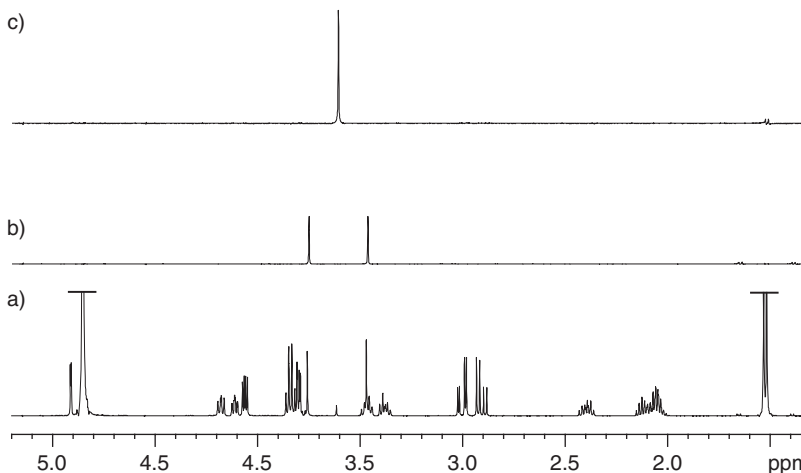
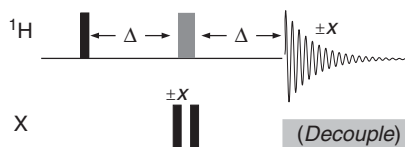


Figure 6.23. The selective observation of protons bound to a carbon-13 label ($2\text{-}^{13}\text{C}$ -glycine) with a gradient-selected 1D HMQC sequence. (a) The 1D proton spectrum and the filtered spectrum recorded (b) without and (c) with carbon-13 decoupling during acquisition.

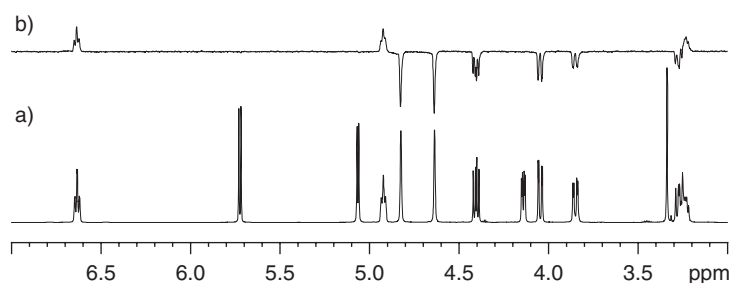


Figure 6.24. Editing of a proton spectrum according to carbon multiplicities. In (b), methylene proton multiplets are inverted and hydroxyl resonances fully suppressed with pulsed field gradients.

sensitivity, because one is again forced to observe the carbon-13 satellites of the conventional proton spectrum. Nevertheless, this editing may prove useful for the rapid characterisation of simple molecules and may provide valuable multiplicity data when small sample quantities preclude the use of carbon-detected 1D editing methods.

6.4. HETERONUCLEAR MULTIPLE-BOND CORRELATION SPECTROSCOPY

All the ^1H -X heteronuclear correlation methods presented so far depend upon the presence of a proton bound to the heteroatom, so they are therefore unable to provide assignments for non-protonated centres and do not produce unambiguous carbon assignments when proton resonances exactly overlap. An alternative approach in such cases is to establish correlations between carbons and neighbouring protons over more than one bond, so-called *long-range* or *multiple-bond* correlations, most commonly through the proton-detected HMBC (Heteronuclear Multiple-Bond Correlation) experiment. In the vast majority of cases, this will involve proton-carbon connectivities through couplings over two or three bonds ($^nJ_{\text{CH}}$, $n=2,3$), since those over greater distances are often vanishingly small (see below). The ability to identify ^1H - ^{13}C correlations across carbon-carbon or carbon-heteroatom linkages presents a wealth of information on the molecular skeleton, providing one of the most powerful approaches to defining an organic structure, perhaps second only to the more difficult to realise direct ^{13}C - ^{13}C correlations provided by the INADEQUATE-based methods of Chapter 5. The HMBC spectrum itself closely resembles that of HMQC in appearance, with the long-range correlations of each proton represented in the column taken at its chemical shift. The abundance of information in such spectra is illustrated in the HMBC spectrum of **6.2** (Fig. 6.25),

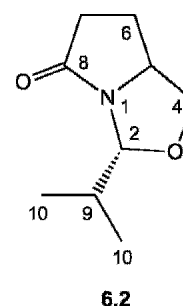
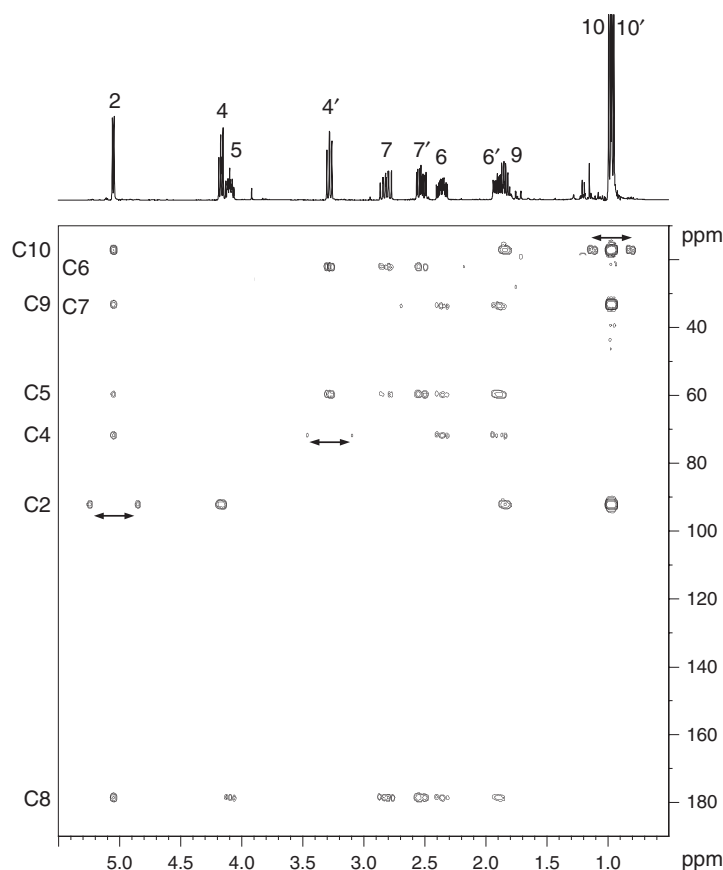


Figure 6.25. The HMBC long-range correlation spectrum of **6.2** recorded with $\Delta_{\text{LR}} = 60$ ms and with gradient selection. The sequence used the most basic first-order low-pass J -filter (Section 6.4.3) to attenuate breakthrough from one-bond correlations (which appear with $^1J_{\text{CH}}$ doublet structure along f_2 (arrowed)). 1 K data points were collected for $256t_1$ increments of eight transients each and the data processed with unshifted sine bells in both dimensions, followed by magnitude calculation. After zero filling once in t_1 , the digital resolution was 4 and 80 Hz/pt in f_2 and f_1 , respectively.

Table 6.3. A summary of the long-range correlations observed in the HMBC spectrum ($\Delta_{LR} = 60$ ms) of **6.2**

Proton	Correlated carbon
2	4, 5, 8, 9, 10
4	2, 5(w), 6(w)
4'	2(w), 5, 6
5	4(w), 7(w), 8
6	4, 5, 7, 8
6'	4, 5, 7, 8
7	5, 6, 8
7'	5, 6, 8
9	2, 10
10	2, 9, 10'
10'	2, 9, 10

Weaker correlations, corresponding to smaller coupling constants, are identified with (w). Not all these are observed in Fig. 6.25 at the contour levels shown.

which should be compared with the HMQC in Fig. 6.4. The complete set of correlations observed in this spectrum is summarised in Table 6.3, but the salient features of the experiment may be appreciated by considering the correlations of proton H2 alone. Firstly, note the breakthrough of the one-bond correlation appearing as the arrowed doublet at 93 ppm, the presence of which is explained in Section 6.4.1. This is equivalent to the correlation seen in Fig. 6.4b and here serves as a reference point, although these are most often an unwelcome complicating factor and attempts are usually made to remove or filter these from HMBC spectra. Nevertheless, the possibility of such peaks appearing should always be borne in mind when interpreting these spectra. Secondly, note that the correlations to C4, C5 and C8 arise from couplings across heteroatoms (N and O), a feature that sometimes seems to surprise those new to the experiment. This type of data can be particularly valuable when proton–proton couplings are absent. Finally, note that H2 shows a correlation to the carbonyl carbon C8, this centre being unobservable in the one-bond correlation experiments. The ability to observe non-protonated centres in this way not only allows their chemical shifts to be determined but also provides valuable connectivity data. Nowadays, the combination of COSY, HMQC/HSQC and then HMBC typically represents the primary techniques to turn to when addressing problems of molecular connectivity in small organic molecules. As for the one-bond correlation methods described above, various extensions to the basic HMBC experiment also exist to enhance or modify information content, and the most significant of these are also presented in the sections that follow.

6.4.1. The HMBC sequence

The HMBC experiment [43, 44] establishes multiple-bond correlations by again taking advantage of the greater sensitivity associated with proton detection, and in essence, the HMQC sequence is ‘tuned’ to detect correlations via small couplings (Fig. 6.26a). Owing to the close similarity of the two, only differences pertinent to HMBC will be presented here, with the application of the experiment being considered in the following section.

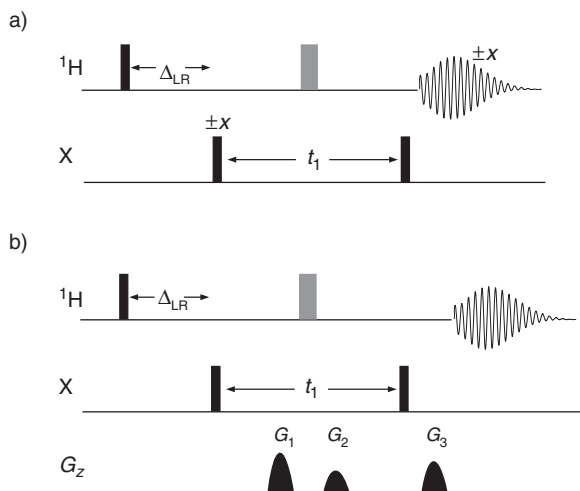


Figure 6.26. The HMBC sequence (a) without and (b) with incorporation of pulsed field gradients. The sequence is closely related to the HMQC experiment and follows a similar coherence transfer pathway but has the coupling evolution period Δ_{LR} optimised for long-range coupling constants.

The tuning of the experiment is achieved by setting the Δ_{LR} preparation period to a sufficiently long time to allow the small, long-range proton–carbon couplings to evolve to produce the anti-phase displacement of vectors required for the subsequent generation of heteronuclear multiple-quantum coherence. Since long-range ^1H – ^{13}C couplings are at least an order of magnitude smaller than one-bond couplings (often $<5\text{ Hz}$), Δ_{LR} should, in principle, be at least 100 ms ($1/2''J_{\text{CH}}$), although shorter delays are often used routinely to avoid relaxation losses. During this long Δ_{LR} period, homonuclear ^1H – ^1H couplings, which are of similar magnitude to the long-range heteronuclear couplings, also evolve and introduce phase distortions to the observed crosspeaks (these distortions are small enough to be ignored in HMQC because of the much smaller Δ periods used). Absolute-value presentations are therefore widely used for HMBC spectra to mask these phase errors, and it has therefore been commonplace to acquire HMBC data sets that are phase-modulated (N-type selection) as a function of t_1 . For sensitivity reasons, the refocusing Δ period of HMQC is omitted in HMBC so that long-range heteronuclear couplings are anti-phase at the start of t_2 , precluding the application of ^{13}C -decoupling. Furthermore, it is generally the case that the use of decoupling in *refocused* HMBC spectra provides no significant benefit and can result in signal reduction from incomplete refocusing of long-range couplings, so its use is not generally recommended.

Phase-sensitive HMBC

Although traditionally acquired as phase-modulated data, the echo–antiecho procedure described in Chapter 5 can be applied to HMBC [45] to produce spectra that are phase-sensitive in f_1 (Fig. 6.27). This approach can yield higher f_1 resolution by producing absorption-mode lineshapes, thus avoiding the need for harsh apodisation associated with the magnitude calculation [46]. It has also been shown to provide a $\sqrt{2}$ sensitivity gain relative to the phase-modulated experiment, so it is gaining in popularity and finds use in other HMBC variants. Due to the appearance of anti-phase heteronuclear coupling and complex proton phase behaviour in f_2 , it remains advantageous to apply magnitude calculation to this dimension for the routine analysis of HMBC correlations. Alternatively, the retention of these anti-phase peaks may enable the measurement of the long-range heteronuclear coupling constants themselves from the f_2 traces; see Section 6.4.4.

Suppressing parent resonances

Without any doubt, the greatest problem associated with the HMBC sequence lies in the suppression of the parent ^1H – ^{12}C signals that may otherwise mask the long-range satellites. Unlike HMQC, the BIRD sequence is not well suited to the removal of these resonances since this is also likely to lead to attenuation of the desired signals. Traditionally, the sequence has relied on phase cycling alone to cancel the intense parent resonance, requiring a very stable spectrometer to be effective. Even so, bands of residual t_1 noise routinely plagued the original HMBC experiment and limited its use as a routine tool in organic chemistry. The introduction of pulsed field gradients [10, 47] has revolutionised the applicability of HMBC, since complete suppression of the parent line is readily achieved, along with a dramatic reduction in the associated t_1 noise. The benefits of the gradient-selected approach are clearly demonstrated in the spectra of Fig. 6.28. Spectrum (a) was recorded with gradient selection and spectrum (b) with conventional phase cycling under otherwise identical conditions (other than a 256-fold increase in receiver amplification in (a) over that in (b)). Four transients were collected per increment, corresponding to the minimum phase cycle (two steps for signal selection and two for suppression of axial peaks). Long-range correlations are clearly identified in (a) but can barely be observed above the t_1 noise bands in (b). Improved results for the phase-cycled version can be achieved by collecting far more transients at the expense of instrument time.

Long-range correlations based on the HSQC sequence are generally less effective. Significant evolution of ^1H – ^1H couplings during the Δ period leads to unwanted COSY-

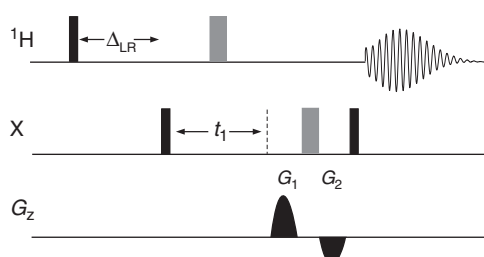
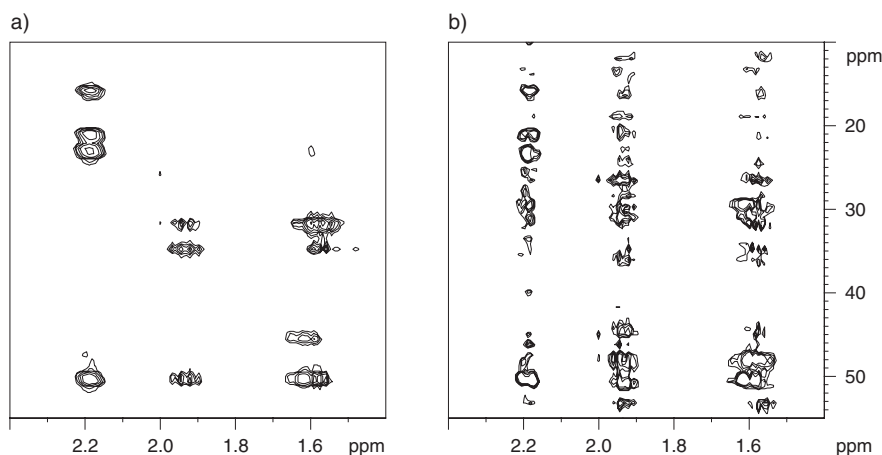


Figure 6.27. The phase-sensitive HMBC experiment with echo–antiecho selection. The gradient ratios G_1 : G_2 are 5:–3 and 3:–5 for odd and even experiments, respectively, when selecting carbon-13.

Figure 6.28. HMBC spectra recorded under identical conditions but with signal selection through (a) pulsed field gradients and (b) phase cycling alone.



type transfers among protons by the second 90° proton pulse of the INEPT sequence, a problem not found with HMBC.

6.4.2. Applying HMBC

The presence of long-range correlations in HMBC spectra is influenced by many factors, both experimental and structural, and an awareness of these points is important for the optimum application and interpretation of the experiment, so they are addressed here before preceding to some illustrative applications. Crosspeak intensities depend upon, among other things, both the magnitude of the long-range coupling and the value selected for Δ_{LR} , which should optimally be set to $1/2^n J_{CH}$ (crosspeak intensity $\propto \sin \pi^n J_{CH} \Delta_{LR}$). Long-range proton–carbon couplings over two or three bonds rarely exceed 25 Hz and in the absence of unsaturation are more often less than 5 Hz [48, 49] (Table 6.4), indicating that Δ_{LR} should be 100 ms or more. Such long delays can lead to relaxation losses prior to detection, especially for larger molecules; so in practice, a compromise is met with Δ_{LR} being set to around 60 ms for routine applications, that is, optimised for ~ 8 Hz coupling. Since small organic molecules in low-viscosity solvents tend to have slower relaxation rates, longer delays can be used successfully in the search for more connectivities through smaller couplings, with Δ_{LR} taking values of up to 200 ms, whereas the faster relaxation associated with large molecules benefits from reduced Δ_{LR} periods perhaps as small as 40 ms. The potentially wide range of $^n J_{CH}$ values present in a molecule can mean correlations are not observed in HMBC when Δ_{LR} is poorly matched to the optimum delays, thus presenting an incomplete set of correlations; more sophisticated HMBC variants aimed at increasing the number of observed correlations are described in Section 6.4.3. When using longer delays, it also becomes possible to detect peaks that arise from four-bond correlations, which are most likely to occur when the coupling pathway contains unsaturation or when it has the planar zigzag (*w*-coupling) configuration, as commonly observed in long-range proton–proton couplings also. One should also recognise that three-bond couplings can be, and often are, greater in magnitude than two-bond couplings, displaying a Karplus-type relationship with the dihedral angle [49–51]. Indeed, one further limitation when using HMBC data is the lack of differentiation between two- and three-bond connectivities, a topic also addressed in Section 6.4.3. Furthermore, the lack of a correlation cannot on its own be taken as evidence for the nuclei in question being distant in the structure, since various factors can contribute to $^n J_{CH}$ being close to zero (for more details, see discussions in reference [48]). The most intense correlations are typically observed for methyl groups since magnetisation is detected on three protons simultaneously and because they display simpler coupling

Table 6.4. Typical values of long-range proton–carbon coupling constants

Coupling pathway	$^2 J_{CH}$	Coupling pathway	$^3 J_{CH}$	Coupling pathway	$^4 J_{CH}$
H–C–C	($\pm$) ≤ 5	H–C–C–C	≤ 5	H–C=C–C=C	($\pm$) ≤ 1
H–C=C	≤ 10	H–C=C–C	$\leq 15^a$	H–C–C–C–C ^b	≤ 1
H–C $\equiv$ C	40–60	H–C $\equiv$ C–C	≤ 5		
H–C(=O)–C	20–25				

Heteroatoms such as O, N may also be included in the coupling pathways illustrated in place of C.

^a *Trans* > *cis*, *cis* usually < 10 Hz.

^b *w*-configuration favoured.

structure, if any, whereas the weakest correlations are generally associated with poorly resolved, complex proton multiplets.

Despite the lack of discrimination, protons can potentially correlate to a great many carbon neighbours within two or three bonds, providing a mass of structural data on an unknown molecule. Connectivities can also be traced across heteroatom linkages (as seen in Fig. 6.25) where proton–proton couplings are usually negligibly small, and in this, the experiment is extremely effective at piecing together otherwise uncorrelated molecular fragments. Thus, the HMBC experiment was able to define the regiochemistry of a dipeptide antibiotic, Tü 1718B, isolated from *Streptomyces* cultures [52]. Two possible structures **6.6a** and **6.6b** were proposed from biosynthetic arguments, but neither proton correlation nor carbon chemical shift data could unambiguously identify which was correct. Confirmation was gained from an analysis of the long-range correlations of protons H4 and H5, and in particular those to the carbonyl groups (Fig. 6.29). H5 was observed to correlate to both whereas H4 correlated only to C6, these data being consistent with **6.6a** only. Connectivities across the oxygen linking neighbouring sugar residues in oligosaccharides likewise provide a useful means of identifying neighbouring residues in these compounds. Naturally, the sequence is not limited to proton–carbon connectivities but can be tailored to any spin- $\frac{1}{2}$ pair. Thus, long-range proton–silicon and proton–carbon correlations established through HMBC were used to confirm the structure of an unexpected product from the rearrangement of epoxydisilanes [53] as the silanol **6.7a** rather than **6.7b**. Additional NOE studies identified the stereochemistries across the alkene.

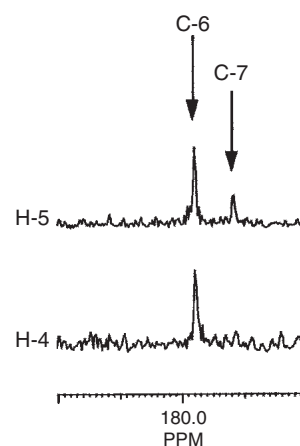
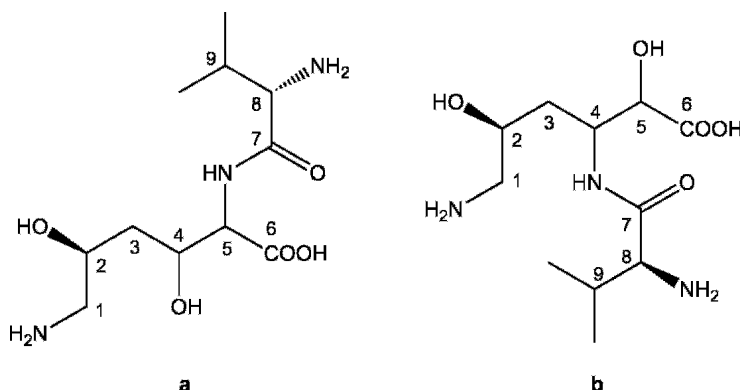
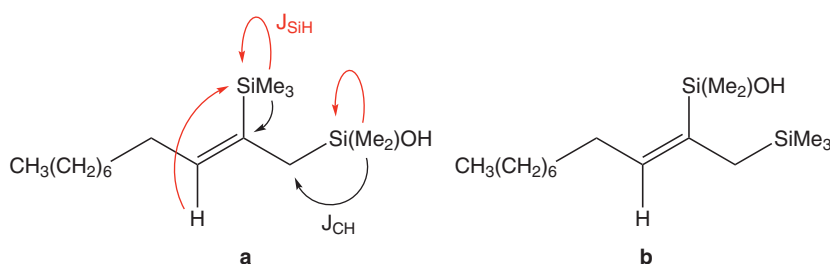


Figure 6.29. Columns taken from the HMBC spectrum of the antibiotic Tü 1718B at the shifts of protons H4 and H5. Only the key correlations to the carbonyl resonances are shown (reproduced with permission from [52], Copyright 1993, Elsevier).



6.6



6.7

Practical set-up

Owing to the relatively small size of long-range couplings and because of the need for long and often non-optimal Δ_{LR} delay periods, the sensitivity of the HMBC experiment is somewhat less than that of its HMQC or HSQC cousins. In the absence of PFGs, it is necessary to acquire many scans for each increment in an attempt to suppress the intense ^1H – ^{12}C signal and to reveal the correlations of interest, making the experiment time-consuming even when large sample quantities are available (spectral quality being highly dependent on spectrometer stability). Gradient-selected versions are considerably quicker to acquire, being dictated by sensitivity and resolution arguments alone, since the suppression of the parent signal is no longer an issue and better use is made of the receiver dynamic range. As a rule of thumb, when using gradient selection, the HMBC experiment will typically take around four times as long to acquire as the corresponding HMQC or HSQC experiment to provide acceptable data. From what was once typically an overnight phase-cycled experiment, high-quality data can now be obtained within a few hours or even tens of minutes; the impact of pulsed field gradients on the HMBC experiment has been profound.

The setting of Δ_{LR} has been described above, and the choice of digital resolution for the two dimensions follows similar arguments as for the one-bond correlation experiments, that is, around 50 Hz/pt in f_1 and around 5 Hz/pt in f_2 should provide acceptable results in most cases. In HMBC, the acquired t_2 FID begins as anti-phase magnetisation with respect to $^nJ_{CH}$ since the refocusing period is omitted, and the signal builds as refocusing occurs during t_2 itself. Optimal window functions providing close to matched filtering are therefore the unshifted sine-bell or squared sine-bell. In contrast, the t_1 interferograms decay from their maximum values and require only sufficient apodisation to avoid truncation errors that may appear under conditions of high signal-to-noise ratio, so a simple exponential decay or shifted sine-bell function suffices. For the phase-modulated experiment, it is commonplace to execute a magnitude calculation on the f_1 dimension for which an unshifted sine-bell function will improve resolution. Such harsh apodisation is not required for the echo–antiecho phase-sensitive version although f_2 magnitude calculation may be employed to sum the anti-phase signal intensity. Improved *digital* resolution of the f_1 dimension can be achieved through zero-filling (at least once is recommended) or the resolution can be increased by the use of forward linear prediction. Finally, the repetition time of the experiment is dictated by the T_1 s of protons *directly* bound to carbon-12 centres. Repetition times are therefore estimated from relaxation times as they would be for *homonuclear* correlation experiments and are longer than those optimal for HMQC or HSQC.

6.4.3. HMBC extensions and variants

The ability to detect two- and three-bond heteronuclear correlations with HMBC plays a hugely important role in structural elucidation and has led to the widespread and routine use of this experiment. Unsurprisingly then, this has also spawned the development of a range of methods that improve the appearance of the correlation spectrum or that add additional information to it. The basic HMBC experiment described so far suffers from a number of limitations ranging from complications arising from the appearance of one-bond coupling correlations, through limited detection of correlations in the presence of a wide range of $^nJ_{CH}$ values and the inability to distinguish two-bond from three-bond correlations. In this section, some of the more significant developments that address these and other issues are presented. We start with methods for the removal of one-bond correlations from HMBC, that is, those correlations observed in HMQC or HSQC, since it is now a standard practice to incorporate this filtration in almost all HMBC experiments. Such filtering is applicable to the other HMBC variants discussed below and its use would be expected, but for reasons of clarity will not be shown in the sequences presented hereafter.

Low-pass J-filtration: removing one-bond correlations

Despite setting the Δ_{LR} period of HMBC according to long-range coupling constants, this may also happen to be a multiple of the appropriate value for selecting one-bond correlations, additionally causing these to appear in the spectrum. Since the FID is acquired without ^{13}C decoupling, these possess distinctive doublet structure in f_2 (as seen in Figs 6.4b and 6.25), which aids their identification. These may be considered useful additions or unwanted interferences, depending on your point of view; although they simultaneously provide one-bond correlation data, they may obscure or become confused with long-range correlations. Their suppression may be achieved, to a greater or lesser degree, by the incorporation of a *low-pass J-filter*; it is called so because it retains or passes only those peaks arising from couplings that are *smaller* than a chosen cut-off value, here the one-bond coupling constant [43, 54]. Typically, these elements are placed at the start of the HMBC sequence prior to the transfer of polarisation onto carbon.

Figure 6.30 illustrates the simplest first-order low-pass filter. Here the Δ_1 period is set to $1/2\ ^1J_{CH}$, whilst Δ_{LR} is set according to the long-range coupling, as above. The first carbon pulse will generate multiple-quantum coherence for the anti-phase, one-bond ^1H – ^{13}C pairs only since Δ_1 is too short for significant evolution of the long-range couplings. Alternation of the phase of this first carbon pulse *without* changing that of the receiver causes these coherences to cancel on alternate scans and so removes one-bond correlations from the final spectrum. In practice, the suppression is often not complete, particularly when a wide range of $^1J_{CH}$ values are present, and improved results can be achieved with a second-order low-pass filter incorporating pulsed field gradients. In this, a second $^1J_{CH}$ evolution delay Δ_2 is included with three purging gradients of ratio 3:–2:–1. For long-range coupled ^1H – ^{13}C pairs, which generate negligible anti-phase components during the short Δ_1 and Δ_2 periods, the three gradient pulses cancel and have no net effect allowing the $^nJ_{CH}$ couplings to act during the subsequent Δ_{LR} delay. In contrast, the multiple-quantum coherence arising from anti-phase $^1J_{CH}$ couplings remains dephased by these gradient pulses and the corresponding correlations are suppressed. The filter delays are set typically to $\Delta_1 = 1/2\ ^1J_{CH(\text{min})}$

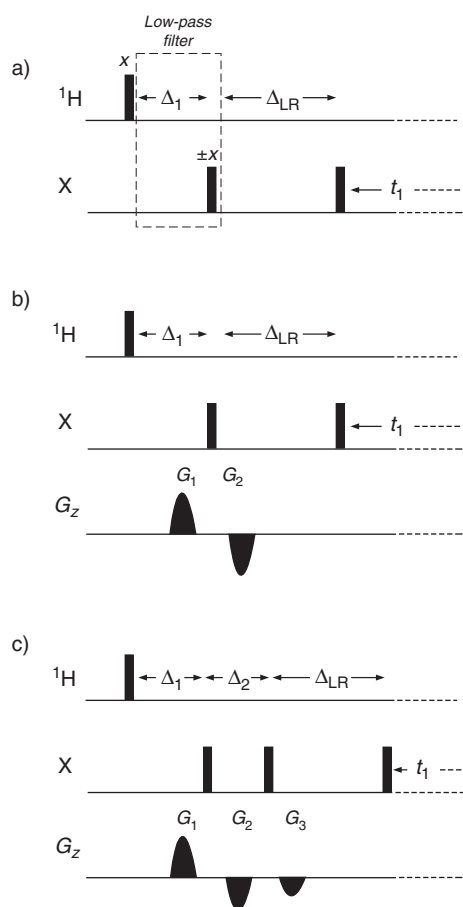


Figure 6.30. The low-pass filter for removing spurious one-bond correlation peaks from HMBC spectra. (a) The basic first-order filter in which inverting the phase of the first X pulse on alternate scans cancels unwanted one-bond contributions, (b) the gradient purged first-order filter ($G_1:G_2 = 1:-1$) and (c) the gradient purged second-order filter ($G_1:G_2:G_3 = 3:-2:-1$). Δ_{LR} is set to $1/2^n J_{\text{CH}}$ whilst Δ_1 and Δ_2 are set for one-bond couplings as described in the text.

and $\Delta_2 = 1/2^1 J_{\text{CH}(\text{max})}$ for the smallest and largest anticipated one-bond couplings, typically 125 and 165 Hz. More elaborate values of $\Delta_1 = 1/2[{}^1 J_{\text{CH}(\text{min})} + 0.146({}^1 J_{\text{CH}(\text{max})} - {}^1 J_{\text{CH}(\text{min})})]$ and $\Delta_2 = 1/2[{}^1 J_{\text{CH}(\text{max})} - 0.146({}^1 J_{\text{CH}(\text{max})} - {}^1 J_{\text{CH}(\text{min})})]$ have been suggested as optimum values [55].

An alternative approach to low-pass filtering that is highly effective even without gradient pulses is to selectively refocus one-bond couplings whilst letting the long-range couplings develop to become anti-phase for transfer to ^{13}C [56]. This can again be achieved by exploiting the large difference in coupling constants by the use of a suitable BIRD pulse cluster at the midpoint of Δ_{LR} (Fig. 6.31). The so-called $\text{BIRD}^{\text{r,X}}$ pulse [57] serves to invert only X-spins (here ^{13}C) and those protons remotely (long-range) coupled to these but not the protons directly attached to the heteronucleus. This achieves the desired refocusing of ${}^1 J_{\text{CH}}$ (since only the ^{13}C experiences a 180° pulse) but defocusing of ${}^n J_{\text{CH}}$ (for which both ${}^1\text{H}$ and ^{13}C experience net 180° pulses). The range of potential ${}^1 J_{\text{CH}}$ values can again reduce the efficiency of this approach, and the addition of a subsequent 180° ^{13}C pulse followed by a short $1/2^1 J_{\text{CH}}$ period Δ_1 aims at refocusing those one-bond couplings that have ‘passed’ through the BIRD filter. Being refocused, these are no longer able to generate multiple-quantum coherence and so do not contribute to the final spectrum. Phase alternation of the 180° pulse then serves to cancel contributions from those components initially refocused by the BIRD that would subsequently become defocused in the following Δ_1 period, giving rise to effective suppression of one-bond correlations. Extension to a second-order filter by the

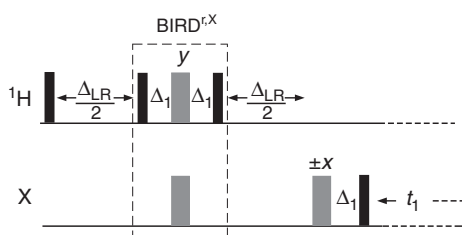


Figure 6.31. An alternative low-pass filtering scheme employing selective refocusing of ${}^1 J_{\text{CH}}$ couplings with a $\text{BIRD}^{\text{r,X}}$ pulse cluster. The 180° X pulses may be replaced advantageously by composite or adiabatic pulses. Δ_1 is set to $1/2^1 J_{\text{CH}}$ and Δ_{LR} to $1/2^n J_{\text{CH}}$.

addition of a second $180^\circ(\text{X})-\Delta_1$ period with the X-pulse phase alternated independently of the first provides still greater suppression.

Constant-time HMBC: eliminating proton–proton coupling in f_1

One of the less desirable features of the HMBC (and HMQC) experiment is the contribution from proton–proton couplings in the X spin f_1 dimension, a consequence of the evolution of ^1H –X multiple-quantum coherence during t_1 . This leads to peak broadening in the f_1 dimension and may limit peak resolution so that, as described above, typical HMBC experiments are recorded with low f_1 resolution so as not to resolve this additional fine structure. In cases where high f_1 resolution is desired, such as in highly crowded ^{13}C spectra, and when broadband sampling of long-range coupling constants is employed (described below), the contribution from proton–proton couplings can be more troublesome. These arise because proton coupling evolution modulates the detected signal as a function of the t_1 period, meaning these can be eliminated from the f_1 dimension if this t_1 -dependent modulation of each FID can be suppressed. This can be achieved through the introduction of the *constant-time* principle [58], in which the *total* period in which proton couplings evolve is held constant throughout the experiment *regardless* of the value of t_1 . In the HMBC experiment, proton–proton coupling evolves throughout the whole sequence following the initial proton excitation, meaning its duration must remain fixed as t_1 increases. One possible approach to this based on the echo–antiecho scheme is illustrated in Fig. 6.32, in which the total period ($\Delta_{\text{CT}}-t_1$) decreases as t_1 is incremented, thus defining the constant time period. The delay Δ_{CT} is set equivalent to the maximum value that the t_1 period will attain, $t_{1(\text{max})}$, ($\Delta_{\text{CT}}=t_{1(\text{max})}$) so that the variable period ($\Delta_{\text{CT}}-t_1$) contracts from Δ_{CT} to zero as t_1 increments from zero to $t_{1(\text{max})}$, with the net result that proton–proton coupling evolution no longer modulates the detected signal.

Owing to the low-resolution conditions appropriate for most routine uses of HMBC, the constant-time approach is arguably of limited use here. However, this can prove more valuable in the band-selective experiment described below, where substantially higher f_1 resolution is employed and where any proton coupling structure may limit crosspeak resolution.

Band-selective HMBC: optimising f_1 resolution

A further potential problem with ^1H – ^{13}C HMBC in particular is the large spectral width that must be indirectly digitised in f_1 . This tends to be a greater problem than with one-bond correlation experiments because of the need to include the carbonyl region in many instances, meaning, as already mentioned, the data are usually collected with rather low f_1 resolution. In regions of the ^{13}C spectrum where many resonances fall close together, such low resolution may prove insufficient and result in crosspeak overlap. One solution to this is to select only the region of the f_1 dimension that contains the most crowded resonances, meaning only these appear in the final spectrum and hence only a small f_1 window need to be digitised at high resolution. In the most basic approach, this can be achieved by selectively exciting only the ^{13}C resonances of interest with a shaped carbon pulse (Chapter 10) in place of the first non-selective carbon pulse of the conventional sequence [59]; selectively acquiring a smaller region of a 2D data set gives rise to the so-called *band-selective* or *semi-selective* variants [60]. However, in such cases of high f_1 resolution, the removal of proton–proton coupling fine structure from f_1 has considerable benefits and the band-selective, constant-time HMBC experiment is then recommended [61]. This may be derived from the constant-time HMBC of Fig. 6.32 by simply replacing the X spin 180° pulse in the gradient echo with a band-selective, shaped 180° pulse. Figure 6.33 compares the carbonyl region of **6.8** from the conventional HMBC with that from the band-selective, constant time variants. The aim was to obtain sequence-specific assignments for each carbohydrate amino acid residue by identifying long-range ^1H – ^{13}C correlations to the carbonyl carbons, thus providing a link between adjacent residues.

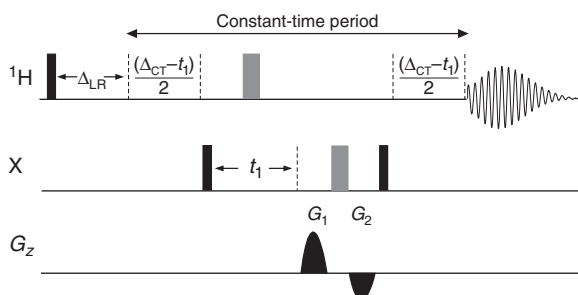


Figure 6.32. The constant-time HMBC experiment based on the echo–antiecho f_1 selection scheme of Fig. 6.27 ($\Delta_{\text{CT}}=t_{1(\text{max})}$). The combined period ($\Delta_{\text{CT}}-t_1$) is decremented as t_1 increases, thus defining a constant-time period for proton coupling evolution that is invariant with changes in t_1 .

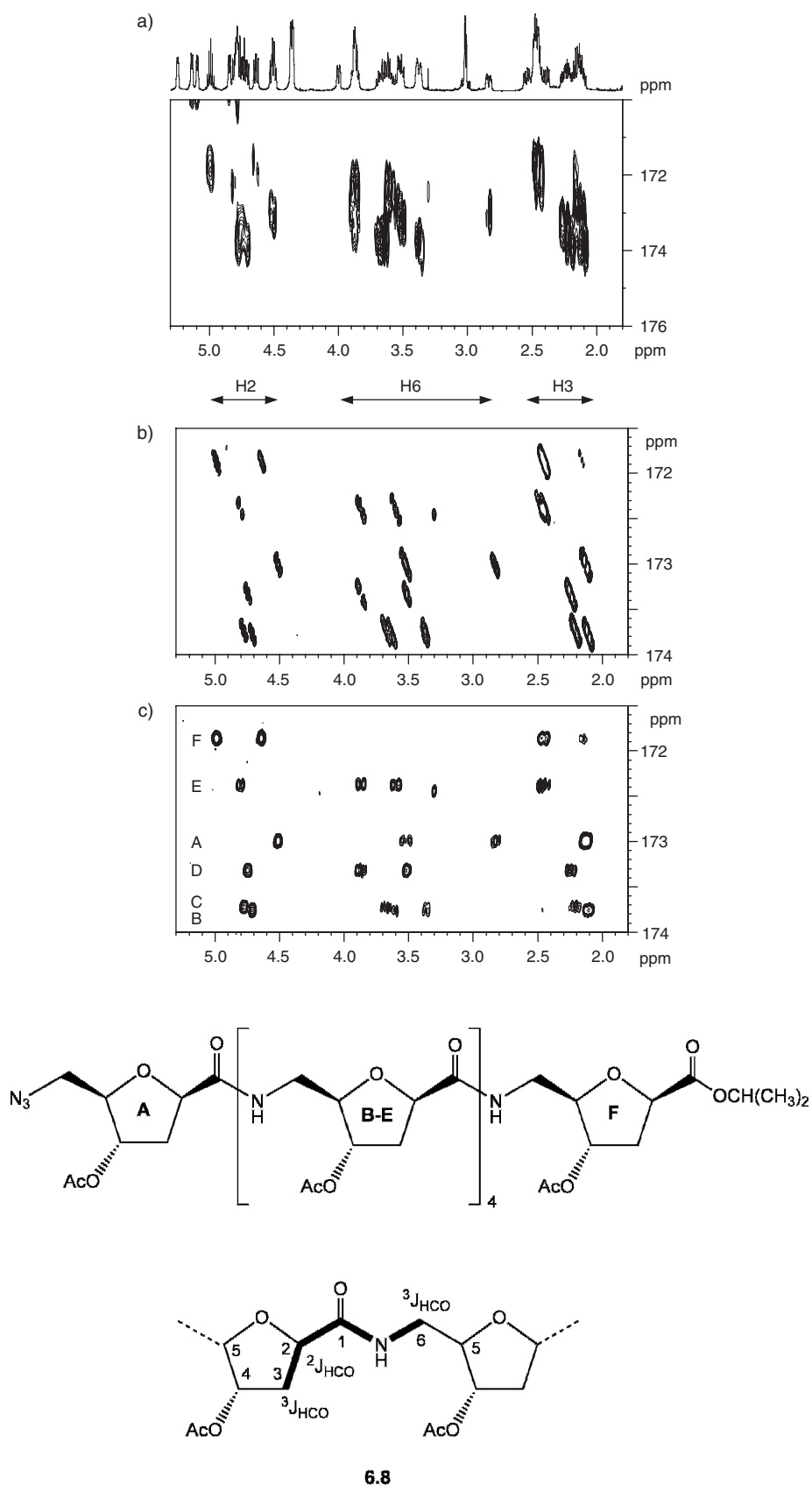


Figure 6.33. Carbonyl regions of the HMBC spectra of **6.8** recorded with (a) the conventional HMBC, (b) the band-selective HMBC and (c) the band-selective, constant-time HMBC sequences. The small carbonyl shift dispersion causes considerable crosspeak overlap with the low f_1 resolution of 45 Hz/pt in (a), whereas the higher resolution of 3 Hz/pt in (b) and (c) removes this limitation. In (c) the proton–proton multiplet structure is also suppressed for optimum peak dispersion (reproduced with permission from [61], The Royal Society of Chemistry).

For Fig. 6.33b and c, the carbonyl region was selected with a 0.5-ms 180° Gaussian pulse and only a 6 ppm ^{13}C window digitised (centred at 172 ppm), providing a 15-fold increase in f_1 resolution relative to the non-selective experiment. The crosspeak skew observed in Fig. 6.33b reveals the presence of the J_{HH} -coupling fine structure, which is suppressed in the constant time variant of Fig. 6.33c resolving peaks sufficient to identify the required $\text{H}3^i$, $\text{H}2^i$ to CO^i and CO^i to $\text{H}6^{i+1}$ long-range correlations and thus establishing connectivities across the amide linkages.

ACCORDION optimisation: broadband $^nJ_{XH}$ detection

One of the limiting features of the HMBC experiment is its inability to sample simultaneously and effectively a wide range of coupling constants, potentially leading to missing long-range correlations in the spectrum. The full range of $^nJ_{CH}$ values is approximately 1–25 Hz whereas the typical HMBC experiment would be optimised for a coupling of around 8 Hz ($\Delta_{LR} \approx 62$ ms). When dealing with complex and/or unknown structures, it can be advantageous to seek a large number of correlations to aid their identification, and the simplest approach here is simply to collect multiple HMBC spectra with differing values of Δ_{LR} . As mentioned previously, the HMBC signal intensity scales with the amount of anti-phase ^1H –X magnetisation generated during Δ_{LR} and so has a $\sin(\pi^nJ_{XH}\Delta_{LR})$ dependence (ignoring relaxation losses), as illustrated in Fig. 6.34. Thus, collecting two experiments optimised for 8 and 4 Hz, corresponding to delays of ~ 62 and 125 ms, respectively, should provide reasonable sampling over a range of 2–14 Hz, which is appropriate for observing many long-range ^1H – ^{13}C correlations. For larger molecules where relaxation losses may be more substantial, it is advantageous to employ delays somewhat shorter than those predicted here, especially when seeking correlations from smaller couplings. The use of the simplest HMBC sequence here has its attractions in that it uses rather few pulses and so suffers minimal signal loss from pulse imperfections. Nevertheless, it is generally undesirable to collect multiple experiments, especially when samples are limited in quantity or even unstable, and a number of sequences have been developed to provide more efficient ‘broadband’ detection over a range of coupling constants in a single experiment, which we shall now consider. Although it appears to have been little used, we shall begin with the originally proposed sequence since it is from this that the more useful variants are derived, many of which have been reviewed [62].

The basis of the original ACCORD-HMBC sequence [63] is illustrated in Fig. 6.35 and differs from the simple HMBC in that the usual ‘static’ delay for the evolution of long-range couplings Δ_{LR} is replaced by a variable delay Δ_{VD} , which is also repeated prior to data acquisition and which serves to sample over a range of coupling constants from $^nJ_{XH(\min)}$ to $^nJ_{XH(\max)}$. Thus, during the experiment, as t_1 is incremented sequentially, the period Δ_{VD} is decremented in a stepwise manner from $\Delta_{VD(\max)}$ to $\Delta_{VD(\min)}$ in steps of $(\Delta_{VD(\max)} - \Delta_{VD(\min)})/n_{t1}$, where n_{t1} represents the total number of t_1 increments used to define the f_1 dimension. The delay $\Delta_{VD(\max)}$ defines the evolution period for the smallest coupling to be sampled ($1/2^nJ_{XH(\min)}$) whilst $\Delta_{VD(\min)}$ is that for the largest ($1/2^nJ_{XH(\max)}$). The stepwise sampling of all intermediate values introduces this new variable time period (in addition to t_1 and t_2) and is termed the ACCORDION approach [64]; it is this that gives rise to the enhanced detection of long-range correlations. Figure 6.36 shows a region of the 8-Hz optimised HMBC and the 2–16-Hz sampled ACCORD-HMBC spectra of the diterpene **6.9** and clearly illustrates the larger number of long-range correlations observed in the latter.

Figure 6.34. The intensity of HMBC correlations as a function of $^nJ_{XH}$ for different values of the Δ_{LR} period. Two experiments optimised for coupling constants of 8 and 4 Hz (Δ_{LR} delays of 62 and 125 ms, respectively) provide reasonable sampling over the range 2–14 Hz.

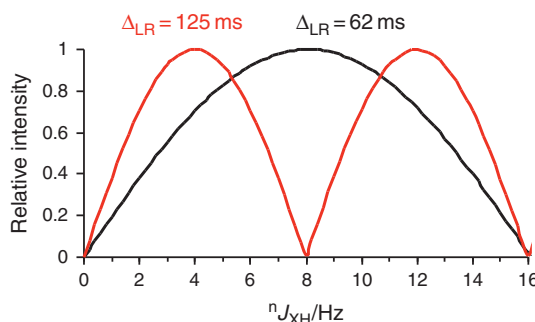
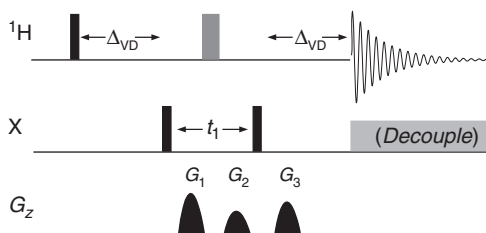


Figure 6.35. The ACCORD-HMBC sequence. The usual delay for the evolution of long-range couplings Δ_{LR} is replaced by a variable delay Δ_{VD} that samples a range of $^nJ_{XH}$ values as t_1 increases.



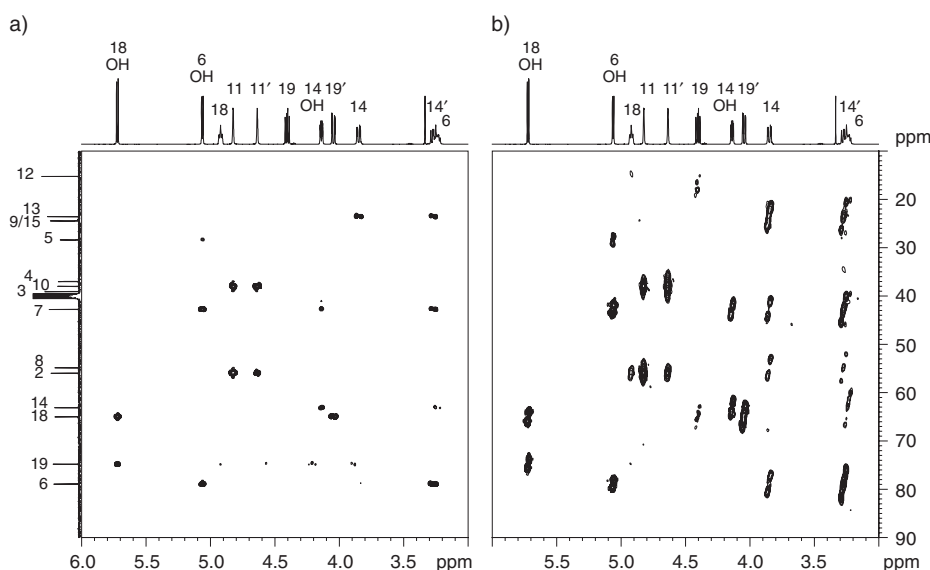


Figure 6.36. A comparison of regions from (a) an 8-Hz HMBC and (b) a 2–16-Hz ACCORD-HMBC spectrum of **6.9**; see text for an explanation of the peak skew observed in (b).

The sequence is held symmetric by the inclusion of a second Δ_{VD} period, which allows for optional X spin decoupling, but is in any case essential to refocus the ^1H chemical shift evolution that occurs during the first Δ_{VD} period. Although this ensures that proton chemical shifts do not modulate the f_1 responses as a function of Δ_{VD} , proton–proton couplings do contribute and are detrimentally scaled in this dimension, giving rise to a characteristic tilting or *skew* of the crosspeak that can compromise peak dispersion, as seen in Fig. 6.36b. The scaling arises from the way in which the Δ_{VD} period alters with each increase in t_1 and is best understood by considering the situation in which Δ_{VD} is *increased* on each t_1 increment. Thus, as t_1 increases, the total evolution time in which J_{HH} is active is increased by t_1 *plus* the associated increase in Δ_{VD} , leading to a greater net coupling evolution than would occur if Δ_{VD} did not alter, that is, if it had remained static as in conventional HMBC. The effect of this greater evolution is to make it *appear* as if larger proton–proton coupling constants had been operative during t_1 , and hence the proton homonuclear couplings appear to be amplified or *scaled* in f_1 . The magnitude of the amplification may be defined by a *scaling factor* J_{scale}

$$J_{\text{scale}} = \frac{2(\Delta_{VD(\text{max})} - \Delta_{VD(\text{min})})}{n_{t1} \delta_{t1}} \quad (6.5)$$

where n_{t1} again represents the number of t_1 increments and δ_{t1} the size of each increment (t_1 dwell time) [65]. From this, it is clear that sampling a wider range of $^nJ_{XH}$ values will lead to increased scaling of J_{HH} in the X spin dimension. Although sampling of the long-range heteronuclear coupling may be improved, this is achieved at the expense of poorer peak separation (Fig. 6.37a). Conversely, the scaling may be diminished by the use of more t_1

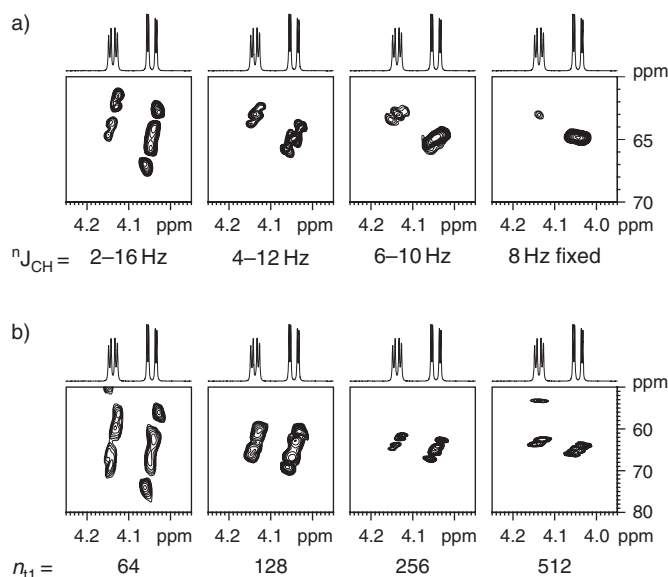
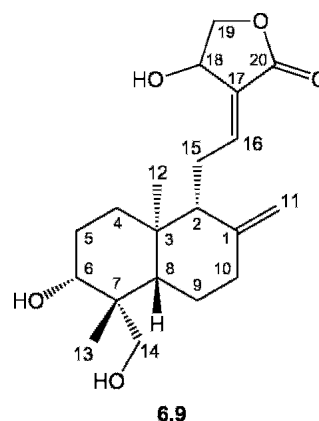


Figure 6.37. The variation in crosspeak skew in ACCORD-HMBC as a function of (a) the $^nJ_{CH}$ range sampled and (b) the number of t_1 increments n_{t1} used to characterise f_1 . For (a) n_{t1} was 256 and for (b) the sampling range was 2–16 Hz.

increments to characterise f_1 (Fig. 6.37b) since the change in Δ_{VD} per increment is then reduced, but this will unavoidably increase the total duration of the experiment. Finally, the need to retain two Δ_{VD} periods necessarily leads to a longer sequence and hence reduced sensitivity relative to the basic HMBC scheme. Thus, whilst the ACCORDION sampling has potential benefits for increased detection of long-range correlations, it suffers from a number of undesirable features that limit its utility in this form.

A more practical implementation of this concept is to be found in the IMPEACH-MBC experiment [66] (improved performance accordion heteronuclear multiple-bond correlation, Fig. 6.38). In this, the variable time delay that permits the broadband sampling of heteronuclear coupling constants Δ_{VD} is preceded by an additional variable period ($\Delta_{CT} - \Delta_{VD}$) such that the sum of these delays remains constant, giving rise to the so-called *constant-time (variable delay)* element. Thus, as Δ_{VD} is decremented on each t_1 increment in the ACCORDION style, the compensating delay is increased by an equivalent amount following the constant-time concept. As described for the constant-time HMBC above, this results in no modulation as a function of t_1 of the detected FIDs by the homonuclear proton–proton couplings and so removes the exaggerated skew that is observed with the ACCORD-HMBC experiment. The only period that modulates the observed signals is t_1 itself, in which homonuclear proton couplings remain active, meaning the IMPEACH-MBC experiment displays J_{HH} structure in f_1 in the manner of the standard HMBC, that is, without further scaling. To ensure that the broadband sampling of the long-range heteronuclear couplings occurs only in the Δ_{VD} period, the compensating element ($\Delta_{CT} - \Delta_{VD}$) is split centrally by a 180° X pulse to refocus the evolution of these heteronuclear couplings during this delay. In the original presentation of this experiment, an equivalent constant-time period was included after t_1 to allow refocusing and X-spin decoupling. However, this extension typically reduces sensitivity and the non-refocused variant presented here may be preferred. Figure 6.39 compares regions of the 8-Hz HMBC with two IMPEACH-MBC experiments. With moderate sampling of 4–12 Hz, an increased number of correlations is observed, such as those highlighted in the red boxes, although the more aggressive 2–16 Hz sampling also leads to the loss of some signals, as in the dashed red box. These losses occur for the broader resonances and are associated with the longer delays required for the sampling of the very small couplings.

A final modification of this broadband sampling concept is found in the elaborately entitled CIGAR-HMBC experiment (constant-time inverse-detection gradient accordion-rescaled HMBC) [67], which now retains the refocusing element after t_1 and can optionally employ X spin decoupling during signal detection. In this method, the constant-time (variable delay) introduced above is allowed to alter its duration as a function of t_1 , but in this case, the magnitude of variation is under complete control of the operator, unlike ACCORD-HMBC. This then allows a user-controllable degree of skew to be reintroduced to the HMBC crosspeak structure with the aim of using the appearance of the skew to differentiate genuine (weak) HMBC peaks from artefacts, including noise (which would not

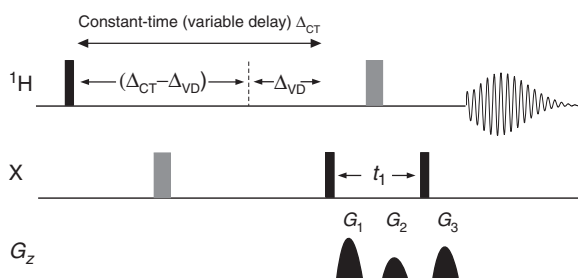


Figure 6.38. The IMPEACH-MBC sequence shown without refocusing of long-range couplings.

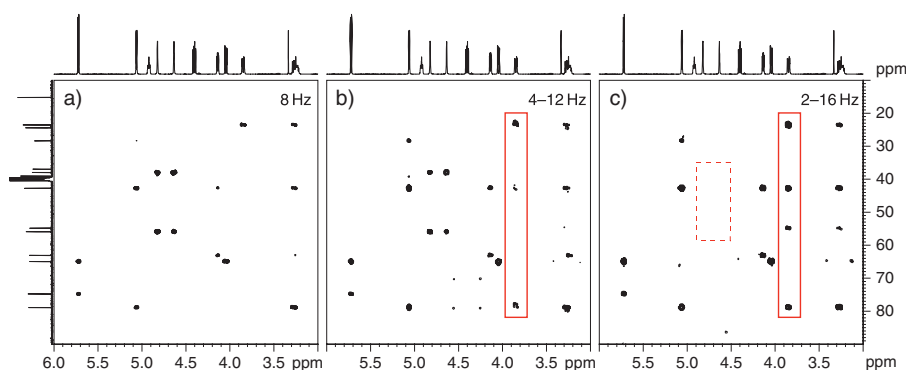


Figure 6.39. A comparison of regions from (a) an 8 Hz HMBC and (b and c) the IMPEACH-MBC recorded with the sequence of Fig. 6.38 on **6.9**.

exhibit the skew). The compensating element of IMPEACH is modified by the introduction of the user-definable J -scaling delay Δ_{JS} to become $(\Delta_{CT} - \Delta_{VD}) + \Delta_{JS}$ and is again applied prior to the Δ_{VD} period with a refocusing 180° X pulse at its midpoint. The additional delay Δ_{JS} is defined as $(J_{\text{scale}} - 1)\delta_{t1}/2$ with the J_{scale} parameter defining the degree of skew. Thus, if $J_{\text{scale}} = 1$, this delay is not used and the resulting experiment produces crosspeaks with an identical appearance to the IMPEACH-MBC method. If $J_{\text{scale}} = 0$, then the delay (now applied twice; this sequence employs a refocusing period) exactly compensates for each increment in t_1 (δ_{t1}), causing the total period from initial proton excitation to detection to become constant time and this then eliminates all J_{HH} couplings from f_1 . If $J_{\text{scale}} > 1$, then a defined scaling of the homonuclear proton couplings arise in f_1 , thus reintroducing the peak skew. Furthermore, the extent of the skew is now independent of the level of f_1 digitisation used, unlike that in ACCORD-HMBC. Note also that, with the exception of $J_{\text{scale}} = 1$, the sequence must retain a refocusing element of equivalent duration after t_1 to refocus proton shift evolution that occurs in Δ_{JS} . Alternatively, but undesirably, an appropriate number of refocusing 180° proton pulses must be introduced judiciously within the new constant-time (variable delay) period if the heteronuclear refocusing period after t_1 is not employed. This is to ensure that the necessary proton shift refocusing is achieved, requiring Δ_{JS} to be increased to $(J_{\text{scale}} - 1)\delta_{t1}$ since it is now used only once in the sequence.

So how might one decide upon a suitable approach when the use of a single HMBC employing a static Δ_{LR} period is deemed insufficient for the sample under scrutiny? Arguably the most direct and pragmatic approach is simply to acquire multiple spectra with differing values of Δ_{LR} and thus make best use of the simplicity and relatively high sensitivity of the basic HMBC sequence. Preferably this would employ the f_1 phase-sensitive version of Section 6.4.1. When this approach is undesirable and a single broadband-optimised experiment is attractive, then the non-refocused IMPEACH-MBC of Fig. 6.38 presents a relatively simple technique. Although an equivalent spectrum should be obtained with the CIGAR-HMBC technique with $J_{\text{scale}} = 1$, the added duration of this when used as the refocused variant makes this a less attractive option. The CIGAR-HMBC may have application when the controllable f_1 skew is required to unambiguously identify weak correlations or perhaps when the complete removal of J_{HH} structure in f_1 is beneficial for reasons of peak resolution, but it would otherwise appear overly complex for routine use and a less attractive alternative to IMPEACH-MBC. When using any of the ACCORDION-based sequences, one also needs to be cautious in how aggressive one is in sampling for small long-range coupling constants since this may encourage the appearance of correlations arising from couplings over four or possibly more bonds. In proton-sparse structures, this may of course be a desirable feature (see reference [68] for examples), whilst for routine structural elucidation, it may introduce additional complication or ambiguity. In cases when two- and three-bond correlations are most desirable, a limited sampling of around 4–12 Hz seems most appropriate; methods aimed at distinguishing two-bond and three-bond correlations in HMBC spectra are the subject of the following section. As indicated earlier, the sequences presented here have not, for reasons of clarity, included explicitly low-pass J -filters for the removal of one-bond correlations, whereas in most applications, these would be incorporated prior to the sampling elements described above, usually as the gradient-optimised filters as in the original publications or alternatively employing the BIRD^{rX} filter also described above [69].

Although most obviously relevant to establishing ^1H – ^{13}C correlations, broadband J_{XH} optimisation has also been suggested as beneficial in the acquisition of ^1H – ^{15}N long-range correlations [70, 71], a topic of increasing interest [72]. Finally, note that ACCORDION optimisation has also been suggested for the HMQC [73] and HSQC [74] experiments when one-bond couplings are expected to vary widely, which is more often the case for nuclides other than ^{13}C and ^{15}N , this having been demonstrated for ^1H – ^{113}Cd correlations [73], for example.

Editing HMBC: differentiating $^2J_{CH}$ and $^3J_{CH}$ correlations

One of the features of HMBC that may lead to ambiguity in structural elucidation is its inability *a priori* to differentiate correlations arising over two-bonds from those over three-bonds. The coupling constants giving rise to such correlations are similar in magnitude and so cannot be separated on the basis of size alone, unlike one-bond correlations that are at least an order of magnitude larger. In fact, rather few methods exist to edit directly HMBC spectra to provide such differentiation, and these have not found widespread use [75, 76]. For example, the $^2J, ^3J$ HMBC sequence [75] is a further variant of the IMPEACH-MBC technique and uses the presence of crosspeak skew to differentiate $^2J_{CH}$ from $^3J_{CH}$ responses. Alternative methods seek to utilise the presence of homonuclear $^3J_{HH}$ vicinal couplings to generate spectra with the appearance of HMBC in which *only* crosspeaks indicative of two-bond pathways appear. These methods exploit quite different transfer pathways by tracing $^1J_{CH}$ and $^3J_{HH}$ couplings and conceptually follow the scheme of an HMQC-COSY experiment in seeking *pseudo*-two-bond

correlations. Provided $^4J_{\text{HH}}$ couplings are negligible, crosspeaks corresponding to three-bond correlations in HMBC remain absent. Since this approach does not exploit the often small $^2J_{\text{CH}}$ couplings, it can offer the further benefit of enhanced intensity for these correlations relative to those observed in HMBC, thus acting to compliment this, as illustrated in the example presented shortly. Note, however, that correlations to non-protonated carbons cannot be observed due to the lack of the necessary vicinal proton–proton coupling pathway. The most recent and refined incarnation of this scheme is the heteronuclear two-bond correlation (H2BC) [77, 78] experiment illustrated in Fig. 6.40. The core of the sequence comprises a constant time period Δ_{CT} during which proton homonuclear couplings evolve after which the 90°_y proton pulse initiates COSY-type transfer to neighbouring protons. Embedded within this period is the HMQC sequence to provide encoding of carbon-13 shifts in the f_1 dimension and so yield a similar appearance to HMBC. Here, the Δ_2 delay is optimised for one-bond heteronuclear couplings in the usual way, and the sequence employs gradient encoding for echo–antiecho signal selection. To ensure that $^1J_{\text{CH}}$ coupling evolution occurs only in the Δ_2 delays within the total Δ_{CT} period, two additional X spin decoupling periods are included in this; these decrease in duration as t_1 increases to maintain the constant time period. The use of a constant-time approach also ensures that J_{HH} couplings do not appear in f_1 . The final element of the sequence is a third-order low-pass J -filter to efficiently suppress one-bond correlations prior to signal detection in the presence of carbon-13 decoupling. Optionally, the addition of a ^{13}C spin-echo within the HMQC segment yields an edited H2BC experiment [78, 79] in which CH_2 responses are inverted relative to those of CH or CH_3 in a manner reminiscent of the edited HSQC. In all cases, the constant time period Δ_{CT} is set to allow the formation of anti-phase homonuclear proton magnetisation arising from the evolution of $^3J_{\text{HH}}$ couplings, and values in the region of 16–22 ms are recommended [77]. A comparison of the HMBC and H2BC spectra of **6.9** is presented in Fig. 6.41 and illustrates the specificity afforded by the H2BC experiment in identifying two-bond correlations. The red

Figure 6.40. The H2BC sequence incorporating a third-order low-pass J -filter. The constant-time period for J_{HH} evolution Δ_{CT} is set typically to 16–22 ms and the heteronuclear delays are $\Delta_1 = 1/2^1J_{\text{CH}(\text{min})}$, $\Delta_2 = 1/(^1J_{\text{CH}(\text{min})} + ^1J_{\text{CH}(\text{max})})$, $\Delta_3 = 1/2^1J_{\text{CH}(\text{max})}$. Gradient selection follows the echo–antiecho protocol and the gradient ratios for the low-pass filter are $G_2:G_3:G_4 = 0.075G_1:0.175G_1:0.75G_1$.

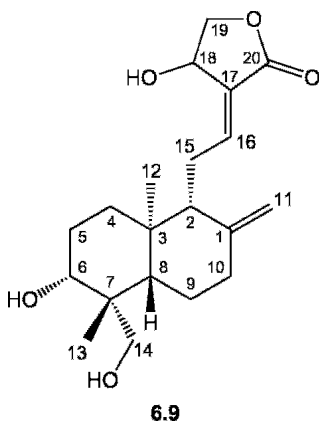
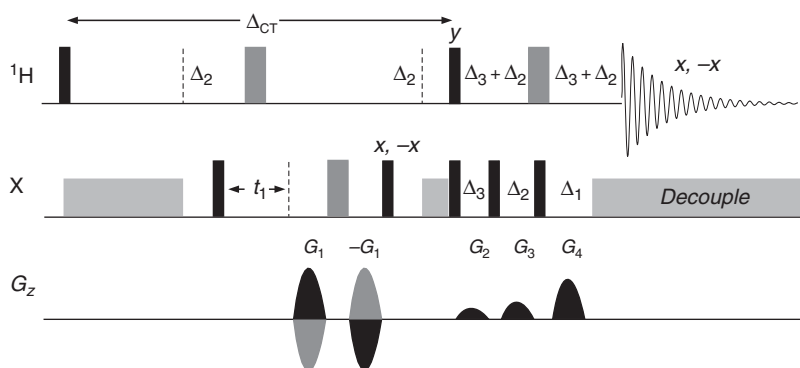
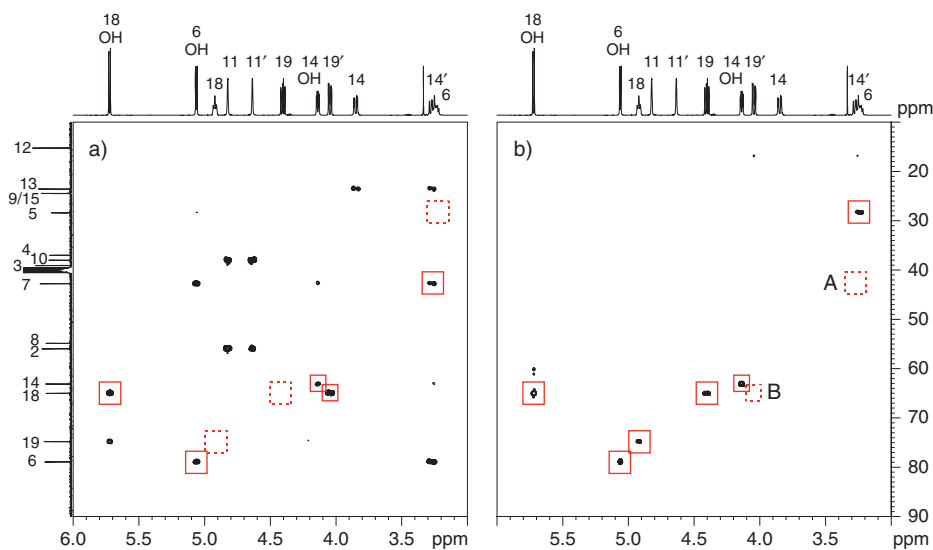


Figure 6.41. A comparison of regions from (a) the 8-Hz HMBC and (b) the H2BC spectra of **6.9** ($\Delta_{\text{CT}} = 22$ ms); see text for discussions.



boxes highlight all two-bond correlations observed within this region, with those shown dashed indicating missing peaks in either spectrum. Clearly the H2BC experiment identifies a number of additional two-bond correlations that were unobservable in HMBC due to vanishingly small long-range heteronuclear couplings, although it also misses two correlations that are apparent in HMBC. That marked **A** cannot be observed in H2BC since it would represent a correlation to a quaternary carbon (H14–C7), whereas that marked **B** is not seen because of the small $^3J_{\text{HH}}$ coupling involved (H19'–H18, <2 Hz). Note that correlations are seen from the hydroxyl protons to adjacent carbons two bonds away.

A final route to the identification of two-bond correlations is through the exploitation of one-bond ^1H – ^{13}C and ^{13}C – ^{13}C couplings to explicitly define ^1H – ^{13}C – ^{13}C relationships [80], as in the refocused 1,1-ADEQUATE experiment described in Section 5.9, which has a display format similar to HMBC. Despite the different coupling pathways employed, crosspeaks in the spectrum will equate to those observed in the H2BC experiment with the further beneficial feature that correlations to quaternary centres may also be observed since $^3J_{\text{HH}}$ couplings are no longer involved in the transfer pathway. This will also avoid the potentially misleading three-bond correlation peaks that can arise in H2BC spectra when $^4J_{\text{HH}}$ values are non-vanishing and may also yield additional correlations that are not observed in HMBC in the presence of vanishingly small $^2J_{\text{CH}}$ values. The significant drawback to this approach is the undoubtedly poorer sensitivity because of the dependence on $^1J_{\text{CC}}$ couplings, but in situations when sample quantities are not limiting, it may provide a further useful approach to unambiguously identifying two-bond correlations.

6.4.4. Measuring long-range $^2J_{\text{CH}}$ coupling constants with HMBC

Finally in this section discussing the HMBC experiment and its variants, we briefly consider its use for the measurement of the magnitudes of the long-range $^2J_{\text{CH}}$ coupling constants themselves. These couplings are of considerable value in the stereochemical assignment of organic molecules owing to the Karplus-like dependence of vicinal couplings on dihedral angles; see discussions in references [81] and [62] and the so-called '*J*-based configurational analysis' method [82, 83] for the definition of stereochemistry, as examples. However, it is fair to say that they are yet to find widespread use in the laboratory despite their considerable potential. This is principally due to the difficulty in measuring these often-small coupling constants and the need to employ typically quite complex pulse sequences to enable their extraction. There have been numerous methods developed over recent years, indeed far too many to describe here, although many of these have been reviewed and compared [81]. In addition to the low natural abundance of carbon-13, the difficulty originates in the potential for many long-range couplings to exist with a single carbon, which, combined with their small magnitude, means they are rarely resolved in either 1D proton or carbon spectra, thus demanding the use of, in most cases, 2D experiments to facilitate their measurement.

The HMBC experiment itself encodes the long-range coupling constants within each crosspeak as an anti-phase splitting superimposed on the usual proton multiplet structure in f_2 since the HMBC FID is recorded without refocusing of heteronuclear couplings. Thus, in principle, these crosspeaks contain the desired $^2J_{\text{CH}}$ information. Unfortunately, however, the multiplets also display complex phase behaviour along f_2 owing to the evolution of proton–proton couplings and proton chemical shifts during the Δ_{LR} $^2J_{\text{CH}}$ evolution period prior to t_1 , so direct extraction of the heteronuclear coupling constants is all but impossible (Fig. 6.42). The extravagant phase behaviour within these peaks explains why HMBC spectra are traditionally displayed after a magnitude calculation to mask this. Nevertheless, it is possible to determine the magnitudes of the heteronuclear couplings, provided one can adequately account for the phase properties arising within each crosspeak structure. The procedure involves the use of a suitable template multiplet that contains the *identical* phase modulation displayed by the HMBC crosspeak but lacks the additional splitting arising from the heteronuclear long-range coupling [84]. This template is then used in a fitting routine that seeks to reproduce the experimental form of the HMBC multiplet, as illustrated in Fig. 6.43. The offset between the template and its inverted twin in the optimally fitted trial multiplet directly reflects the magnitude of the heteronuclear coupling $^2J_{\text{CH}}$ responsible for the appearance of the crosspeak; see the original reference for details of the fitting procedure [84].

There are a number of approaches to generate suitable template multiplets, the simplest of which is to take these from a 1D proton spectrum recorded with a delay equal to Δ_{LR} immediately after the excitation pulse (Fig. 6.44a) so as to precisely simulate the phase evolution occurring in the corresponding HMBC delay. Experimentally, this can in fact be achieved by simply collecting a standard proton acquisition and removing the first part of the FID equivalent to Δ_{LR} by left-shifting the FID data points. Figure 6.44b shows an optimised

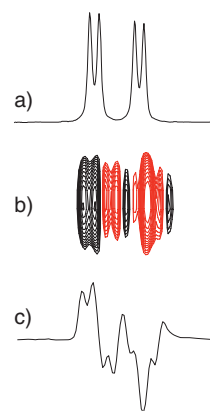


Figure 6.42. Multiplet structure from HMBC. (a) One-dimensional proton multiplet, (b) HMBC crosspeak and (c) the f_2 (horizontal) trace through the crosspeak displaying the additional anti-phase heteronuclear coupling combined with complex phase modulation.

Figure 6.43. Schematic illustration of the template-fitting routine for the extraction of ${}^nJ_{\text{CH}}$ values from HMBC traces. The trial coupling constant value J_{trial} is varied to produce best fit of the trial multiplet with the experimental HMBC multiplet. The conventional 1D proton multiplet is shown boxed for reference purposes only.

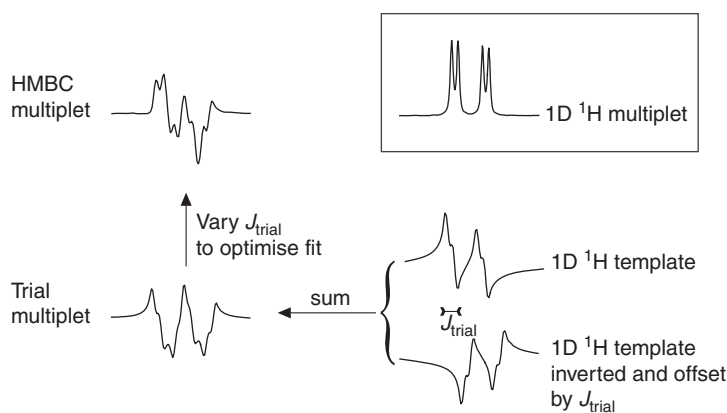
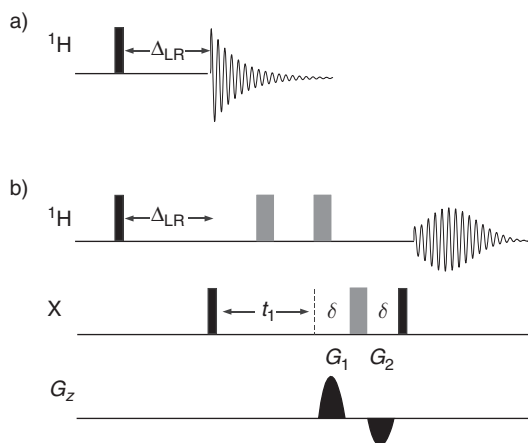


Figure 6.44. The phase-sensitive HMBC sequence for measuring ${}^nJ_{\text{CH}}$ coupling constants. (a) A possible scheme for producing reference proton template multiplets (see text) and (b) the HMBC experiment.



form of the phase-sensitive HMBC containing only one additional proton 180° pulse that produces crosspeaks with f_2 phase that precisely match those obtained from the 'delayed' 1D proton spectrum of Fig. 6.44a (strictly, a delay of $\Delta_{\text{LR}} + 2\delta$ must then be used in the 1D template when gradient pulses are employed for signal selection in HMBC). Alternatively, the one-bond correlation peaks that often appear in HMBC spectra recorded without low-pass filtration also provide template multiplets since each satellite peak of the ${}^1J_{\text{CH}}$ doublets has the identical phase properties of the long-range correlations but itself cannot have the long-range heteronuclear coupling (since the associated proton is attached directly to carbon-13 in this case). These may have the advantage of greater separation when multiplets are buried in the 1D proton spectrum and thus not available as templates. Sequences optimised for the retention of these satellites have also been presented [84] and software suitable for performing the template-fitting procedures has also been made available [85]. Typically, the HMBC experiment is recorded with higher digital resolution in the proton dimension than would be employed for routine HMBC acquisitions so that multiplet fine structure is better defined; a corresponding increase in the t_2 acquisition time by a factor of 4 is usually sufficient. When using multiplets from the 1D spectrum as templates, the 1D digital resolution should match that of the HMBC traces to enable the fitting procedure to operate.

One of the significant advantages of the HMBC approach is its ability to measure coupling constants to both protonated and non-protonated centres, whereas many alternative approaches are limited to protonated carbons only due to the transfer processes involved. Figure 6.45 illustrates the application of HMBC to measure three-bond coupling constants to carbonyl C20 in **6.9**, as highlighted in fragment **6.9a**. The complexity of the HMBC multiplet structures precludes the direct measurement of the long-range heteronuclear couplings, but these may be extracted via the multiplet fitting procedure.

Finally we note that there exist techniques for the measurement of long-range coupling constants derived from the HSQC experiment that aim at producing multiplet structures devoid of the complex phase properties associated with the HMBC method [86]. In the ideal case, crosspeak multiplets contain only in-phase proton homonuclear couplings and the anti-phase heteronuclear couplings of interest such that ${}^nJ_{\text{CH}}$ values can be measured directly from these anti-phase splittings. A number of refinements of the HSQC experiment are required for this to be the case [87, 88], and whilst the direct

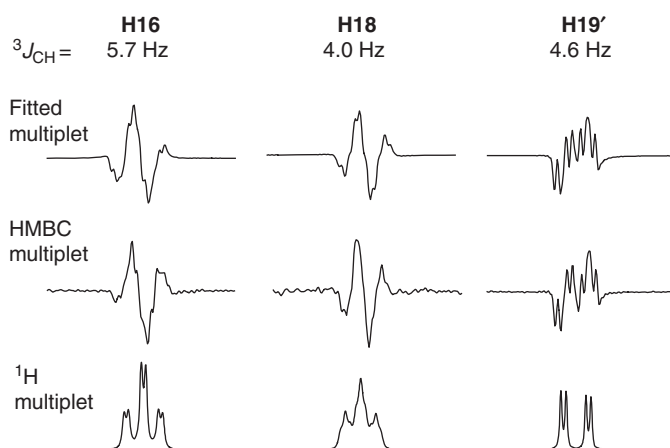
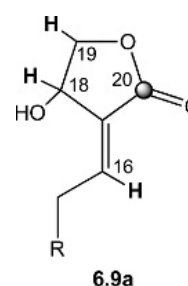


Figure 6.45. Example of experimental and fitted multiplet structures for the long-range ^1H - ^{13}C correlations to carbonyl C20 occurring in the structural fragment **6.9a**. The HMBC spectrum was acquired using the sequence of Fig. 6.44b and the fitting performed as described in [84].

measurement of coupling constants is possible for simple multiplets, more complex structures may still benefit from the fitting procedure described above. Nevertheless, these methods may present attractive alternatives when fitting protocols are neither desirable nor available.

6.5. HETERONUCLEAR X-DETECTED CORRELATION SPECTROSCOPY

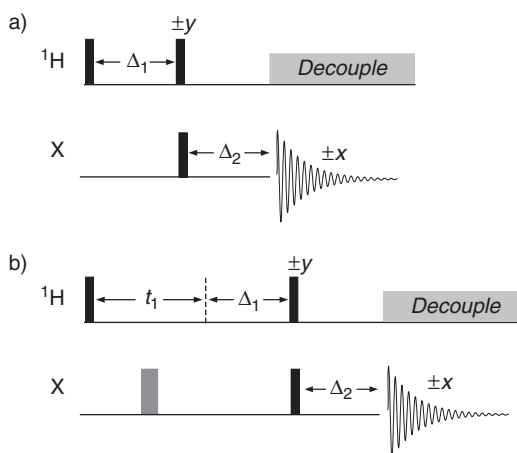
Traditional methods of heteronuclear shift correlation follow the scheme of Fig. 6.1b, relying on the direct observation of the X spin and with the source nucleus (typically protons) detected indirectly. As discussed at the beginning of this chapter, this general approach is less sensitive than that involving the direct observation of the proton, and this has led to the general decline in popularity of the X spin-observed techniques. However, one should not shun their existence completely as they have one major advantage over the proton-detected counterparts and that is the ability to record the X spin with high digital resolution. With proton-detected methods, the often large X spin chemical shift range is measured in the indirect dimension, and recording this with high resolution entails collecting many t_1 incremented data sets, making the experiments time-consuming. With X spin detection, high resolution may be achieved by simply collecting more data points per FID, making a minimal difference to the total duration of the experiment. When heteronuclear correlations are required for a crowded carbon spectrum, complex aromatic systems, for example, the ^{13}C detected approach may thus prove superior. A more mundane reason for turning to these methods may be due to purely practical considerations in as much as your spectrometer may be unable to operate in the 'inverse' mode, that is, it cannot be configured to observe protons but still be able to provide pulsing and decoupling of the heteronucleus. This will be a constraint only for older instruments rather than for those of modern architecture. The sensitivity of these methods can also be increased by the use of microcell or microtube techniques [89] in place of standard NMR tubes and is aided considerably by cryogenic probes with enhanced carbon sensitivity. The following experiments are described in the context of ^1H - ^{13}C but again can be tailored to other spin pairs.



6.5.1. Single-bond correlations

Methods for establishing heteronuclear connectives are based on the general idea of polarisation transfer from the proton to carbon and as such can be understood with reference to the previously encountered 1D INEPT experiment of Section 4.4.2. The refocused INEPT sequence allows the transfer of proton populations (polarisation) onto the attached carbon by the application of simultaneous proton and carbon pulses after the heteronuclear coupling has evolved for a period Δ_1 . Following the transfer, the carbon magnetisation thus created is anti-phase with respect to $^1J_{\text{CH}}$, so it is allowed to evolve under the influence of the heteronuclear coupling for a period Δ_2 until the carbon vectors have realigned. At this point, proton decoupling is applied and the carbon FID recorded (Fig. 6.46a; here the 180° pulses at the midpoint of Δ_1 and Δ_2 , which serve to remove chemical shift evolution, are omitted for simplicity). To produce the 2D shift

Figure 6.46. (a) The 1D-refocused INEPT experiment, shown in simplified form with the refocusing pulses at the midpoints of Δ_1 and Δ_2 removed for clarity. The 2D shift correlation experiment, hetero-COSY (HETCOR) in (b) is derived from INEPT by the addition of the t_1 evolution period to encode proton chemical shifts prior to polarisation transfer. The 180° carbon pulse at the midpoint of t_1 refocuses heteronuclear coupling evolution and thus provides carbon decoupling in f_1 .



correlation experiment [90], the variable t_1 evolution period is added immediately after the initial proton excitation and prior to the polarisation transfer step so that the detected ^{13}C signal becomes modulated by the proton chemical shift as a function of t_1 (Fig. 6.46b). During this, ^1H – ^1H and ^1H – ^{13}C coupling will also evolve, leading to the appearance of both homonuclear and heteronuclear splittings in f_1 , thereby reducing signal intensities. The removal of ^1H – ^{13}C coupling can be achieved by refocusing $^1J_{\text{CH}}$ with the insertion of a 180° carbon pulse at the midpoint of t_1 (preferably applied as a composite pulse to reduce resonance offset effects) (Fig. 6.46b). Because there is no net CH coupling evolution in t_1 , the anti-phase magnetisation required for polarisation transfer develops only during the subsequent Δ_1 period, which is therefore optimised for $\Delta_1 = 1/2^1J_{\text{CH}}$ precisely as for INEPT (typically 3.5 ms for ^1H – ^{13}C correlations). The resulting spectrum displays crosspeaks correlating carbon chemical shifts in f_2 and proton shifts in f_1 , which are further spread by homonuclear proton couplings in f_1 . Figure 6.47 displays a part of the carbon–proton shift correlation spectrum of the palladium complex **6.10**. Despite the extensive crowding in the aromatic region, the carbon shifts are sufficiently dispersed to resolve all correlations (note that some resonances are broadened by restricted dynamic processes within the molecule and some are split by coupling to phosphorus).

The scheme of Fig. 6.46b has been widely used to produce absolute-value shift correlation spectra and is often referred to as HETCOR or hetero-COSY. Conversion to the preferred phase-sensitive equivalent (of which various forms have been investigated [91])

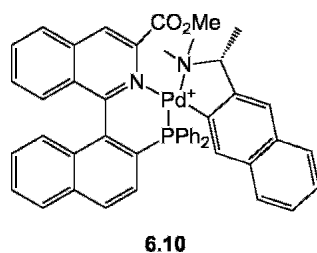
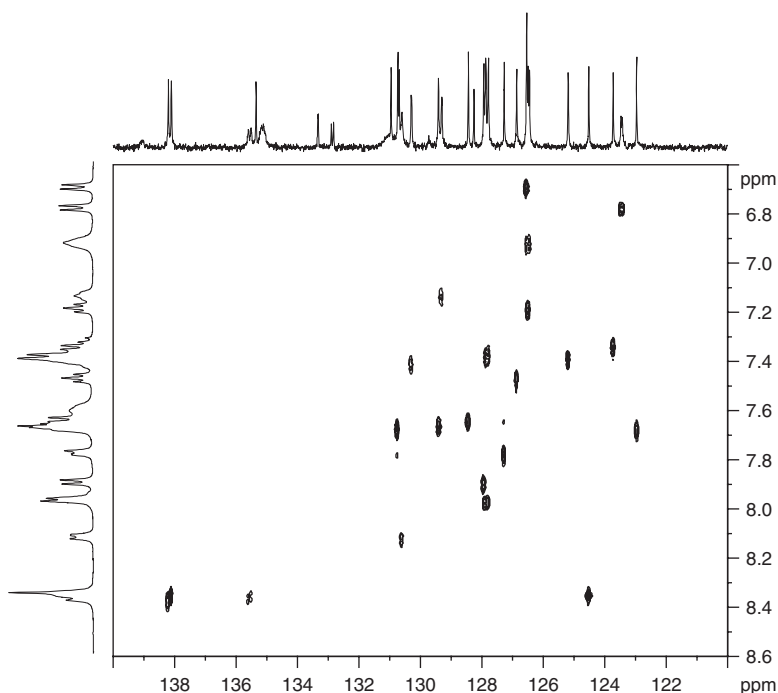


Figure 6.47. The aromatic region of the absolute-value ^{13}C – ^1H hetero-COSY (HETCOR) spectrum (500 MHz) of the palladium complex **6.10**. 2 K data points were collected for $256t_1$ increments of 128 transients each for a 40×2.6 -ppm window. Unshifted sine bells were applied in both dimensions and after zero filling once in each, the digital resolution was 2.5 and 5.0 Hz/pt in f_2 and f_1 , respectively.



requires the reintroduction of the simultaneous $180^\circ(^1\text{H}, ^{13}\text{C})$ pulses into the midpoints of both Δ_1 and Δ_2 to remove chemical shift evolution during these periods, exactly as in the full refocused INEPT. In addition, the incorporation of the States or TPPI phase cycling of the 90° proton pulse of the polarisation transfer step is required. Suppression of axial peaks is through the phase alternation of the final proton pulse together with the receiver (which is equivalent to that used in INEPT to suppress contributions from the natural X spin magnetisation). As for the INEPT sequence, editing of the phase-sensitive spectrum is possible by the judicious choice of the Δ_2 refocusing delay, for example, setting $\Delta_2 = 1/1.5J_{\text{CH}}$ produces a spectrum in which responses from CH_2 groups are inverted. For routine application, selecting $\Delta_2 = 1/3J_{\text{CH}}$ (typically 2.3 ms for $^1\text{H}-^{13}\text{C}$) provides positive intensities for all protonated carbons.

Since polarisation transfer is employed, the experiment repetition rate is dictated by the recovery of the faster-relaxing proton spins, and repetition times should be around 1.3 times the proton T_1 s. Resolution in the proton dimension can be quite low for routine applications since one does not usually wish to resolve the proton fine structure, and because the homonuclear coupling is in-phase, there is no fear of signal cancellation; f_1 digital resolution may therefore be as low as 10 Hz/pt or so, requiring rather few t_1 increments. The number of scans per increment should be set such that the carbon resonances of interest can just be observed in the spectrum of the first-recorded FID (which is equivalent to the 1D INEPT experiment).

Homonuclear decoupling in f_1

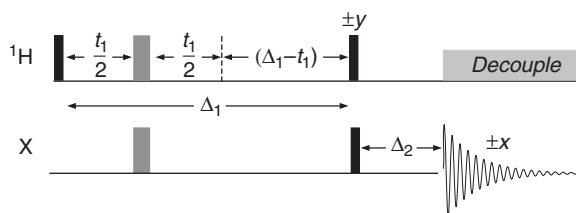
By introducing a slight modification to the sequences presented above, it is possible to achieve (almost complete) removal of homonuclear proton couplings from f_1 also, thus removing the multiplet spread and increasing both sensitivity and f_1 resolution. To do this, an approach similar to that for the removal of the *heteronuclear* coupling in f_1 described above is employed, that is, for each coupled spin pair, one must invert, at the midpoint of t_1 , *only one* of the two spins so that the coupling evolution is refocused at the end of t_1 . For the heteronuclear case, this is trivial, since one can apply a 180° carbon pulse confident in the knowledge that its proton partner will be unaffected. However, applying a single-proton 180° pulse will fail since both protons experience this pulse and the coupling will continue to evolve, as for a homonuclear spin-echo. Still worse, proton chemical shifts will be refocused during t_1 , destroying the proton shift dimension altogether (although undesirable in this application, such an approach can be useful if one wishes to analyse only coupling patterns in f_1 ; see Chapter 7 on *J*-resolved spectroscopy). What is required then is a means of inverting only the *remote J*-coupled neighbours of a *directly* ^{13}C -bound proton (which is ultimately involved in the polarisation transfer step). The solution to this seemingly impossible task lies in the application of the BIRD sequence [17] already introduced in Section 6.3.3 as a means of selectively inverting ^{12}C -bound protons whilst leaving ^{13}C -bound protons unaffected [92, 93]. The differentiation of protons in the context of f_1 decoupling relies on the very different magnitudes of the one-bond and long-range couplings ($^1J_{\text{CH}} \gg ^{2/3}J_{\text{CH}}$) such that the long-range couplings can be considered negligible. Hence, placing the BIRD cluster at the midpoint of t_1 refocuses proton–proton coupling whilst the carbon 180° pulse of BIRD also serves to refocus proton–carbon coupling, as above (hence BIRD; bilinear rotational *decoupling*). The f_1 resonances then appear as singlets with the exception of those from non-equivalent geminal protons, which retain their mutual coupling (as neither proton experiences the inversion since both are bound to ^{13}C) [94].

6.5.2. Multiple-bond correlations and small couplings

Numerous X-detected sequences have been presented over the years for establishing long-range $^1\text{H}-^{13}\text{C}$ correlations [95] prior to the widespread adoption of the proton-detected counterparts. This section presents the most widely used sequence (COReLation through LOng-range Coupling, COLOC) and briefly mentions more recent sequences that have superior performance in most instances.

A direct way to obtain long-range correlations is to optimise the *J*-delay periods found in the one-bond HETCOR sequence above for the much smaller long-range couplings. The potential drawback of this approach is the total length of the resulting sequence because of the small couplings and the associated signal losses due to spin relaxation. An early modification, known as COLOC, places the t_1 period *within* the Δ_1 delay and thus reduces the overall length of the sequence (Fig. 6.48) [96, 97]. The t_1 period is now defined by a pair of $180^\circ(\text{H}, \text{C})$ pulses that move through Δ_1 as t_1 is incremented, presenting another example of the ‘constant-time’ experiments. The detected signal is modulated by proton shifts because, although these are refocused during t_1 , they continue to evolve in the *remainder* of the Δ_1 period, which itself is

Figure 6.48. The COLOC sequence for establishing long-range correlations through X spin observation.



dependent on t_1 , thus allowing characterisation of the proton chemical shifts. In contrast, homonuclear proton couplings evolve for the whole of the fixed Δ_1 period regardless of the position of the moving 180° proton pulse, meaning these couplings cause no modulation as a function of t_1 and the f_1 dimension conveniently displays only proton chemical shifts. Transfers due to one-bond couplings may also appear in the COLOC spectrum if the Δ_1 and Δ_2 periods happen to be multiples of the appropriate $^1J_{\text{CH}}$ values, and, if desired, the inclusion of a low-pass J -filter, as described for HMBC above, may help attenuate these [98].

A greater problem with this sequence is the influence of one-bond couplings during the Δ_2 refocusing period, which can lead to the disappearance of a long-range crosspeak even when the Δ_1 and Δ_2 delays are optimised for the long-range couplings [99]. Following the polarisation transfer step, the vectors associated with the *one-bond* couplings of the carbon nuclei are in-phase (they are *passive* couplings since they have not been responsible for the polarisation transfer process), and during the subsequent Δ_2 period, they will pass in-phase and out-of-phase a number of times under the influence of $^1J_{\text{CH}}$. If these happen, coincidentally, to have an anti-phase disposition, when the acquisition starts and proton decoupling is applied, the vectors will cancel and the crosspeak will be lost. In any case, the detected long-range correlation intensity will be modulated by the one-bond H–C coupling of the carbon. To remove these effects, one must ensure that these vectors are returned to their original in-phase disposition at the end of Δ_2 but at the same time allow the initially anti-phase vectors due to *long-range* couplings to continue to evolve and become in-phase. Once again, this can be achieved by positioning a BIRD sequence at the midpoint of Δ_2 , which inverts the carbon and its long-range coupled proton but not the directly coupled proton [100, 101]. The use of the BIRD cluster in this manner is recommended for all X-detected long-range correlation methods.

In general, optimisation of the Δ_1 and Δ_2 delays for the COLOC experiment can be problematic. In addition to the one-bond modulation effects already described, the transfer efficiency is also influenced by homonuclear proton couplings evolving during Δ_1 . The fixed Δ_1 period also limits $t_{1(\text{max})}$ ($t_{1(\text{max})}/2 \leq \Delta_1$), which may dictate fewer t_1 increments be collected than would otherwise be desirable, thus reducing the digital resolution of the f_1 dimension. These problems have been addressed in some detail in 1D sequences presented for parameter optimisation [102]. For routine use, it has been suggested [95] that optimising delays for $^nJ_{\text{CH}} = 10$ Hz should provide most responses of interest, particularly when using the BIRD sequence within the Δ_2 refocusing delay. This corresponds to delays of 50 ms ($1/2^n J_{\text{CH}}$) and 33 ms ($1/3^n J_{\text{CH}}$) for Δ_1 and Δ_2 , respectively.

Various other sequences that generally show improved performance over COLOC have been proposed over the years. If heterospin-detected methods are best suited to the study of your molecules, two sequences to note are XCORFE [103] and FLOCK [104], details of which can be found in the original literature.

6.6. HETERONUCLEAR X–Y CORRELATIONS

The methods presented so far in this chapter have focussed principally on the correlation of two nuclei, typically ^1H and a single heteronucleus X. There are, however, areas of chemistry in which it proves advantageous to correlate, or at least utilise, coupling interactions between two heteronuclei, such experiments being often referred to as *heteronuclear X–Y correlations*. Most examples of this come from studies of organometallic chemistry, in which correlations with a metal centre are sought, or from non-metallic organoelement compounds, in which multiple NMR-active heteroatoms exist to be correlated. Likewise, most examples have both X and Y as spin- $1/2$ nuclei. Methods suitable for such studies may be classified into two groups: those that employ the direct observation of one of the heteroatoms and those that employ proton detection, most often for reasons of sensitivity, both of which will be considered briefly below. Both classes require the spectrometer to possess (at least) three rf channels and a triple-resonance probe that can be tuned to all the nuclei involved and hence are not accessible on standard two-channel instruments. However, with increasing access to three-channel spectrometers and the wider range of triple-resonance probes available, such methods can be expected to provide

additional routes to structural determination and the study of chemical transformations. Probe configurations such as ^1H - ^{13}C -Broadband, ^1H - ^{31}P -Broadband or custom-designed ^1H -X-Y combinations are suitable for such work. Some of the practical considerations of these methods and the range of techniques adapted for such studies have been reviewed extensively and numerous examples given [105–107].

6.6.1. Direct X–Y correlations

Direct observation of correlations between two different nuclides can, in principle, be achieved using the 2D methods already described in this chapter [108] and do not necessarily require proton detection, as long as the observed nucleus exists at high abundance. Indeed, such methods may become necessary in proton-sparse systems and may even provide greater sensitivity by utilising large J_{XY} coupling constants in situations when only small, long-range protons couplings to the heteroatoms exist. In such cases, the HMQC experiment can be employed with the observation of the nuclide of greater sensitivity X (combined with optional Y spin decoupling), with indirect detection of nucleus Y and, typically, with broadband proton decoupling operating throughout the sequence (Fig. 6.49). As an illustration, Fig. 6.50 shows a phosphorus-detected ^{31}P - ^{13}C correlation of **6.11** acquired in this manner but without carbon-13 decoupling during the detection period; the correlation thus appears as a $^1J_{\text{CP}}$ doublet in f_2 . The use of ^{31}P as the observed nucleus X is well documented due to its favourable properties and many examples of this may be found, including ^{31}P correlations with ^{103}Rh , ^{109}Ag , ^{119}Sn and ^{183}W to name a few [109]. Inverse-correlation methods can therefore provide access to chemical shift data of nuclei that are otherwise too insensitive to be observed directly. This has particular significance for the indirect observation of metals, many of which have very low intrinsic sensitivity but can nevertheless be detected through their spin-coupled neighbours on adjacent ligands [107]. The indirect observation of ^{57}Fe , a notoriously difficult nucleus to observe directly, is one more example [110–112] (Fig. 6.51).

The HMQC sequence in particular is favoured for such correlations because of its robustness and because the phase-cycled version can be collected without the need for 180° pulses on the Y-channel. This is beneficial when the Y nucleus chemical shifts cover a wide range, notably the case for metals again, where off-resonance losses from the 180° Y pulse could be highly detrimental. When pulsed field gradients are desirable for optimal signal suppression, the use of composite or adiabatic 180° pulses within the gradient echoes may prove beneficial for both HMQC and HSQC. When the heteronuclear couplings vary widely in magnitude, optimal detection of all correlations can be problematic and the ACCORDION-

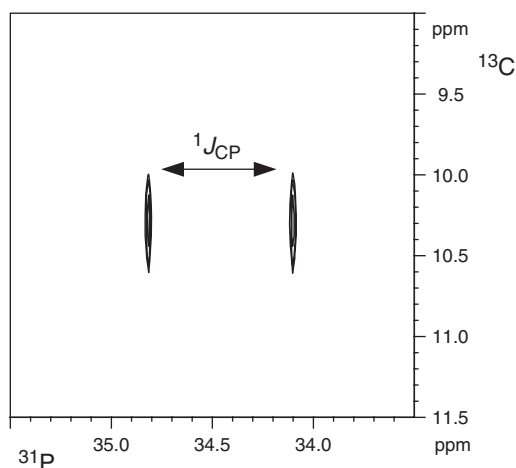
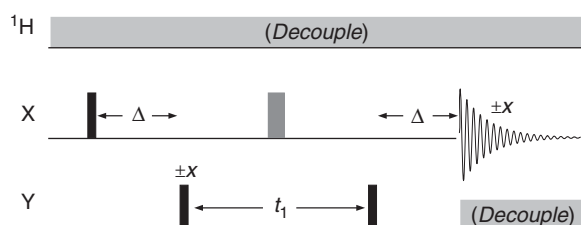
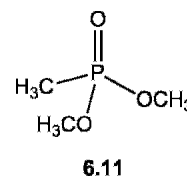
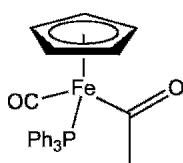


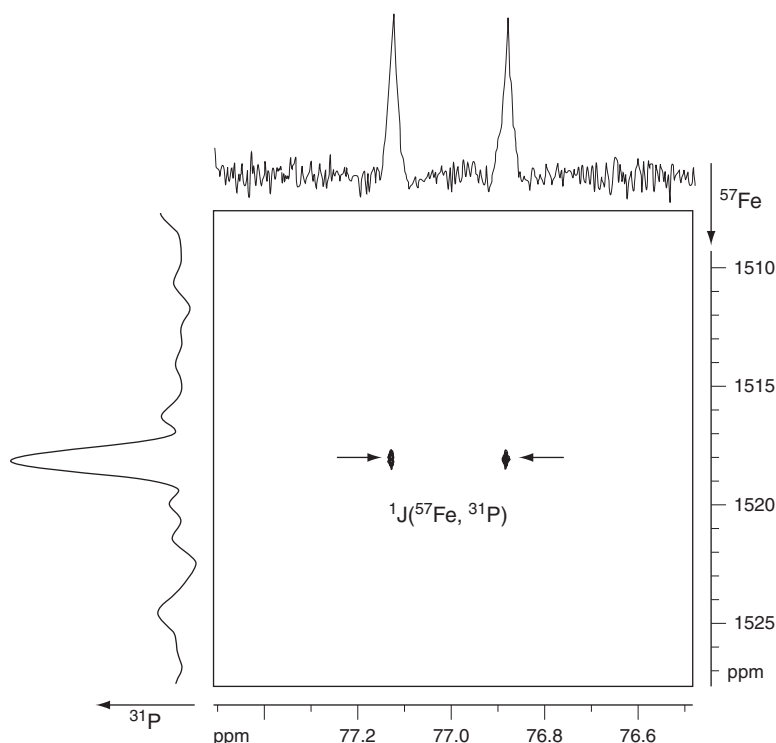
Figure 6.49. The HMQC sequence for X–Y correlations in the presence of broadband proton decoupling. Pulsed field gradients may be incorporated as for the standard 2D HMQC experiment.

Figure 6.50. Phosphorus-detected ^{31}P - ^{13}C HMQC spectrum of **6.11**. Proton decoupling was applied throughout but no carbon-13 decoupling was used during detection of the phosphorus FID. These data were acquired using a home-built third channel and a probe modified for triple-channel operation.

Figure 6.51. Phosphorus-detected, proton-decoupled ^{31}P - ^{57}Fe HMQC spectrum of **6.12**. The traces are projections taken from the 2D spectrum (reproduced with permission from [112]).



6.12

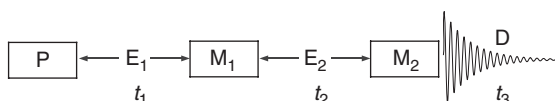


optimised HMBC sequences described previously may then be adapted for X–Y correlations. One such example has been the application of the IMPEACH-MBC experiment in the observation of ^{19}F – ^{15}N correlations where the $^{2/3}J_{\text{FN}}$ couplings ranged between 4 and 50 Hz [113].

6.6.2. Indirect ^1H -detected X–Y correlations

It is perhaps not surprising that more recent developments for identifying X–Y correlations have incorporated proton detection and pulsed field gradients for signal selection, with many of the techniques being derived from the triple-resonance ^1H – ^{13}C – ^{15}N techniques originally designed for the study of biological macromolecules [114]. These were conceived as 3D methods that can be derived conceptually from their 2D counterparts by the incorporation of another variable evolution time period and associated mixing steps (Fig. 6.52). The additional evolution period is incremented in a stepwise manner independently of the others, thus yielding three independent time domains t_1 , t_2 and t_3 , which ultimately transform to three frequency domains f_1 , f_2 and f_3 . Such methods result in 3D data cubes in which crosspeaks are dispersed throughout according to the three chemical shifts of the participating spins and so have enormous power in dispersing the numerous correlation peaks that arise in the spectra of isotopically labelled proteins. Although 3D sequences have been presented for ^1H – ^{13}C – ^{31}P correlations, these find little use in the study of smaller organic molecules or their complexes for two principal reasons. Firstly, the need to sample two *indirect* time domains for 3D spectra invariably leads to longer experiment times and, secondly, since there are typically rather few heteroatom centres in small molecules, there is little need for the additional dispersion provided by 3D experiments. Rather, most ^1H –X/Y correlation experiments are recorded as 2D spectra correlating ^1H and Y with the X heteroatom acting in a spectrum editing or magnetisation relay capacity, signified by the notation ^1H –(X)–Y. Such methods may be derived from their 3D parent by simply making static one of the incremented time

Figure 6.52. The general scheme for 3D pulse sequences, by analogy with that shown for 2D sequences in Fig. 5.1 (Chapter 5). In the 3D techniques, there exist two indirectly measured time domains t_1 and t_2 and a third t_3 in which the FID is recorded.



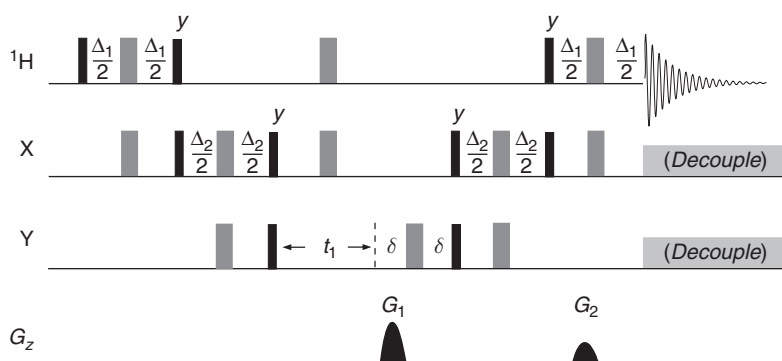
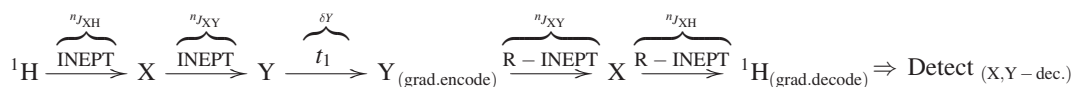


Figure 6.53. A triple-resonance sequence for recording 2D ^1H –(X)–Y heteronuclear correlations. The delays are set to $\Delta_1 = 1/2^n J_{\text{HX}}$ and $\Delta_2 = 1/2^n J_{\text{XY}}$ with gradient strengths set as $G_2 = G_1(\gamma_{\text{Y}}/\gamma_{\text{H}})$.

delays, thus using the corresponding nucleus for the relaying of coherence rather than for the indirect sampling of its chemical shift.

One such approach that illustrates this principle is shown in Fig. 6.53, which may be viewed as an extended HSQC experiment now employing two transfer steps between nuclei [115, 116]. In this case, the transfers are made with INEPT stages sequentially from ^1H to X and then onto Y for encoding in t_1 . Gradient selection is made on the Y nucleus (which may also provide for echo–antiecho selection for f_1 quadrature detection), and then reverse INEPT stages transfer magnetisation back onto X and finally onto ^1H for gradient decoding and then detection, optionally with X and/or Y spin decoupling. Following the notation used for HSQC in Section 6.3.2 above, the overall transfer process may be summarised as follows:



The 2D spectrum therefore correlates ^1H with the Y spin via a pathway involving both nJ_{XH} and nJ_{XY} couplings, where n is 1 or greater according to the available coupling pathways. The ‘relay’ nucleus is frequently ^{13}C or ^{31}P with the Y-nucleus representing a more exotic spin, including, for example, ^{15}N , ^{183}W [116] and ^{195}Pt [115]. Fig. 6.54 illustrates the ^1H –(^{13}C)– ^{195}Pt correlation spectrum of a mixture of the *E*- and *Z*-isomers of the thiourea platinum complexes **6.13** using the sequence of Fig. 6.53. Magnetisation transfer steps made use of $^1J_{\text{CH}}$ and $^4J_{\text{CPt}}$ couplings, and the assignment of the *E*- and *Z*-isomers was based on the observation that significant $^4J_{\text{CPt}}$ couplings of $\sim 30\text{ Hz}$ existed only when the carbon of the *N*-alkyl branch existed in a favourable ‘W’ configuration with the Pt centre, as represented in bold in the structures (any possible $^5J_{\text{HPt}}$ couplings were not observed and thus could not be employed in

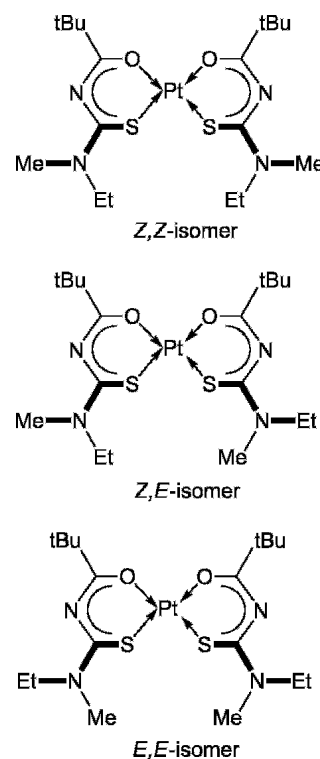
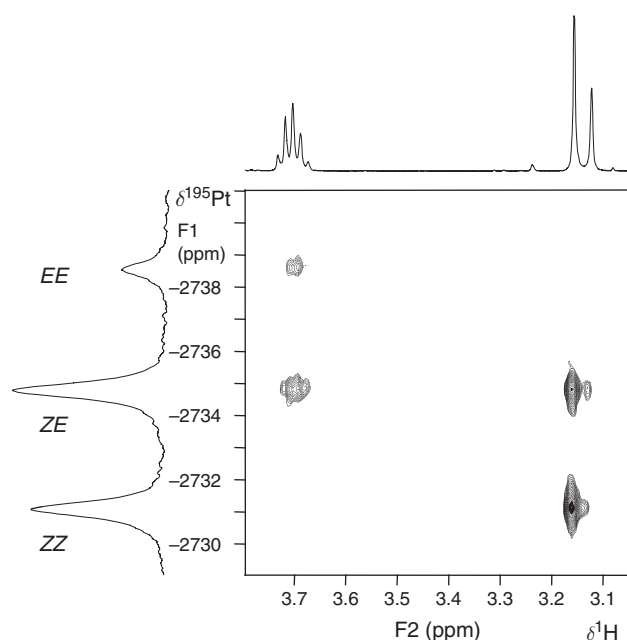
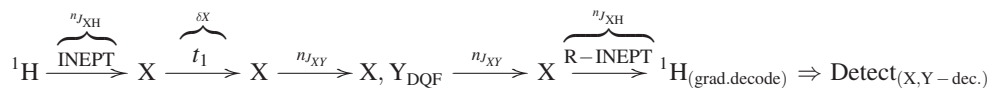


Figure 6.54. The ^1H -detected ^1H –(^{13}C)– ^{195}Pt correlation spectrum of *E/Z* mixtures of the thiourea platinum complexes of **6.13** (reproduced with permission from [115], Copyright 2003, John Wiley & Sons Limited).

HMBC-based experiments). From the 2D spectrum it was possible to directly assign the platinum resonances of the different isomers and hence determine the isomer ratios. These data were collected on a triple-channel instrument equipped with a ^1H - ^{13}C -Broadband probe for which the broadbanded channel had been tuned to ^{195}Pt .

An alternative approach, referred to as gsSELTRIP (*selective triple resonance*) [117], correlates ^1H and X spins with the Y spin serving to *edit* the resulting 2D spectrum according to the presence of X–Y coupling. Thus, one may produce a ^1H - ^{13}C correlation spectrum ($\text{X} = ^{13}\text{C}$) with the same appearance as a standard ^1H - ^{13}C HSQC in which correlations appear only for those carbons that are themselves *also* coupled to ^{31}P ($\text{Y} = ^{31}\text{P}$). The transfer process for this may be summarised as follows:



where X, Y_{DQF} represents a gradient-based filter element that selects for double-quantum X–Y coherence. This provides one potential approach to simplifying the often crowded aromatic region of the ^1H - ^{13}C correlation spectra of aromatic organometallic phosphorus complexes, for example. In this regard, it should also be noted that the ^1H - ^{31}P HMBC experiment provides a very sensitive method to aid in the assignment of proton spectra by utilising long-range proton–phosphorus couplings and benefiting from the high natural abundance of ^{31}P .

REFERENCES

- [1] L. Müller, *J. Am. Chem. Soc.*, 1979, **101**, 4481–4484.
- [2] A. Bax, R.H. Griffey and B.L. Hawkins, *J. Magn. Reson.*, 1983, **55**, 301–315.
- [3] A. Bax and S. Subramanian, *J. Magn. Reson.*, 1986, **67**, 565–569.
- [4] J. Cavanagh and J. Keeler, *J. Magn. Reson.*, 1988, **77**, 356–362.
- [5] G. Bodenhausen and D.J. Ruben, *Chem. Phys. Lett.*, 1980, **69**, 185–189.
- [6] W.F. Reynolds, S. McLean, L.-L. Tay, M. Yu, R.G. Enriquez, D.M. Estwick and K.O. Pascoe, *Magn. Reson. Chem.*, 1997, **35**, 455–462.
- [7] T. Parella, *Magn. Reson. Chem.*, 1998, **36**, 467–495.
- [8] J. Ruiz-Cabello, G.W. Vuister, C.T.W. Moonen, P.v. Gelderen, J.S. Cohen and P.C.M. v. Zijl, *J. Magn. Reson.*, 1992, **100**, 282–302.
- [9] G.W. Vuister, J. Ruiz-Cabello and P.C.M. v. Zijl, *J. Magn. Reson.*, 1992, **100**, 215–220.
- [10] W. Willker, D. Leibfritz, R. Kersebaum and W. Bermel, *Magn. Reson. Chem.*, 1993, **31**, 287–292.
- [11] J.R. Tolman, J. Chung and J.H. Prestegard, *J. Magn. Reson.*, 1992, **98**, 462–467.
- [12] A.L. Davis, J. Keeler, E.D. Laue and D. Moskau, *J. Magn. Reson.*, 1992, **98**, 207–216.
- [13] J. Boyd, N. Soffe, B. John, D. Plant and R. Hurd, *J. Magn. Reson.*, 1992, **98**, 660–664.
- [14] A.G. Palmer, J. Cavanagh, P.E. Wright and M. Rance, *J. Magn. Reson.*, 1991, **93**, 151–170.
- [15] L.E. Kay, P. Keifer and T. Saarinen, *J. Am. Chem. Soc.*, 1992, **114**, 10663–10665.
- [16] J. Schleucher, M. Schwendinger, M. Sattler, P. Schmidt, O. Schedletsky, S.J. Glaser, O.W. Sørensen and C. Griesinger, *J. Biomol. NMR*, 1994, **4**, 301–306.
- [17] J.R. Garbow, D.P. Weitekamp and A. Pines, *Chem. Phys. Lett.*, 1982, **93**, 504–508.
- [18] G. Otting and K. Wüthrich, *J. Magn. Reson.*, 1988, **76**, 569–574.
- [19] W.F. Reynolds, M. Yu, R.G. Enriquez and I. Leon, *Magn. Reson. Chem.*, 1997, **35**, 505–519.
- [20] W.F. Reynolds and R.G. Enriquez, *Magn. Reson. Chem.*, 2003, **41**, 927–932.
- [21] D. Jeannerat, *Magn. Reson. Chem.*, 2000, **38**, 415–422.
- [22] D. Jeannerat, *Magn. Reson. Chem.*, 2003, **41**, 3–17.
- [23] D. Jeannerat, *J. Magn. Reson.*, 2007, **186**, 112–122.
- [24] H. Kessler, P. Schneider and M. Kurz, *J. Magn. Reson.*, 1989, **85**, 400–405.
- [25] T. Parella, F. Sánchez-Ferrando and A. Virgili, *J. Magn. Reson. Ser. A*, 1995, **117**, 78–83.
- [26] S.-I. Tate, Y. Masui and F. Inagaki, *J. Magn. Reson.*, 1991, **94**, 625–630.
- [27] T. Parella, F. Sánchez-Ferrando and A. Virgili, *J. Magn. Reson.*, 1997, **126**, 274–277.
- [28] T. Parella, J. Belloc, F. Sánchez-Ferrando and A. Virgili, *Magn. Reson. Chem.*, 1998, **36**, 715–719.
- [29] T. Domke and D. Leibfritz, *J. Magn. Reson.*, 1990, **88**, 401–405.
- [30] J. Stelten and D. Leibfritz, *J. Magn. Reson.*, 1992, **99**, 170–177.
- [31] J. Stelten and D. Leibfritz, *Magn. Reson. Chem.*, 1996, **34**, 951–954.
- [32] M. Liu, R.D. Farrant, J.K. Nicholson and J.C. Lindon, *J. Magn. Reson. Ser. B*, 1995, **106**, 270–278.
- [33] J.M. Schmidt and H. Rüterjans, *J. Am. Chem. Soc.*, 1990, **112**, 1279–1280.
- [34] H. Kessler, P. Schneider and H. Oshkinat, *J. Am. Chem. Soc.*, 1990, **112**, 8599–8600.
- [35] U. Wollborn and D. Leibfritz, *Magn. Reson. Chem.*, 1991, **29**, 238–243.
- [36] L. Lerner and A. Bax, *J. Magn. Reson.*, 1986, **69**, 375–380.
- [37] D.G. Davis, *J. Magn. Reson.*, 1989, **84**, 417–424.
- [38] T. Domke, *J. Magn. Reson.*, 1991, **95**, 174–177.
- [39] B.K. John, D. Plant, S.L. Heald and R.E. Hurd, *J. Magn. Reson.*, 1991, **94**, 664–669.
- [40] G. Mackin and A.J. Shaka, *J. Magn. Reson. Ser. A*, 1996, **118**, 247–255.
- [41] R.C. Crouch, A.O. Davis and G.E. Martin, *Magn. Reson. Chem.*, 1995, **33**, 889–892.

- [42] T. Parella, F. Sánchez-Ferrando and A. Virgili, *J. Magn. Reson. Ser B*, 1995, **109**, 88–92.
- [43] A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, 1986, **108**, 2093–2094.
- [44] M.F. Summers, L.G. Marzilli and A. Bax, *J. Am. Chem. Soc.*, 1986, **108**, 4285–4294.
- [45] D.O. Cicero, G. Barbato and R. Bazzo, *J. Magn. Reson.*, 2001, **148**, 209–213.
- [46] A. Bax and D. Marion, *J. Magn. Reson.*, 1988, **78**, 186–191.
- [47] P.L. Rinaldi and P.A. Keifer, *J. Magn. Reson. Ser A*, 1994, **108**, 259–262.
- [48] P.E. Hansen, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1981, **14**, 179–296.
- [49] J.L. Marshall, *Carbon-Carbon and Carbon-Proton NMR Couplings: Applications to Organic Stereochemistry and Conformational Analysis*, VCH, Florida, 1983.
- [50] V.F. Bystrov, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1976, **10**, 41–81.
- [51] R.H. Contreras and J.E. Peralta, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2000, **37**, 321–425.
- [52] J.E. Baldwin, T.D.W. Claridge, K.C. Goh, J.W. Keeping and C.J. Schofield, *Tetrahedron Lett.*, 1993, **34**, 5645–5648.
- [53] D.M. Hodgson and P.J. Comina, *Tetrahedron Lett.*, 1996, **37**, 5613–5614.
- [54] H. Kogler, O.W. Sørensen, G. Bodenhausen and R.R. Ernst, *J. Magn. Reson.*, 1983, **55**, 157–163.
- [55] A. Meissner and O.W. Sørensen, *Magn. Reson. Chem.*, 2000, **38**, 981–984.
- [56] R. Burger, C. Schorn and P. Bigler, *Magn. Reson. Chem.*, 2000, **38**, 963–969.
- [57] D. Uhrin, T. Liptaj and K.E. Kover, *J. Magn. Reson. Ser A*, 1993, **101**, 41–46.
- [58] K. Furihata and H. Seto, *Tetrahedron Lett.*, 1998, **39**, 7337–7340.
- [59] H. Kessler, P. Schmeider, M. Köck and M. Kurz, *J. Magn. Reson.*, 1990, **88**, 615–618.
- [60] C. Gaillat, C. Lequart, P. Debeire and J.-M. Nuzillard, *J. Magn. Reson.*, 1999, **139**, 454–459.
- [61] T.D.W. Claridge and I. Pérez-Victoria, *Org. Biomol. Chem.*, 2003, **1**, 3632–3634.
- [62] G.E. Martin, *Annu. Rep. NMR. Spectrosc.*, 2002, **46**, 37–100.
- [63] R. Wagner and S. Berger, *Magn. Reson. Chem.*, 1998, **36**, S44–S46.
- [64] G. Bodenhausen and R.R. Ernst, *J. Am. Chem. Soc.*, 1982, **104**, 1304–1309.
- [65] G.E. Martin, C.E. Hadden, R.C. Crouch and V.V. Krishnamurthy, *Magn. Reson. Chem.*, 1999, **37**, 517–528.
- [66] C.E. Hadden, G.E. Martin and V.V. Krishnamurthy, *J. Magn. Reson.*, 1999, **140**, 274–280.
- [67] C.E. Hadden, G.E. Martin and V.V. Krishnamurthy, *Magn. Reson. Chem.*, 2000, **38**, 143–147.
- [68] R. Araya-Maturana, T. Delgado-Castro, W. Cardona and B.E. Weiss-Lopez, *Curr. Org. Chem.*, 2001, **5**, 253–263.
- [69] J. Furrer, *Magn. Reson. Chem.*, 2006, **44**, 845–850.
- [70] G.E. Martin and C.E. Hadden, *Magn. Reson. Chem.*, 2000, **38**, 251–256.
- [71] M. Kline and S. Cheatham, *Magn. Reson. Chem.*, 2003, **41**, 307–314.
- [72] G.E. Martin and C.E. Hadden, *J. Nat. Prod.*, 2000, **63**, 543–585.
- [73] K. Zangger and I.M. Armitage, *Magn. Reson. Chem.*, 2000, **38**, 452–458.
- [74] C.E. Hadden and D.T. Angwin, *Magn. Reson. Chem.*, 2001, **39**, 1–8.
- [75] V.V. Krishnamurthy, D.J. Russell, C.E. Hadden and G.E. Martin, *J. Magn. Reson.*, 2000, **146**, 232–239.
- [76] T. Sprang and P. Bigler, *Magn. Reson. Chem.*, 2003, **41**, 177–182.
- [77] N.T. Nyberg, J.O. Duus and O.W. Sørensen, *J. Am. Chem. Soc.*, 2005, **127**, 6154–6155.
- [78] N.T. Nyberg, J.Ø. Duus and O.W. Sørensen, *Magn. Reson. Chem.*, 2005, **43**, 971–974.
- [79] B.O. Petersen, E. Vinogradov, W. Kay, P. Würtz, N.T. Nyberg, J.Ø. Duus and O.W. Sørensen, *Carbohydr. Res.*, 2006, **341**, 550–556.
- [80] M. Kock, B. Reif, W. Fenical and C. Griesinger, *Tetrahedron Lett.*, 1996, **37**, 363–366.
- [81] B.L. Marquez, W.H. Gerwick and R.T. Williamson, *Magn. Reson. Chem.*, 2001, **39**, 499–530.
- [82] N. Matsumori, D. Kaneno, M. Murata, H. Nakamura and K. Tachibana, *J. Org. Chem.*, 1999, **64**, 866–876.
- [83] G.J. Sharman, *Magn. Reson. Chem.*, 2007, **45**, 317–324.
- [84] R.A.E. Edden and J. Keeler, *J. Magn. Reson.*, 2004, **166**, 53–68.
- [85] J. Keeler, HMBC fitting routine, <http://www-keeler.ch.cam.ac.uk>, 2004
- [86] R.T. Williamson, B.L. Marquez, W.H. Gerwick and K.E. Kover, *Magn. Reson. Chem.*, 2000, **38**, 265–273.
- [87] H. Koskela, I. Kilpelainen and S. Heikkinen, *J. Magn. Reson.*, 2003, **164**, 228–232.
- [88] K.E. Kövér, G. Batta and K. Fehér, *J. Magn. Reson.*, 2006, **181**, 89–97.
- [89] R.W. Dykstra, *J. Magn. Reson. Ser A*, 1995, **112**, 255–257.
- [90] A. Bax and G.A. Morris, *J. Magn. Reson.*, 1981, **42**, 501–505.
- [91] K.A. Carpenter and W.F. Reynolds, *Magn. Reson. Chem.*, 1992, **30**, 287–294.
- [92] A. Bax, *J. Magn. Reson.*, 1983, **53**, 517–520.
- [93] J.A. Wilde and P.H. Bolton, *J. Magn. Reson.*, 1984, **59**, 343–346.
- [94] V. Rutar, *J. Magn. Reson.*, 1984, **58**, 306–310.
- [95] G.E. Martin and A.S. Zektzer, *Magn. Reson. Chem.*, 1988, **26**, 631–652.
- [96] H. Kessler, C. Griesinger, J. Zarbock and H.R. Loosli, *J. Magn. Reson.*, 1984, **57**, 331–336.
- [97] H. Kessler, C. Griesinger and J. Lautz, *Angew. Chem. Int. Ed.*, 1984, **23**, 444–445.
- [98] M. Salazar, A.S. Zektzer and G.E. Martin, *Magn. Reson. Chem.*, 1988, **26**, 28–32.
- [99] M.J. Quast, A.S. Zektzer, G.E. Martin and R.N. Castle, *J. Magn. Reson.*, 1987, **71**, 554–560.
- [100] C. Bauer, R. Freeman and S. Wimperis, *J. Magn. Reson.*, 1984, **58**, 526–532.
- [101] V.V. Krishnamurthy and J.E. Casida, *Magn. Reson. Chem.*, 1987, **25**, 837–842.
- [102] M. Perpich-Dumont, R.G. Enriquez, S. McLean, F.V. Puzzuoli and W.F. Reynolds, *J. Magn. Reson.*, 1987, **75**, 414–426.
- [103] W.F. Reynolds, D.W. Hughes, M. Perpich-Dumont and R.G. Enriquez, *J. Magn. Reson.*, 1985, **63**, 413–417.
- [104] W.F. Reynolds, S. McLean, M. Perpich-Dumont and R.G. Enriquez, *Magn. Reson. Chem.*, 1989, **27**, 162–169.
- [105] D. Gudat, *Annu. Rep. NMR. Spectrosc.*, 1999, **38**, 139–202.

- [106] D. Gudat, *Annu. Rep. NMR. Spectrosc.*, 2003, **51**, 59–103.
- [107] J.A. Iggo, J. Liu and G. Overend, *Annu. Rep. NMR. Spectrosc.*, 2008, **63**, 191–274.
- [108] S. Berger, T. Facke and R. Wagner, *Magn. Reson. Chem.*, 1996, **34**, 4–13.
- [109] F. Lopez-Ortiz and R.J. Carbajo, *Curr. Org. Chem.*, 1998, **2**, 97–130.
- [110] R. Benn, H. Brenneke, A. Frings, H. Lehmkuhl, G. Mehler, A. Rufunski and T. Wildt, *J. Am. Chem. Soc.*, 1988, **110**, 5661–5668.
- [111] E.J.M. Meier, W. Kozminski, A. Linden, P. Lustenberger and W.v. Phillipsborn, *Organometallics*, 1996, **15**, 2469–2477.
- [112] D. Nanz, A. Bell, W. Kozminski, E.J.M. Meier, V. Tedesco and W.v. Phillipsborn, *Bruker Rep.*, 1996, **143**, 29–31.
- [113] D.J. Russell, C.E. Hadden, G.E. Martin and K. Krishnamurthy, *Magn. Reson. Chem.*, 2002, **40**, 207–210.
- [114] J. Cavanagh, W.J. Fairbrother, A.G. Palmer, N.J. Skelton and M. Rance, *Protein NMR Spectroscopy: Principles and Practice*, 2nd ed., Academic Press (Elsevier), San Diego, 2006.
- [115] D. Argyropoulos, E. Hoffmann, S. Mtongana and K.R. Koch, *Magn. Reson. Chem.*, 2003, **41**, 102–106.
- [116] D. Gudat, *Magn. Reson. Chem.*, 2003, **41**, 253–259.
- [117] R. Wagner and S. Berger, *J. Magn. Reson. Ser A*, 1996, **120**, 258–260.

Chapter 7

Separating shifts and couplings: *J*-resolved spectroscopy

7.1. INTRODUCTION

Unlike the techniques encountered in the previous two chapters that exploit scalar (*J*) couplings to *correlate* the chemical shifts of interacting spins, '*J*-resolved' experiments aim to separate (or resolve) chemical shifts from scalar couplings, thus allowing the chemist to examine one parameter without complications arising from the other. For example, the analysis of crowded proton spectra is often complicated by the overlap of neighbouring multiplets, making the extraction of coupling constants or the accurate measurement of chemical shifts difficult or even impossible. This overlap clearly results from the similarities in chemical shifts of the corresponding protons; so if the individual multiplets could in some way be displayed *independently* of their shifts the overlap would be removed and, in principle, the coupling patterns analysed. In practice, *J*-resolved methods are subject to a number of technical difficulties that may limit their effectiveness in this respect, and as such the methods have found far less use in routine structural work than the shift correlation experiments. That said, one of the major sources of complication when using *J*-resolved methods (strong coupling between spins) can be eliminated in many instances, or perhaps reduced to an acceptable level, by the use of higher magnetic field strengths. As these become more routinely available to the research chemist, the *J*-resolved methods are perhaps more likely to re-establish themselves as useful tools in the chemist's armoury, rather than fall further into relative obscurity. The methods are all based on 2D spectroscopy in which the shift and coupling parameters are resolved by presenting chemical shifts in f_2 and *only spin couplings* in f_1 , which may be heteronuclear or homonuclear couplings, depending on the details of the experiment. The principal techniques of this chapter are summarised in Table 7.1. These techniques were the most widely studied methods in the early development of 2D NMR spectroscopy and may be understood with reference to the vector model, being based on simple spin-echoes. As such, I would recommend familiarity with spin-echoes before proceeding (see Section 2.2).

7.2. HETERONUCLEAR *J*-RESOLVED SPECTROSCOPY

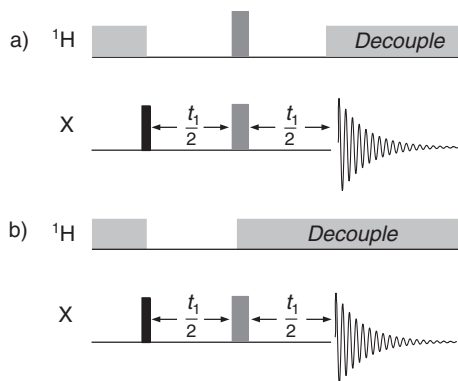
In the heteronuclear version of the experiment, the chemical shift of the X spin is presented in f_2 whilst couplings to a second nucleus, typically protons, are presented in f_1 . The f_1 dimension therefore enables an analysis of resonance multiplicity as well as measurement of the heteronuclear coupling constants (J_{XH}) themselves, as described further below.

To reduce the f_1 information content of the 2D spectrum to only couplings, it is necessary to make the detected FIDs insensitive to chemical shift evolution during the t_1 period, which is readily achieved by the use of a spin-echo during t_1 (Fig. 7.1a) [1, 2]. Thus, following initial X spin excitation, simultaneous 180° proton and carbon pulses are applied at the midpoint of t_1 , such that X spin chemical shifts will refocus but the heteronuclear coupling will continue to evolve. There is no 'mixing' step in *J*-resolved experiments because magnetisation or coherence transfer to other spins is not employed. The sequence

Table 7.1. The principal applications of the main techniques described in this chapter

Technique	Principal applications
Heteronuclear <i>J</i> -resolved	Separation of heteronuclear couplings (usually $^1\text{H-X}$) from chemical shifts. Used to determine the multiplicity of the heteroatom or to provide direct measurement of heteronuclear coupling constants.
Homonuclear <i>J</i> -resolved	Separation of homonuclear couplings (usually $^1\text{H-}^1\text{H}$) from chemical shifts. Used to provide direct measurement of homonuclear coupling constants or to display resonance chemical shifts without homonuclear coupling fine structure (e.g. 'proton-decoupled' proton spectra).
'Indirect' homonuclear <i>J</i> -resolved	Separation of proton homonuclear couplings according to chemical shift of attached carbon centre. Used to provide direct measurement of homonuclear coupling constants.

Figure 7.1. The (a) spin-flip and (b) gated decoupling techniques for recording heteronuclear J -resolved spectra. In (b) coupling evolution occurs for half of the t_1 period only, so splittings observed in f_1 appear with half their true $^1J_{\text{XH}}$ values.



is the exact 2D analogue of the J -modulated 1D editing sequence presented in Section 4.3.1, with the fixed coupling evolution period Δ here being replaced with variable period t_1 . Since proton decoupling will invariably be applied during the detection period, heteronuclear X–H couplings do not appear in f_2 . The detected signals therefore experience the desired pure amplitude modulation according to the evolution of the heteronuclear couplings in t_1 (Fig. 7.2). In addition, the spin-echo refocuses field inhomogeneity effects since these are merely another source of chemical shift differences within the sample, meaning the resonances in f_1 possess *natural* line widths, that is, they are governed by the T_2 rather than T_2^* of the heterospin [3].

The scheme of Fig. 7.1a is referred to as the ‘spin-flip’ or ‘proton-flip’ method because of the use of the 180° proton pulse. A widely used alternative is the ‘gated decoupler’ method [4] (Fig. 7.1b) in which this pulse is omitted and instead proton decoupling is applied for one-half of the evolution period (again analogous to the 1D spectrum-editing method). Chemical shifts are again refocused by the X-pulse, but the heteronuclear couplings now evolve for only half the total evolution time, resulting in the f_1 splittings being reduced by a factor of two (be sure which version you are using if you intend to measure J_{XH} !). Note that if neither the proton decoupling nor proton 180° pulse were applied in t_1 , the effect of the lone X(180°) would be to refocus the heteronuclear coupling in addition to the chemical shifts, thus preventing the operation of the experiment altogether. The attractiveness of the gated decoupler method lies in the simplicity of implementation because no $^1\text{H}(180^\circ)$ calibrations are required (a point of greater significance when these methods were initially developed), the results are better behaved in the presence of strong coupling (see below) and no artefacts are introduced by inaccuracies in the proton pulse.

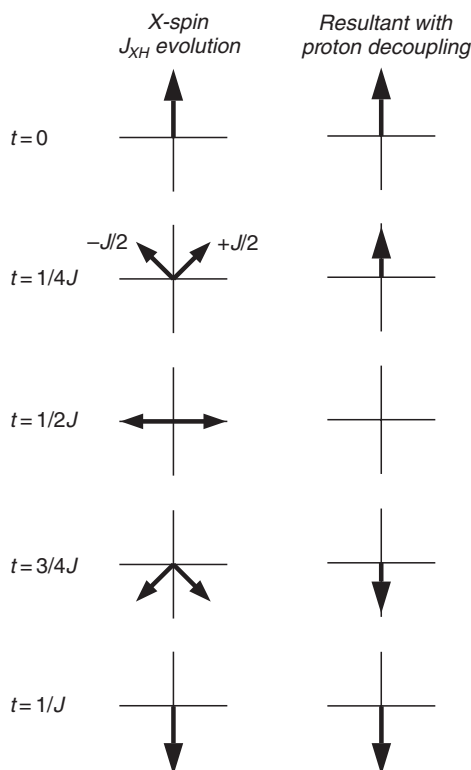


Figure 7.2. Amplitude modulation of the X spin signal by heteronuclear coupling evolution during t_1 , illustrated for a coupled X–H pair. The doublet vectors evolve throughout t_1 but are frozen during t_2 by the application of proton decoupling; so the resultant X spin signal displays pure amplitude modulation according to the heteronuclear coupling constant.

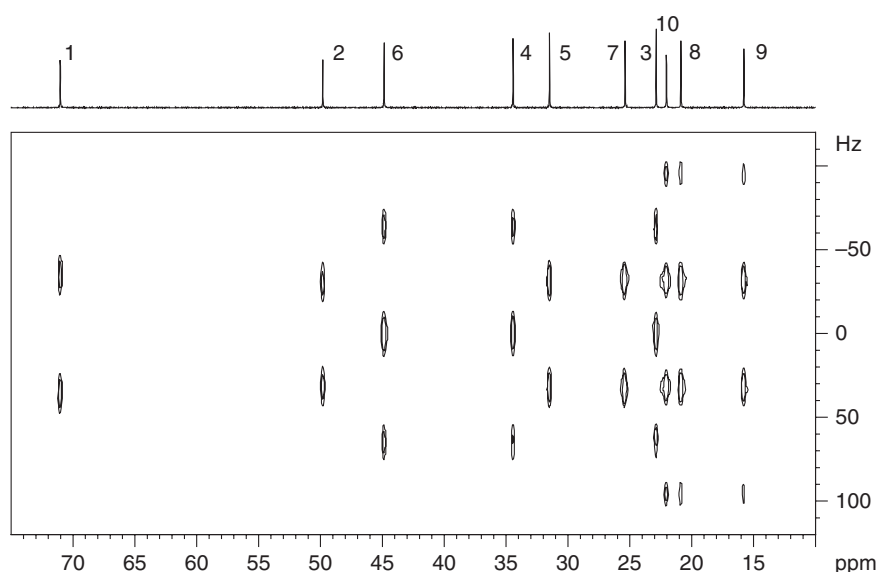
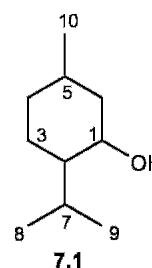


Figure 7.3. The 125 MHz ^1H – ^{13}C heteronuclear *J*-resolved spectrum of menthol **7.1**, above which is the conventional 1D carbon spectrum. The gated decoupler method of Fig. 7.1b was used and the splittings in f_1 are therefore half their true $^1J_{\text{CH}}$ values.

Figure 7.3 shows the ^1H – ^{13}C *J*-resolved spectrum of menthol **7.1**. The projection onto the f_2 axis would produce the usual proton-decoupled 1D carbon spectrum in which resonances appear as singlets, whilst in f_1 the carbon multiplicities from $^1J_{\text{CH}}$ are clearly delineated. Such a spectrum represents a straightforward way of determining multiplicities, but this approach is rarely used nowadays since the 1D spectrum-editing methods of Chapter 4 offer a more rapid alternative and the edited heteronuclear shift correlations of Chapter 6 are also more informative. The *J*-resolved spectrum may prove useful when unusually large $^1J_{\text{CH}}$ values exist and produce ambiguous results from the spectrum-editing methods. Recall that these rely on delays being set according to the estimated *J* values and may fail when the timings used are far from optimum, whereas *J*-resolved methods have no such requirement. A more attractive use for the *J*-resolved experiment lies in the measurement of heteronuclear coupling constants, and in particular the measurement of long-range proton–carbon couplings, since these can be of considerable use in conformational or configurational studies. Whilst in principle it is possible to record the f_1 dimension with sufficient digital resolution to resolve the small long-range couplings in the presence of the far greater one-bond couplings (which are typically at least an order of magnitude larger), a more efficient approach is to eliminate $^1J_{\text{CH}}$ from f_1 and retain only $^{2/3}J_{\text{CH}}$, thus reducing the f_1 spectral width and allowing finer digitisation for more accurate measurements. A number of approaches towards this are introduced in the following section.

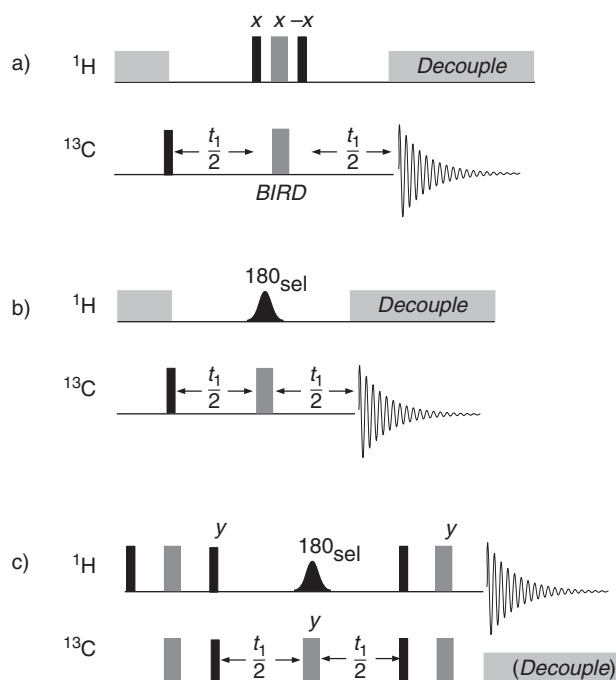
Refinements to the basic sequences of Fig. 7.1 include the use of polarisation transfer sequences prior to the spin-echo to prepare transverse X spin magnetisation. INEPT, DEPT [5] and PENDANT [6] have been proposed for this purpose. In addition to sensitivity gains, the polarisation transfer sequences allow repetition rates to be dictated by the T_1 s of the faster-relaxing protons. They may also be favoured when studying nuclei that experience a loss of intensity from the ^1H –X NOE generated by proton decoupling, that is, those X spins that have negative magnetogyric ratios, for example ^{15}N or ^{29}Si (see Chapter 8). Avoiding the use of proton decoupling during the relaxation delay minimises NOE build-up and hence signal losses, whilst the addition of the polarisation transfer step enhances sensitivity.



7.2.1. Measuring long-range proton–carbon coupling constants

The use of long-range ^1H – ^{13}C coupling constants in the definition of molecular configuration or conformation is an increasingly active area, with numerous methods for measuring these being developed in recent years [7]. One technique based on the HMBC experiment has already been presented in Section 6.4.4. In principle, *J*-resolved methods are well suited to such measurements, but tend to suffer from the presence of the far greater $^1J_{\text{CH}}$ couplings, as mentioned above. The common aim of the sequences described below is to eliminate these less informative one-bond ^1H – ^{13}C couplings from f_1 and thus reduce the corresponding spectral width. Since $^1J_{\text{CH}}$ values are greater than ~ 125 Hz, whilst long-range couplings are typically less than 10 Hz, substantial reductions in the f_1 spectral width can be made, which allows higher digital resolution and provides accurate characterisation of the remaining $^nJ_{\text{CH}}$ values without the need to collect prohibitively large or

Figure 7.4. Heteronuclear J -resolved sequences for the measurement of long-range proton–carbon coupling constants; (a) semi-selective, (b) selective and (c) selective with proton observation (see text).



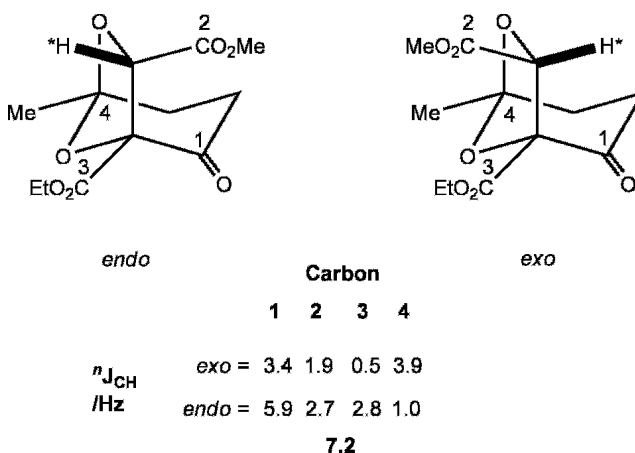
time-demanding data sets. The descriptions are deliberately brief but serve to illustrate the most useful approaches.

Semi-selective

By placing a BIRD cluster (Section 6.3.3) with proton pulse phases x , x , $-x$ at the midpoint of t_1 in place of the simultaneous 180° pulses of the spin-flip method (Fig. 7.4a), it is possible to invert only those protons that share long-range couplings, while leaving those directly bound to a ^{13}C spin unaffected. This results in the selective refocusing of the one-bond couplings by the $^{13}\text{C}(180^\circ)$ pulse, and thus their removal from f_1 , whereas the long-range couplings remain [8, 9]. The f_1 traces display *all* $^nJ_{\text{CH}}$ values associated with each carbon, and may thus possess quite complex fine structures since each carbon is likely to be coupled to many protons. Such complexity may itself preclude the measurement of the coupling constants, and no information on which spin-pair gives rise to a specific coupling is available.

Selective

To simplify the fine structure appearing in f_1 , it is possible to selectively invert only *one* proton resonance during t_1 , taking care to avoid inverting its one-bond ^{13}C satellites. This requires the application of a selective $^1\text{H}(180^\circ)$ pulse in place of the usual ‘hard’ pulse in the spin-flip method (Fig. 7.4b). In this case, *all* couplings will be refocused and therefore removed from f_1 with the exception of those to the selectively inverted proton(s) [10]. The f_1 dimension therefore displays only simple multiplets (doublets, triplets or quartets depending on the selected proton group) and the coupling pairs giving rise to each are readily identified. This was one approach used for measuring long-range couplings to the bridgehead proton in a series of structures related to 7.2 in an attempt to define the unknown



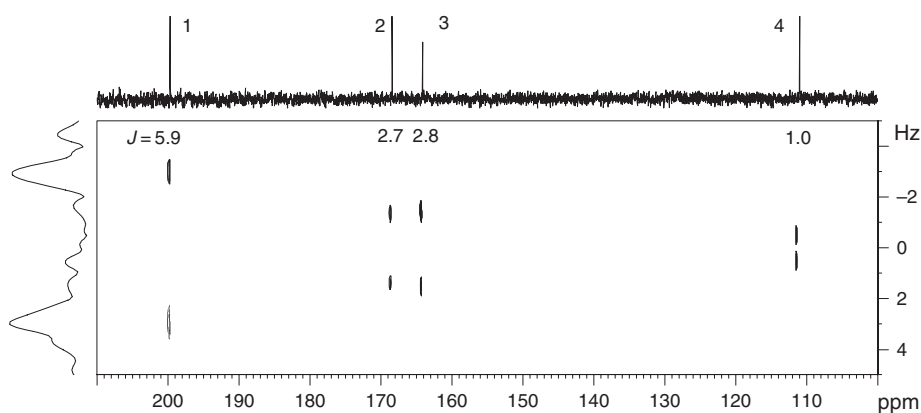


Figure 7.5. A region of the selective heteronuclear J -resolved spectrum of 7.2. Long-range heteronuclear couplings to the selected proton appear as splittings in f_1 , the values of which are shown. The vertical trace is taken through the resonance at 199 ppm. $4K t_2$ data points were collected for 32 t_1 -increments over spectral widths of 100 ppm and 14 Hz, respectively. A 100 ms Gaussian 180° pulse was used to select the proton resonance. The final f_1 resolution after zero-filling was 0.1 Hz/pt.

bridgehead stereochemistry and thus differentiate *endo* and *exo* products. A lack of NOEs to the bridgehead protons did not provide an unambiguous definition; so long-range couplings in the unknown products were compared with those in structures of known configuration. Figure 7.5 shows a section of one such spectrum in which these couplings were measured from the f_1 doublet splittings. This requires the proton(s) of interest to be sufficiently resolved, and numerous experiments will be required if many ${}^nJ_{\text{CH}}$ values are to be determined. This has, nevertheless, been employed extensively in the measurement of long-range coupling constants in monosaccharide units [11], and other variants have been incorporated in the INEPT [12] and DEPT [13] sequences for sensitivity enhancement.

Selective with proton detection

Long-range couplings can also be observed through the observation of the participating proton, leading to significant sensitivity gains. An elegant way of performing the proton-detected experiment is based on a simple modification of the HSQC sequence presented in Section 6.3.2, and may be performed as the phase-sensitive experiment with or without pulsed field gradients [6] (Fig. 7.4c). The non-selective ${}^1\text{H}(180^\circ)$ inversion pulse within t_1 (which serves to refocus heteronuclear couplings in HSQC) has been made selective here, and a simultaneous non-selective ${}^{13}\text{C}(180^\circ)$ pulse added, producing a spin-echo during t_1 analogous in design and operation to the selective experiment above. The polarisation transfer step following t_1 transfers magnetisation back onto protons for detection through their one-bond proton-carbon coupling. The resulting spectrum thus displays the normal ${}^1\text{H}$ spectrum in f_2 and ${}^nJ_{\text{CH}}$ doublets along f_1 . These couplings are from the selectively inverted proton to the carbon attached to the proton at the f_2 chemical shift (Fig. 7.6); thus measurements are restricted to long-range couplings to protonated carbons only. Once again, the spin pair giving rise to the coupling can be identified, assuming the target proton is sufficiently well resolved to be inverted selectively.

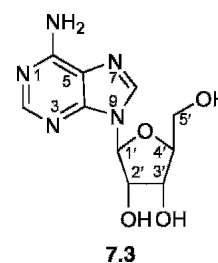
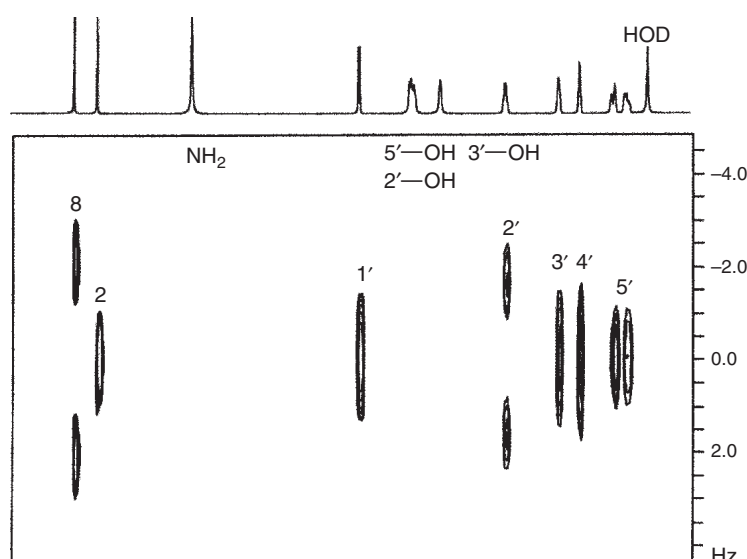


Figure 7.6. The proton-detected selective heteronuclear J -resolved spectrum of adenosine 7.3. The $\text{H}1'$ proton has been selectively inverted and the doublet splittings in f_1 record its long-range couplings to C8 and C2' (reproduced with permission from [6], Copyright 1995, Elsevier).

7.2.2. Practical considerations

The requirements for sign discrimination in f_1 for the J -resolved experiments of Fig. 7.1 are readily met in a *first-order system* because the multiplets are symmetrical about their midpoints and thus folding about $f_1 = 0$ causes no confusion. Phase cycling for f_1 quadrature detection is, therefore, unnecessary. Since, in the presence of broadband decoupling, the detected signals experience pure amplitude modulation, pure phase spectra may also be obtained. However, when the system is no longer first order, that is, in the presence of strong coupling *between protons* (more precisely, between those protons giving rise to ^{13}C satellites and others), the H–X multiplets lose their symmetry and the detected signals experience some phase modulation, which in turn introduces dispersion-mode contributions to the spectrum. In this case, the 2D spectrum may be presented in the absolute-value mode to mask the phase distortions or, alternatively, following the f_2 transform, columns may be extracted from the 2D data set at the chemical shift of a resonance of interest and processed separately as 1D traces. Despite the overall dispersive contribution to the 2D lineshape, a single column displays absorption-mode characteristics. Further complications arise in the presence of strong coupling for the spin-flip method [1].

The quality of J -resolved spectra can be seriously compromised by deficiencies in the accuracy of the pulses (especially the 180° pulses), arising from pulse miscalibration, rf inhomogeneity and off-resonance effects. The result is the appearance of a variety of additional weak resonances at esoteric positions within the spectrum, referred to as ‘ghosts’ and ‘phantoms’. The ghosts arise from imperfections in the 180° refocusing pulse, such that some transverse magnetisation fails to experience the effect of the pulse and thus fails to be refocused. Phantoms arise from combined deficiencies in the 90° and 180° pulses causing residual longitudinal magnetisation that exists after the 90° pulse to become transverse following the imperfect 180° pulse. These spurious responses may be eliminated with the EXORCYCLE phase cycle [14], which is widely employed in sequences that utilise spin-echoes. This involves stepping the phase of the X spin refocusing pulse through $x, y, -x, -y$ whilst the receiver inverts, that is, steps $x, -x, x, -x$. Stepping the refocusing pulse by 90° causes the echo to shift in phase by 180° , as explained in Section 2.2.4; hence receiver inversion follows the echo, whereas the unwanted responses ultimately cancel. Deficiencies in the proton 180° inversion pulse [15] can be avoided by the use of the gated decoupler method, but where this is not possible, as in the case of the homonuclear J -resolved experiments that follow, use of a composite 180° pulse such as $90_x 240_y 90_x$ [16], and better still the more recent frequency-swept broadband inversion pulses (BIPs) (as in Section 7.3.3), is advantageous.

Experimental set-up

Digitisation of the data, in particular the number of t_1 increments that are needed, will be dictated by the information required of the spectrum. If one simply wishes to determine multiplicities, low digital resolution will suffice, allowing few increments and rapid data collection. For carbon-13 the widest multiplets arise from the quartets of methyl groups; so the f_1 spectral width needs be about 3.2 times $^1J_{\text{CH}}$ (although it is possible to reduce the spectral width further and deliberately fold in the outer lines of the quartets). This requirement can beneficially be reduced by a factor of two for the gated decoupler method to around 200 Hz, assuming a coupling constant of 125 Hz.

To merely characterise the multiplet structure, a digital resolution of around 20 Hz/pt in f_1 should suffice, requiring as little as 20 increments. If one wished to *measure* the value of $^1J_{\text{CH}}$ from the multiplet structure, a digital resolution of somewhat less than 5 Hz/pt would be more appropriate. Some 200 increments would be required for 2 Hz/pt, leading to a significantly longer experiment, particularly if many scans are required per increment for reasons of sensitivity. If even finer digitisation were required for the measurement of *long-range* couplings, this approach becomes impossible. In contrast, when using one of the selective methods for $^nJ_{\text{CH}}$, one may only need digitise a 10 Hz window, requiring only 40 increments for 0.5 Hz/pt. Following the f_2 transform, columns may be extracted from the 2D data set and treated as 1D FIDs, including the use of zero-filling, to further enhance measurements.

7.3. HOMONUCLEAR J -RESOLVED SPECTROSCOPY

The homonuclear version of the J -resolved experiment [17] is most frequently applied in proton spectroscopy, although, again, it is suitable for any abundant nuclide. In principle, the separation of δ and J should reveal proton multiplets in f_1 free from overlap and thus available for analysis, and singlets in f_2 at the corresponding chemical shifts, such that the f_2 projection

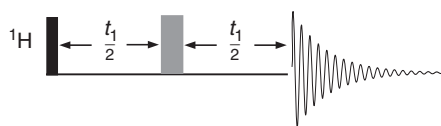


Figure 7.7. The homonuclear J -resolved experiment. The 180° pulse at the midpoint of t_1 refocuses proton shifts but not homonuclear couplings, implying that these appear only in f_1 .

represents the 'broadband proton-decoupled proton spectrum'. The possibility of generating such a spectrum has obvious appeal, allowing the accurate measurement of chemical shifts in even the most heavily crowded proton spectra. However, a number of technical difficulties must be overcome if one is to achieve this goal, which, alas, is not readily accomplished although more recent methods incorporating gradient pulses have improved matters. A second possible application is in the measurement of homonuclear coupling constants, which is possible within the limitations of certain caveats detailed shortly.

The homonuclear sequence (Fig. 7.7) closely resembles the heteronuclear methods (although restricted to the spin-flip version only), and utilises the EXORCYCLE scheme. The appearance of the homonuclear spectrum is fundamentally different from its heteronuclear equivalent in that both chemical shifts *and couplings* appear in f_2 (because one cannot simultaneously *broadband* decouple and observe the proton spectrum). Thus, rather than lying parallel to the f_1 axis, the proton multiplets appear along a slope of -1 (in units of hertz), or, in other words, sit at 45° to either axis (assuming identical plot scaling for both dimensions, Fig. 7.8). Columns parallel to f_1 do not, therefore, display the expected proton multiplets, and the f_2 projection displays both chemical shifts and scalar couplings. To overcome these shortcomings, post-processing techniques are routinely applied, as described in the following section. Furthermore, unlike the heteronuclear case, the detected signals unavoidably experience *phase modulation* as a function of t_1 (Fig. 7.9), resulting in phase-twist lineshapes. The spectra are therefore usually presented as absolute-value data following strong resolution enhancement and a magnitude calculation. Further complications arise in the presence of strong coupling between protons in the form of additional responses (see below); so the J -resolved experiment is most suitable for overlapped spectra that are still first order.

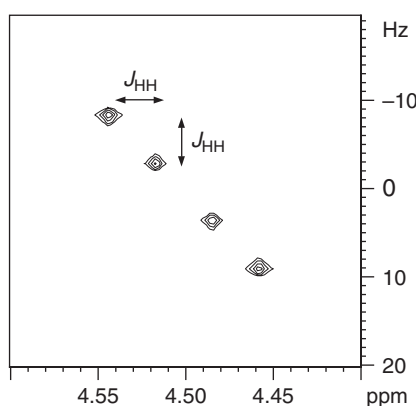


Figure 7.8. A multiplet from the homonuclear J -resolved experiment showing the characteristic tilting brought about by the presence of proton couplings in both f_1 and f_2 .

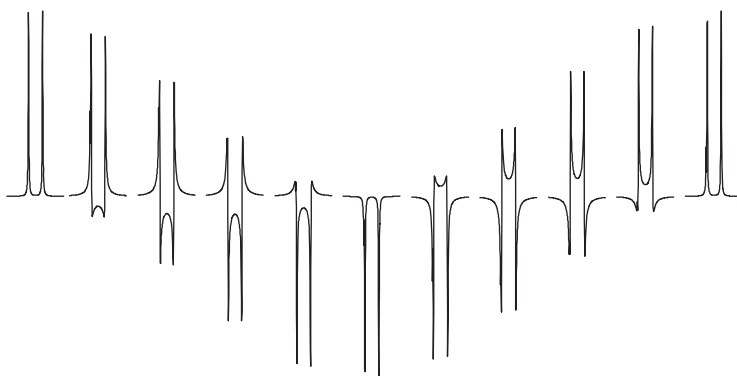


Figure 7.9. Phase modulation of a proton doublet as a function of time. The signals retain full intensity at all times (i.e. they do not experience amplitude modulation) but each line experiences a net 360° phase change over the sequence. The results are shown for a 10 Hz doublet with evolution times from 0 to 200 ms in 20 ms increments.

7.3.1. Tilting, projections and symmetrisation

To reach the ultimate goal of retaining only chemical shifts in f_2 , it is possible to eliminate the couplings from this dimension by 'tilting' (or 'shearing') the multiplets through an angle of 45° about their midpoints [18], as illustrated schematically in Fig. 7.10. Software routines for this process are common to NMR-processing packages nowadays. The resulting spectrum then has an appearance similar to the heteronuclear analogue, with columns parallel to f_1 reproducing the multiplet structures (provided magnitude calculation has been performed), and the projection onto the f_2 axis producing the broadband-decoupled proton spectrum (Fig. 7.11). Figure 7.12 compares traces taken from this J -resolved spectrum with the equivalent multiplets from the 1D proton spectrum and illustrates the fine resolution of multiplet structure that can be obtained in the f_1 dimension.

Further improvements in the form of t_1 -noise reduction may also be achieved with additional post-processing. Prior to the tilt procedure, bands of t_1 -noise lie parallel to the f_1 axis, as for all

Figure 7.10. A schematic illustration of the tilting procedure for eliminating homonuclear couplings from the f_2 dimension. (a) The original multiplet structure and (b) that following the tilt procedure.

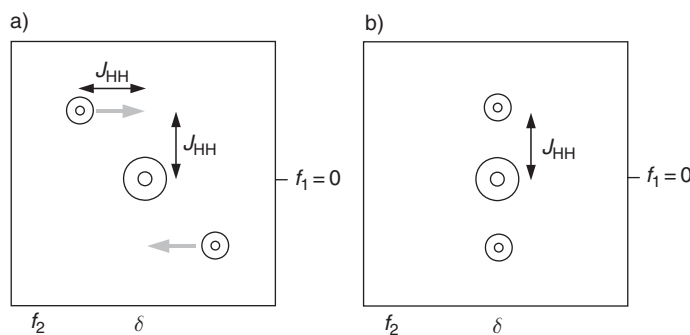
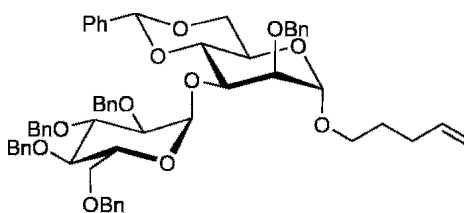
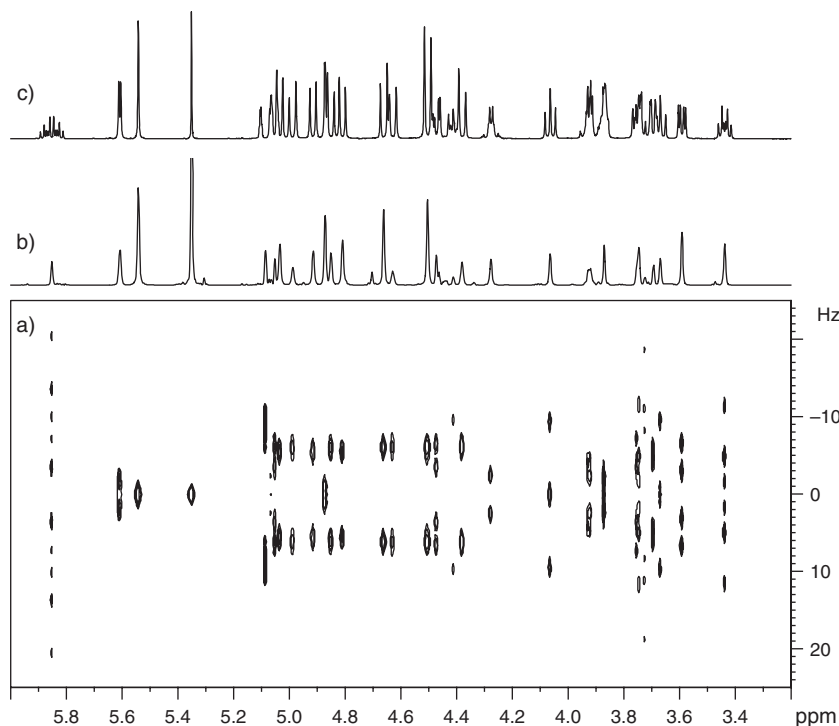


Figure 7.11. (a) The 500 MHz proton homonuclear J -resolved spectrum of **7.4** (after tilting and symmetrisation). The f_2 projection (b) approximates to the 'proton-decoupled proton spectrum' and is considerably less complex than the conventional 1D spectrum (c). 4K t_2 data points were acquired for 64 t_1 increments over spectral widths of 5 ppm and 60 Hz, respectively. The final f_1 resolution after zero-filling was 0.5 Hz/pt. Data were processed with unshifted sine-bell windows in both dimensions and are presented in magnitude mode.



7.4

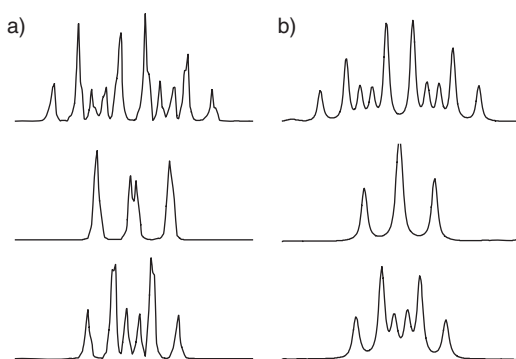


Figure 7.12. (a) Selected f_1 traces taken from the *J*-resolved spectrum of Fig. 7.11(a) and (b) the equivalent multiplets from the 1D proton spectrum.

2D experiments, whilst after the tilt they sit at 45° to it. In first- and higher-order systems, the multiplets will themselves be symmetrical about the line $f_1 = 0$ Hz after tilting [19]. If the whole data set were symmetrised about this line, that is, the lower intensity point for symmetrically related data points replacing the higher, the multiplets would be retained whereas the contributions from the sloping t_1 -noise would be diminished [20]. The procedure is similar to that introduced in Section 5.3.3 for t_1 -noise reduction in absolute-value COSY spectra, although a different symmetrisation procedure is of course required, and again one should be aware of the possibility of introducing artefacts into the spectrum as a result of the symmetrisation routine itself. Again, these routines are common to modern processing packages.

7.3.2. Applications

Perhaps the most obvious application of the homonuclear *J*-resolved experiment is the separation of overlapping multiplets so that the fine structure within each may be analysed. There is however a strict requirement for the spin-systems to be first-order for the separation of shifts and couplings to be successful [19] because strong coupling causes unwanted additional responses to appear midway between the shifts of the strongly coupled protons. To reduce the degree of strong coupling within spectra, the use of the highest available field strength is recommended for *J*-resolved spectroscopy wherever possible. Figure 7.13 shows simulated spectra for a three-spin system at field strengths of 200 and 600 MHz. At the lower field (Fig. 7.13a), the coupled protons at 3.7 and 3.8 ppm ($J = 12$ Hz) experience strong coupling, as evidenced in the ‘roofing’ of their 1D resonances, which gives rise to the additional responses between them in the *J*-resolved spectrum. No such artefacts are associated with the resonance at 4.5 ppm, which experiences only first-order coupling to its partners. In contrast, the higher-field spectrum (Fig. 7.13b) shows no extra responses since all couplings are now (approximately) first-order.

The homonuclear *J*-resolved spectrum can also assist in the measurement of proton heteronuclear couplings, notably when the coupling exists to a high abundance heteronucleide such as ^{31}P or ^{19}F . Since only the proton of a coupled heteronuclear pair will experience the 180° pulse, the heteronuclear coupling is refocused in t_1 and therefore absent in f_1 . This coupling will nonetheless be operative during detection of the proton FID and will thus appear in f_2 . Since this heteronuclear splitting sits *parallel* to the f_2 axis rather than at 45° to it, it is *not* removed from this dimension by the tilting process and may thus be examined without interference from homonuclear proton couplings along f_2 . Figure 7.14 demonstrates this approach for the palladium phosphine complex **7.5**. The f_2 projection of the tilted spectrum contains splittings for the ^1H – ^{31}P couplings only at each proton shift, as

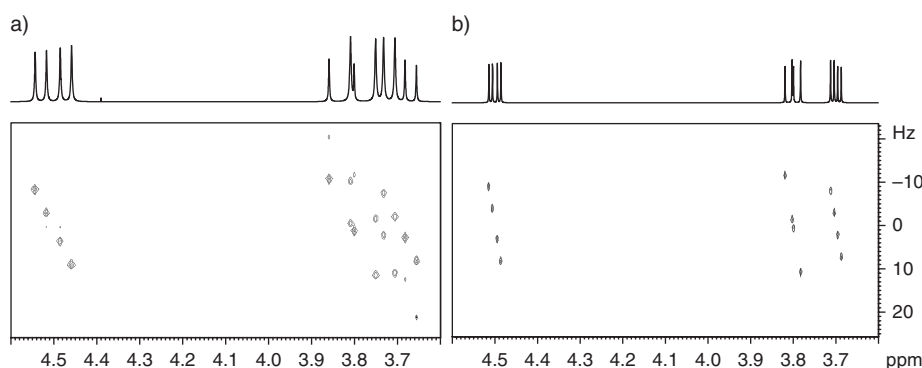
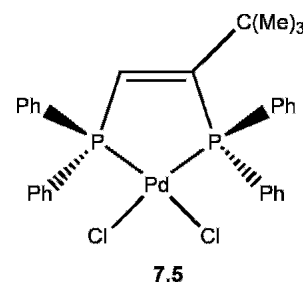
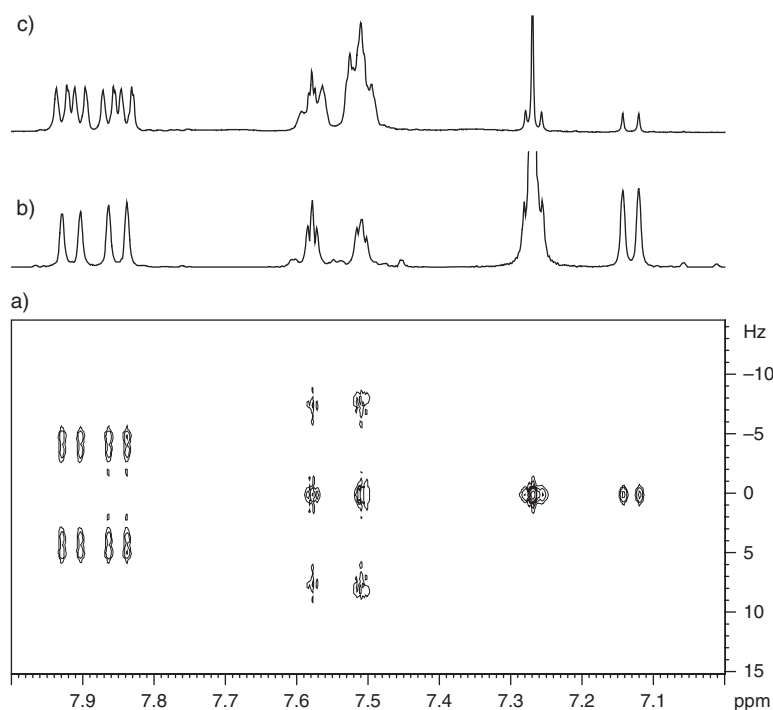


Figure 7.13. Simulated 2D homonuclear *J*-resolved and 1D spectra for a three-spin proton system at (a) 200 and (b) 600 MHz. The additional artefacts in (a) arise from strong coupling between the two low-frequency protons (no tilting has been applied).

Figure 7.14. Measurement of proton heteronuclear couplings from the tilted homonuclear J -resolved spectrum (a). Spectrum (b) is the f_2 projection of (a) and displays only proton shifts and ^1H – ^{31}P coupling. Spectrum (c) is the conventional 1D spectrum displaying shifts and both ^1H – ^1H and ^1H – ^{31}P couplings.



is most clearly seen for the phenyl *ortho*-protons at 7.85 and 7.92 ppm. These data can be used to complement the 1D X spin-decoupled proton spectrum in which proton–proton couplings are observed without interference from H–X spin couplings (see Fig. 4.14 of the same complex for comparison and Section 4.2.3). If desired, the heteronuclear couplings could also be eliminated from the J -resolved spectrum by the application of broadband X spin decoupling during the acquisition time. For the measurement of the heteronuclear couplings, rows taken parallel to f_2 through the multiplet components provide better resolution than the projection itself.

7.3.3. Broadband-decoupled ^1H spectroscopy

The possibility of recording ‘broadband homonuclear decoupled spectra’ has been discussed above and the most basic approach illustrated in Fig. 7.11. The simple J -resolved experiment tends to be of limited success in this respect owing to interferences from strong coupling, the poor lineshapes and limited resolution obtained, and because the signal intensities in the projected spectrum bear little relation to signal intensities in the conventional 1D spectrum. Greater success is again expected on higher-field instruments but in any case caution in interpretation of such data is required. Whilst more sophisticated data processing based on pattern recognition procedures has been proposed to improve spectrum quality [21,22], the algorithms required have not gained widespread availability. A more recent experimental method offers a practical approach to achieving absorption-mode lineshapes and hence high-resolution broadband-decoupled spectra and will be described briefly. It has the further advantage of producing faithful reproductions of multiplet structures in f_1 , which is typically not the case for traditional J -resolved spectra possessing phase-twist lineshapes.

It is the presence of the phase-twist lineshape containing a broad dispersive component that limits resolution in the homonuclear J -spectra presented thus far, an issue usually addressed by the application of a strong (pseudo-echo) window function to attenuate this. The key to producing pure absorption-mode lineshapes is to cancel any contribution from the dispersive element whilst retaining the absorptive component. This is the approach employed in the ‘echo–antiecho’ f_1 processing of gradient-selected 2D data sets collected as separate P- and N-type data (Section 5.5.2) and a similar approach may be employed for J -resolved spectra [23]. Thus, if two data sets are collected in which magnetisation had followed opposing coherence transfer pathways (which have been termed the J - and *anti*- J spectra [23], Fig. 7.15), they may be combined to eliminate the undesirable dispersive component. Multiplet peak positions in the J and *anti*- J spectra are related by reflection about $f_1 = 0$ (as for P- and N-type data), but crucially have their phase-twists in opposite senses. Conceptually, one may imagine generating the *anti*- J spectrum simply by the addition of a 180° inversion pulse at the end of t_1 to invert the selected pathway (dashed

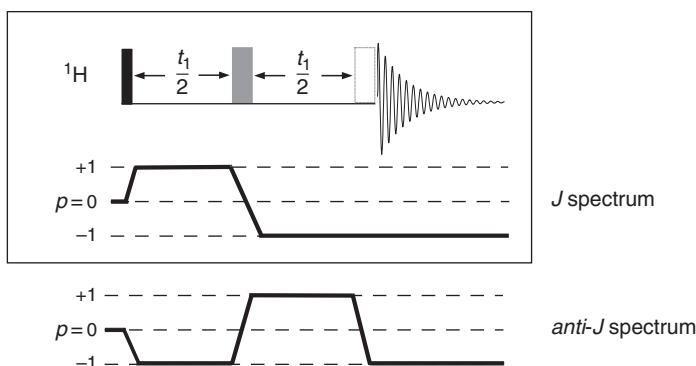


Figure 7.15. The coherence transfer pathways required for the *J* and *anti-J* data sets. The conventional sequence provides the *J*-spectrum (boxed) whilst an additional inversion is required from a further 180° pulse (dashed) to generate the *anti-J* spectrum (see text).

pulse in Fig. 7.15) although in reality such an approach fails and the dispersive elements would not cancel. The failure stems from the fact that all spins would be inverted by this additional 180° pulse, whereas it is necessary to invert *only* the active spins (those contributing directly to each 2D peak) whilst leaving all other coupled spins (the passive spins) unaffected. One may imagine applying a selective 180° pulse to each individual multiplet in turn to achieve this although the efficiency of the 2D approach is then lost.

It turns out that the desired inversion can be achieved by the simultaneous application of a weak field gradient and selective 180° pulse (the so-called 'Zangger–Sterk pulse element' [24]). The weak z -gradient pulse imposes a frequency spread of resonances along the length of the sample so that when the frequency-selective 180° pulse is applied, different regions of the proton spectrum are inverted for molecules that sit in different regions of the NMR sample. This concept is illustrated schematically in Fig. 7.16 for two molecules A and B of identical structure and demonstrates how the inversion of only a single spin per molecule may be achieved simultaneously for all resonances in the spectrum. Since molecule A experiences a greater local field than B due to the applied field gradient, its spins will resonate at relatively higher frequencies than the equivalent nuclei in molecule B; this is shown as an offset of the corresponding spectra along the frequency axis. Application of the frequency-selective inversion pulse will thus act simultaneously on *different* spins for the two molecules, as illustrated by the red box, yet will have no influence on the coupled passive spin resonances that sit outside the inversion bandwidth (those outside the red box). When all signals from the selectively excited region are retained (through appropriate phase cycling), each detected active spin experiences the required inversion whilst none of its passive partners do so. Hence, appending the Zangger–Sterk element to the end of the conventional *J*-resolved sequence produces the required *anti-J* spectrum. To produce signals of similar intensity, it is also necessary to include this element within the *J*-spectrum sequence, in this case prior to the t_1 period (Fig. 7.17) so as to allow proper combination of the two data sets through echo–antiecho processing. The resulting 2D spectrum may be titled and projected to yield the absorption-mode broadband-decoupled spectrum whilst the f_1 projections present exceptionally clean, high-resolution multiplet structures. The application of this technique is illustrated for the complex multiplets of menthol **7.1** (Fig. 7.18) and demonstrates the desired decoupling for all but the mutually coupled and closely spaced resonances of protons 3 and 4 (see below).

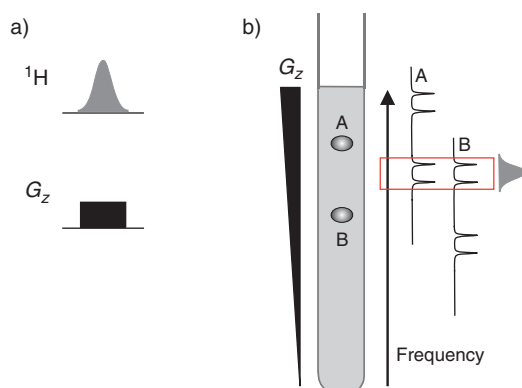


Figure 7.16. Operation of the Zangger–Sterk pulse element. (a) The selective pulse/gradient combination and (b) schematic illustration of selective inversion of only a single spin per molecule by this element; see text for discussion.

Figure 1 displays three ^1H NMR spectra of poly(2-vinylpyridine) in CDCl_3 . The x-axis represents the chemical shift in ppm, ranging from 2.3 to 1.1. The spectra are labeled (a), (b), and (c). Spectrum (a) is the experimental spectrum, showing peaks labeled 1 through 7. Spectrum (b) is a simulated spectrum, showing peaks labeled 1 through 7. Spectrum (c) is another simulated spectrum, showing peaks labeled 2 through 7. The peaks are assigned to specific protons in the polymer structure: 1 (backbone methine), 2 (backbone methylene), 3 (vinyl methine), 4 (vinyl methylene), 5 (pyridine ring), 6 (pyridine ring), and 7 (pyridine ring).

The unavoidable disadvantage of the experiment is the reduced sensitivity that arises since only spins from the selected frequency slice contribute to the final J -spectrum, which can result in only $\sim 1\%$ of the signal being retained. The greater the selectivity of the pulse, the narrower the slice and the weaker the detected signal. The frequency bandwidth of the selective 180° pulse is dictated by the frequency separation of the most closely spaced, mutually coupled spins to be decoupled; so some compromise between the complete decoupling of neighbouring multiplets and sensitivity losses might be required. Indeed, in Fig. 7.18b, it is apparent that the decoupling of H3 from H4 of menthol is incomplete because the frequency separation was small (24 Hz) relative to the bandwidth of the selective Q3 pulse employed (~ 68 Hz) and thus illustrates one potential limitation of the method. The strength of the gradient pulse is itself chosen such that the frequency spread imposed on the sample is comparable to the width of the proton spectrum. The frequency spread $\Delta\nu$ across a sample of active length l within the probe rf coil is $\Delta\nu = \gamma G l$ for a gradient strength G , from which this may be defined. Thus, at 500 MHz a 10 ppm spectrum covers 5 kHz; so for a typical rf coil length of 1.6 cm a gradient strength of $\sim 0.7 \text{ G cm}^{-1}$ is appropriate ($\gamma_{\text{H}} = 4258 \text{ Hz G}^{-1}$). The experiment further benefits from the use of broadband 180° pulses at the midpoint of t_1 such as an adiabatic swept pulse or a time-efficient broadband inversion pulse (BIP, described in Section 10.2). In this case the pulse must be employed twice in the sequence such that phase errors imposed by one pulse are refocused by the second, as indicated in Fig. 7.17.

Many of the practical considerations given above for heteronuclear J -spectroscopy are equally applicable to the homonuclear case, and the selection of digital resolution in f_1 follows similar lines of thinking as before. Most proton multiplets will rarely exceed a width of 50 Hz (although those of other nuclides with many homonuclear couplings may do), so at least for proton spectroscopy, an f_1 spectral width equal to this will suffice. Using 64 and 128

t_1 increments will therefore provide an f_1 resolution of 1.6 and 0.8 Hz/pt, respectively. Using many more increments than these causes t_1 to be rather long, implying that relaxation losses become significant. Because of the phase-twist lineshapes and the use of magnitude calculation with the conventional experiment, strong resolution enhancement functions such as unshifted sine-bells need to be applied in both dimensions. Such severe treatment is not required for the absorption mode spectra described in Section 7.3.3 however.

7.4. 'INDIRECT' HOMONUCLEAR J-RESOLVED SPECTROSCOPY

An alternative approach to resolving proton multiplets is to disperse them according to the chemical shift of the carbon nucleus to which the protons are attached, rather than those of the proton themselves [25]. The advantage of this approach lies in the typically greater dispersion of the carbon chemical shifts, although one must tolerate the reduced sensitivity of carbon observation.

The sequence that achieves this (Fig. 7.19) is a simple variant on the INEPT-based heteronuclear shift correlation sequence of Fig. 6.46 (HETCOR); so the loss in sensitivity is compensated somewhat by the use of a polarisation-transfer step. In fact, the only difference between the two lies in the net evolution of *only shifts* or *only couplings* for the whole of t_1 . The addition of a proton 180° pulse at the midpoint of t_1 here serves to refocus proton chemical shifts and heteronuclear coupling constants (so the X spin 180° pulse of HETCOR becomes redundant) but leaves the proton homonuclear couplings free to evolve. The resulting spectrum therefore contains only proton multiplets in f_1 dispersed by the corresponding X spin shifts in f_2 (Fig. 7.20).¹

Once again, strong coupling, this time between proton satellites, causes problems. Furthermore, because one must consider the *satellites* of each proton rather than the parent resonance itself (since only the satellites can contribute to the polarisation transfer), the multiplet patterns observed for high-order systems may differ from those of the parent resonance in the 1D spectrum. Other complications occur in the case of non-equivalent geminal protons since each may possess different multiplet patterns, yet both will be

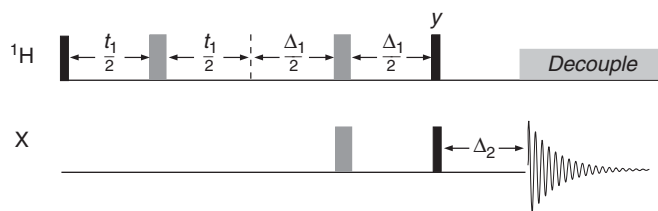


Figure 7.19. The indirect homonuclear J-resolved sequence. Proton couplings are effective during t_1 , after which polarisation transfer leads to X spin observation.

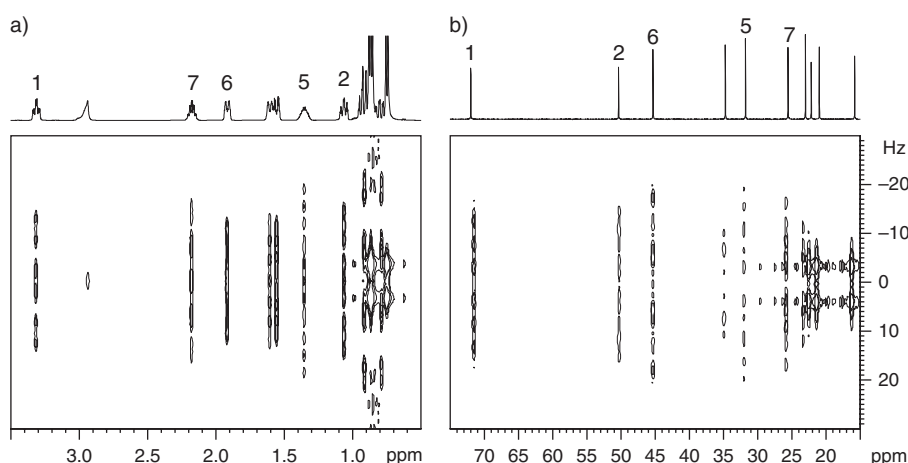


Figure 7.20. The (a) direct homonuclear J-resolved spectrum and (b) indirect homonuclear J-resolved spectrum of menthol 7.1. Both experiments present homonuclear couplings in f_1 dispersed by either the proton or the carbon chemical shift.

¹ Notice the similarity here with the approach used in sequence 7.4c in which the basic HSQC shift correlation sequence was adapted for the measurement of long-range heteronuclear couplings. In both cases, the chemical shift evolution period of the shift correlation sequence was converted into one in which only couplings were active (through the use of spin-echoes) so producing the J-resolved experiment. Often, subtle changes involving the addition or removal of one or two strategically placed pulses are all that is required to alter the characteristics of the resulting spectrum, producing a 'new' pulse sequence (which may or may not be useful to the chemist). This, in part, contributes to the plethora of NMR pulse sequences found throughout the chemical literature.

observed at the same carbon chemical shift, leading to a potentially complex multiplet overlap (this is apparent for the H6 protons in the indirect spectrum of Fig. 7.20) The most likely use of this variant is in the separation of the multiplet patterns of protons having coincidentally degenerate chemical shifts, which are not, therefore, separable in the conventional homonuclear version.

REFERENCES

- [1] G. Bodenhausen, G.A. Morris, R. Freeman and D.L. Turner, *J. Magn. Reson.*, 1977, **28**, 17–28.
- [2] L. Müller, A. Kumar and R.R. Ernst, *J. Magn. Reson.*, 1977, **25**, 383–390.
- [3] G. Bodenhausen, R. Freeman, R. Niedermeyer and D.L. Turner, *J. Magn. Reson.*, 1976, **24**, 291–294.
- [4] R. Freeman, G.A. Morris and D.L. Turner, *J. Magn. Reson.*, 1977, **26**, 373–378.
- [5] V. Rutar and T.C. Wong, *J. Magn. Reson.*, 1983, **53**, 495–499.
- [6] M.L. Liu, R.D. Farrant, J.M. Gillam, J.K. Nicholson and J.C. Lindon, *J. Magn. Reson. (B)*, 1995, **109**, 275–283.
- [7] B.L. Marquez, W.H. Gerwick and R.T. Williamson, *Magn. Reson. Chem.*, 2001, **39**, 499–530.
- [8] A. Bax, *J. Magn. Reson.*, 1983, **52**, 330–334.
- [9] V. Rutar, *J. Magn. Reson.*, 1984, **56**, 87–100.
- [10] A. Bax and R. Freeman, *J. Am. Chem. Soc.*, 1982, **104**, 1099–1100.
- [11] C. Morat, F.R. Taravel and M.R. Vignon, *Magn. Reson. Chem.*, 1988, **26**, 264–270.
- [12] T. Jippo, O. Kamo and K. Nagayama, *J. Magn. Reson.*, 1986, **66**, 344–348.
- [13] D. Uhrin, T. Liptaj, M. Hricovini and P. Capek, *J. Magn. Reson.*, 1989, **85**, 137–140.
- [14] G. Bodenhausen, R. Freeman and D.L. Turner, *J. Magn. Reson.*, 1977, **27**, 511–514.
- [15] G. Bodenhausen and D.L. Turner, *J. Magn. Reson.*, 1980, **41**, 200–206.
- [16] R. Freeman and J. Keeler, *J. Magn. Reson.*, 1981, **43**, 484–487.
- [17] W.P. Aue, J. Karhan and R.R. Ernst, *J. Chem. Phys.*, 1976, **64**, 4226–4227.
- [18] K. Nagayama, P. Bachmann, K. Wüthrich and R.R. Ernst, *J. Magn. Reson.*, 1978, **31**, 133–148.
- [19] G. Bodenhausen, R. Freeman, G.A. Morris and D.L. Turner, *J. Magn. Reson.*, 1978, **31**, 75–95.
- [20] J.D. Mersh and J.K.M. Sanders, *J. Magn. Reson.*, 1982, **50**, 171–174.
- [21] M. Woodley and R. Freeman, *J. Magn. Reson. (A)*, 1994, **111**, 225–228.
- [22] M. Woodley and R. Freeman, *J. Magn. Reson. (A)*, 1994, **109**, 103–112.
- [23] A.J. Pell and J. Keeler, *J. Magn. Reson.*, 2007, **189**, 293–299.
- [24] K. Zangger and H. Sterk, *J. Magn. Reson.*, 1997, **124**, 486–489.
- [25] G.A. Morris, *J. Magn. Reson.*, 1981, **44**, 277–284.

Chapter 8

Correlations through space: The nuclear Overhauser effect

8.1. INTRODUCTION

The previous three chapters in this book have all been concerned with *scalar couplings* between nuclei, that is, the indirect couplings that are transmitted through intermediate electron spins such as those in intervening chemical bonds. We have seen that by application of the appropriate techniques, the chemist is able to exploit this coupling information and piece together molecular fragments and, ultimately, gross molecular structures. In this chapter, we shall be concerned with a fundamentally different form of interaction between nuclear spins, that of the direct, through-space magnetic interactions (*dipolar couplings*) that give rise to the nuclear Overhauser effect (NOE). This brings about changes in resonance intensities and is, as we shall discover, intimately related to nuclear spin relaxation. The NOE is typically employed during the later stages of a structural investigation when the gross structure of the molecule has been (largely) defined through the application of the various techniques described in the four preceding chapters. The importance of the NOE in modern structure elucidation can hardly be overstated. It is unique in its ability to provide the chemist with information on 3D molecular geometry. Such information can be obtained because the NOE depends on, amongst other factors, internuclear separations such that only those spins that are 'close' in space are able to demonstrate this effect.

The NOE also finds widespread use as a means of sensitivity enhancement of low- γ spin- $\frac{1}{2}$ nuclei, so widespread in fact that it is often taken for granted and its contribution to experiments often overlooked. As described in Chapter 4 and below, the use of broadband proton decoupling during the recording of carbon spectra contributes as much as a three-fold increase in resonance intensity by virtue of the proton to carbon NOE. However, the principal aim of this chapter is to develop an appreciation of the NOE as a tool in structural analysis, where it has a unique role to play. The interpretation of NOE measurements does, however, require more care than for those methods that exploit scalar couplings and is generally more susceptible to erroneous conclusions being drawn. As part of this, it is also important to be conscious of the nature of the NOE measurement being taken, and in particular whether it is a *steady-state* or a *transient* protocol that is used. The first of these is exemplified by the NOE difference experiment whilst the second is best known as the NOESY technique, executed as either the selective 1D or the 2D variants. The nature of the measurement has fundamental implications for how the data should be *interpreted* and indeed *reported*. As such, these two fundamentally different approaches to NOE measurements will be treated separately for much of the chapter, although they both share in the same underlying theory. The principal techniques described in this chapter are summarised in Table 8.1.

Which one of these two approaches is adopted in the laboratory may be dictated by the motional properties of the molecule(s) under study and more specifically the rates at which the molecules tumble in solution. Pre-empting what is to follow, it will be shown that the steady-state experiments are appropriate only for molecules that tumble 'rapidly' in solution (we shall also see what defines 'rapidly' in this context). Such measurements have traditionally been the home territory of small organic molecules in relatively non-viscous solutions. In contrast, very much larger molecules that tumble 'slowly' in solution (or smaller molecules in very viscous solutions) can be meaningfully studied only with the transient NOE techniques, which may also be suitable for small-molecule studies. Between these two extremes of molecular tumbling rates, the conventional NOE can become weak and vanishingly small, a condition most likely to occur for those molecules with masses of around 1000–2000 daltons. It is here that rotating-frame NOE (ROE) measurements play a vital role, and these shall also be described.

The chapter is presented in two parts, the first covering the essential theory that underlies the NOE and the second addressing the practicalities of how one measures NOE enhancements, the experimental steps required to optimise such measurements and how to correctly

Table 8.1. The principal applications of the main techniques described in this chapter. The molecular masses mentioned provide only approximate ranges over which the experiments are applicable (see main text)

Technique	Principal applications
NOE difference	Establishing NOEs and hence spatial proximity between protons. Suitable only for 'small' molecules ($M_r << 1000$), for which NOEs are positive. Observes <i>steady-state</i> or <i>equilibrium</i> NOEs generated from the <i>saturation</i> of a target.
NOESY(2D or 1D)	Establishing NOEs and hence spatial proximity between protons. Suitable for 'small' ($M_r << 1000$) and large molecules ($M_r > 2000$) for which NOEs are positive and negative respectively, but may fail for mid-sized molecules (zero NOE). Observes <i>transient</i> NOEs generated from the <i>inversion</i> of a target. Estimates of internuclear separations can be obtained in favourable cases.
ROESY(2D or 1D)	Establishing NOEs and hence spatial proximity between protons. Suitable for any molecule but often essential for mid-sized molecules; NOEs are positive for all molecular sizes. Observes <i>transient</i> NOEs in the rotating-frame, but is prone to interference from other mechanisms so requires cautious interpretation. Estimates of internuclear separations can be obtained in favourable cases.
HOESY	Establishing heteronuclear NOEs and hence spatial proximity between different nuclides, for example, ^1H – ^{13}C . Can provide useful stereochemical information when homonuclear NOEs are insufficient or inappropriate. Suffers from low sensitivity but ^1H detected variants can help.
EXSY (2D)	Qualitative mapping of exchange pathways in dynamic systems when exchange rates are slow on the NMR chemical shift timescale, meaning separate resonances are observed for each exchanging species. Quantitative data on exchange kinetics can be obtained in favourable cases.

1D, one-dimensional; 2D, two-dimensional; NOE, nuclear Overhauser effect; ROESY, rotating-frame spectroscopy

interpret the data. In keeping with the style of this book, mathematical equations are kept to a minimum and are introduced only when they serve to illustrate a point of fundamental importance. Likewise, the equations are generally presented rather than being derived, and the interested reader is encouraged to read dedicated texts on these topics for further elaboration [1].

Part I: Theoretical aspects

8.2. DEFINITION OF THE NOE

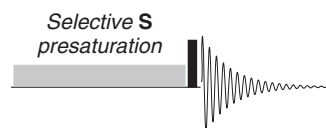
The NOE may be defined as the change in intensity of one resonance when the spin transitions of another are somehow perturbed from their equilibrium populations. The perturbation of interest usually corresponds to either *saturating* a resonance, that is, equalising the spin population differences across the corresponding transitions or *inverting* it, in other words, inverting the population differences across the transitions. The magnitude is expressed as a relative intensity change between the equilibrium intensity, I_0 , and that in the presence of the NOE, I , such that

$$\eta_I\{S\} = \frac{I - I_0}{I_0} \times 100(\%) \quad (8.1)$$

where $\eta_I\{S\}$ indicates the NOE observed for spin I when spin S is perturbed, which shall also be referred to as the NOE from spin S to spin I . The use of the symbols I and S stems from the original publications on the phenomenon (which, in fact, observed the NOE from electron spins to nuclear spins in a metal) and have become the recognised nomenclature when describing the NOE. However, both S and I have been used to define the *perturbed* spin over the years and even across modern texts both definitions are encountered, so one should always be clear as to the terminology in use. Herein, S will always refer to the perturbed (or Source) spin and I to the enhanced (or Interesting) spin. The intensity changes brought about by the NOE can be either positive (an increase) or negative (a decrease) as dictated by the motional properties of the molecules and by the signs of the magnetogyric ratios of the participating spins. Throughout the chapter, the term 'enhancement' will be used to refer to the intensity changes.

8.3. STEADY-STATE NOES

In laying down the background to how the NOE arises and what factors dictate its sign and magnitude, we shall restrict our discussion to steady-state NOEs in which the perturbation is brought about by *saturating* S spin transitions by the selective application of weak rf irradiation to the S resonance (Fig. 8.1). It is this form of the NOE that is observed with the popular NOE difference method, which has had such an enormous impact on structural

**Figure 8.1.** The general experimental scheme for observing steady-state NOE enhancements.

organic chemistry. Further discussions relating to other forms of NOE measurement then logically follow from this background material. We begin by considering the simple case of a homonuclear two-spin system and then progress to consider more realistic multispin systems.

8.3.1. NOEs in a two-spin system

Origin of the NOE

Consider a system comprising only two homonuclear spin- $\frac{1}{2}$ nuclei, I and S, that exist in a rigid molecule, which tumbles isotropically in solution, that is, it has no preferred axis about which it rotates. The two nuclei do not share a scalar coupling ($J_{IS}=0$) but are sufficiently close to share a dipolar coupling. This is the direct, through-space magnetic interaction between the two spins such that one spin is able to sense the presence of its dipolar-coupled partner. This coupling may be viewed as being analogous to the interaction one witnesses when two bar magnets are brought close together, and follows the idea introduced in Chapter 2 that nuclear magnetic dipoles can be viewed as microscopic bar magnets (see below).

The energy level diagram for an ensemble of $4N$ molecules is shown in Fig. 8.2. Since we are considering a homonuclear system, the energies of the I and S transitions will be essentially identical (chemical shift differences are negligible relative to Larmor frequencies), and we can therefore assume that the populations of the $\alpha\beta$ and $\beta\alpha$ states are equal at equilibrium. According to the Boltzmann distribution, there will then exist an excess of nuclei in the lower energy $\alpha\alpha$ orientation, and a deficit in the higher energy $\beta\beta$ state. We shall ultimately be interested in the population differences across transitions as it is these that dictate the intensity of the NMR resonances, so we shall simply symbolise the population excess as Δ and the deficit as $-\Delta$ relative to those of the $\alpha\beta$ and $\beta\alpha$ states. Note that dipolar couplings do not produce observable splittings in solution spectra (see below), so the two transitions associated with each spin are of identical energy. The spectrum in the absence of perturbation therefore contains two singlet resonances of equal intensity (Fig. 8.2a).

Now suppose we instantaneously saturate the S resonance forcing the population differences across the S transitions to zero. The new spin populations are indicated in Fig. 8.2b. Clearly, the system has been forced away from the equilibrium population differences, so will attempt to regain this by altering its spin populations. The changes of spin states required to achieve this are brought about by longitudinal spin relaxation processes, so we need to consider which relaxation pathways are now available to the spins. Ignoring, for the moment, the mechanism by which these changes may occur, we see that six possible pathways can be identified for a two-spin system (Fig. 8.3). Four of these correspond to the single-quantum transitions, involving

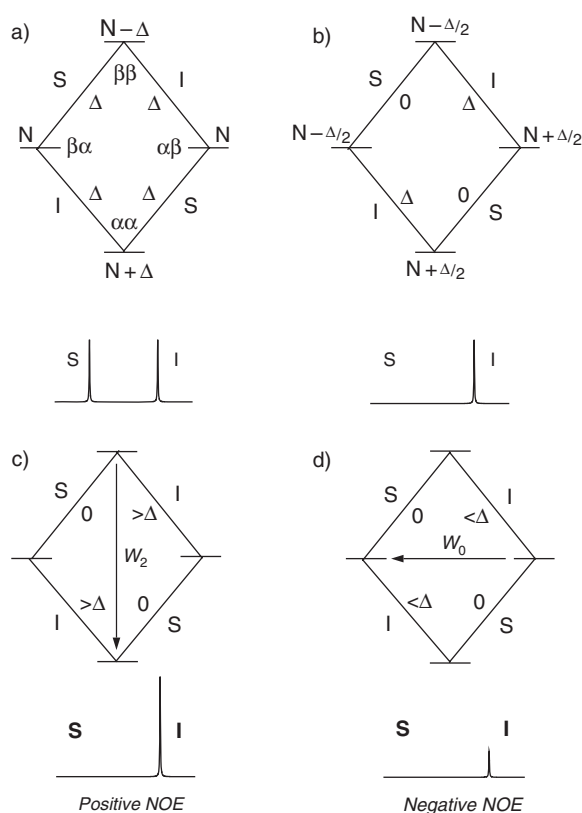


Figure 8.2. Schematic energy level diagrams and population differences for two spins, S and I, which share a dipolar coupling; (a) at equilibrium, (b) after instantaneous saturation of the S spins, (c) after relaxation via W_2 processes and (d) after relaxation via W_0 processes. Below each are the corresponding schematic spectra.

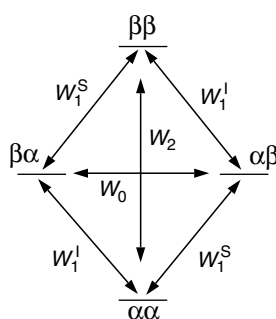


Figure 8.3. The six possible transitions in a two-spin system.

the flip of a single spin, for example, $\alpha\alpha \rightarrow \beta\alpha$. The W labels represent the 'transition probabilities,' for each, or in other the words, the rates at which the corresponding spin flips occur, and the subscripts the magnetic quantum number of the transition. The two other transitions, $\alpha\beta \rightarrow \beta\alpha$ and $\alpha\alpha \rightarrow \beta\beta$, involve the simultaneous flipping of both S and I spins. Although these transitions do occur, they cannot be directly *observed* in an NMR experiment, unlike single-spin flips, because the overall change in magnetic quantum, ΔM , does not equal one. They are said to be 'forbidden' by quantum mechanical selection rules. The $\alpha\beta \rightarrow \beta\alpha$ W_0 process is referred to as the zero-quantum transition ($\Delta M = 0$) whilst the $\alpha\alpha \rightarrow \beta\beta$ W_2 process is the double-quantum transition ($\Delta M = 2$) (notice the same terminology was used when describing the transitions of scalar-coupled spins in Chapter 5). Both are able to act as relaxation pathways and, in fact, it is only these two that are responsible for the NOE itself. Collectively, they are referred to as *cross-relaxation* pathways, a term suggestive of the simultaneous participation of both spins.

Returning to Fig. 8.2, we are now in a position to consider how the various relaxation pathways may be used to re-establish the equilibrium condition, noting that throughout the W_1^S transitions remain saturated by the continuous rf energy. The population differences across the I spin transitions are still Δ , as they were at equilibrium, so the W_1^I processes will play no part in re-establishing equilibrium and thus have no role to play in producing the NOE. The W_2 process will act to remove spins from the $\beta\beta$ state and transfer them to the $\alpha\alpha$ state in an attempt to recover the population differences across the S transitions. In doing so, this will *increase* the population *difference* across the two I transitions (Fig. 8.2c). Thus, relaxation via the W_2 process will result in a net increase in the I spin resonance intensities in the spectrum; this is then a *positive NOE*. Likewise, the W_0 process will act to transfer spins from the $\beta\alpha$ to the $\alpha\beta$ state, again in an attempt to recover the population differences across the S transitions. In this case, the result will be a *decrease* in the population *difference* across the two I transitions (Fig. 8.2d) so that relaxation via the W_0 process will result in a net reduction in the I spin resonance intensities in the spectrum; this is then a *negative NOE*.

From these qualitative considerations, we can already say a fair amount about how we might expect the NOE to appear. Clearly, the W_2 and W_0 cross-relaxation processes compete with one another, with the dominant pathway dictating the sign of the observed NOE. In addition, the W_1^I pathways will act to re-establish the equilibrium population differences for the I transitions as soon as the NOE begins to develop, so will tend to act against the build-up of the NOE. Thus, if the relaxation mechanisms for the W_1^I pathways happen to be rather more efficient than those of the W_2 and W_0 pathways, then a measurable NOE may never develop; it is, in effect, bypassed altogether. This can have a significant bearing on the experimental measurement of NOEs, as we shall see in due course. The NOE therefore results from a balance between a number of competing relaxation pathways. Saturating the S transitions for a period of time that is long relative to the relaxation times allows a new *steady-state* of populations to arise as a result of this competition, and it is this one eventually measures. A full consideration of the various rate processes involved in the population changes leads to the so-called Solomon equation, which for the steady-state NOE can be used to derive the expression:

$$\eta_I\{S\} = \frac{\gamma_S}{\gamma_I} \left[\frac{W_2 - W_0}{W_0 + 2W_1^I + W_2} \right] \equiv \frac{\gamma_S}{\gamma_I} \left[\frac{\sigma_{IS}}{\rho_{IS}} \right] \quad (8.2)$$

The term σ_{IS} represents the *cross-relaxation rate constant* for the two spins, whilst ρ_{IS} is the total *dipolar longitudinal relaxation rate constant* of spin I. The magnetogyric ratios (γ_S and γ_I) are included to take account of the different equilibrium populations that would exist for spins with differing γ s; for a homonuclear spin system as considered thus far, these values would obviously be equal and may be ignored. This fundamental expression contains within it the qualitative arguments arrived at above; $W_2 - W_0$ dictates the sign of the NOE whereas W_1^I processes make no contribution to this but serve to reduce its magnitude. To appreciate the size and sign of the NOE, how this relates to molecular motion and, indeed, how this can be related in any way to internuclear distances, it is necessary to define what factors influence the participating rate constants, and for this one needs to consider the spin relaxation processes involved.

Spin relaxation and dipolar coupling

The NOE arises as a result of the redistribution of spin populations and hence flips between spin states. Such redistributions occur as a result of longitudinal spin relaxation (Chapter 2), which do not occur spontaneously but require a suitable stimulus to induce the transitions. This stimulus is a magnetic field fluctuating at the frequency of the corresponding transition (both here and below 'frequency' corresponds to the energy of the transition rather than the rate at which the spin flips occur). Here exists an analogy with the pulse excitation of a spin system initially at equilibrium. The time-dependent magnetic component of the electromagnetic rf

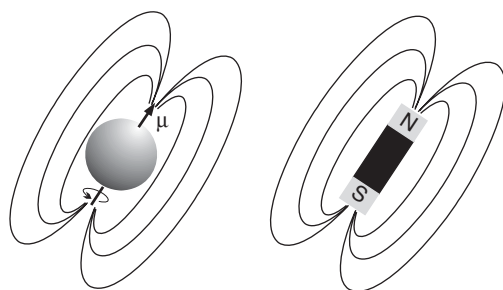


Figure 8.4. The bar magnet analogy for a spin- $\frac{1}{2}$ nucleus in which the magnetic dipole is viewed as possess a magnetic north and south pole.

radiation interacts with nuclear magnetic moments and is thus able to tip the bulk magnetisation vector into the transverse plane. Only if the rf has magnetic components oscillating at the Larmor frequencies of the spins does excitation occur; this is why one is able to apply excitation pulses to protons whilst leaving, say, carbon spins unaffected.

The magnetic field of relevance to the NOE is the local field experienced by a spin as a result of dipolar interactions with neighbouring magnetic nuclei. These interactions may be visualised using the microscopic bar magnet analogy for spin- $\frac{1}{2}$ nuclei in which they are considered to possess a magnetic north and a south pole (Fig. 8.4). Depending on the relative orientation of the two nuclei to one another, the field generated by a neighbouring spin will either reinforce or counteract the applied magnetic field, with the time-dependent fluctuation produced by the rotational motion of the molecule in which these nuclei sit (Fig. 8.5). It is precisely this that provides the mechanism for relaxation, known as *longitudinal dipole–dipole relaxation*, and this requirement for an interaction with a neighbouring spin intuitively fits with the arguments presented above for the NOE arising from *mutual* spin flips (the W_2 and W_0 processes). The magnitude of dipolar coupling between spins is acutely sensitive to the internuclear separation r , being proportional to r^{-3} , and it is in this that the NOE itself ultimately has a distance dependence.

One should note here that despite providing an important relaxation mechanism, dipolar couplings do not usually produce observable splittings in solution state NMR spectra. This is because, although the couplings have a finite value at any instant in time, they are averaged precisely to zero on the NMR timescale by the rapid isotropic tumbling of a molecule.

To induce the spin transitions we have been considering, the molecule must tumble at the appropriate frequency to provide a suitable fluctuating field. The rate at which a molecule tumbles or rotates in solution is typically defined by its rotational *correlation time*, τ_c . This is usually taken to define the average time required for the molecule to rotate through an angle of 1 radian about any axis, meaning rapidly tumbling molecules possess small correlation times whilst slowly tumbling molecules have large correlation times. A *very rough* estimate of this time for a molecule of mass M_r may be obtained from the relationship:

$$\tau_c \approx M_r \times 10^{-12} \text{ s} \quad (8.3)$$

The power available within a molecular system to induce transitions by virtue of its molecular tumbling is referred to as the *spectral density* $J(\omega)$ (Section 2.5), and this provides a measure of how the relaxation rates W_0 , W_1 and W_2 vary as a function of tumbling rates. This is illustrated schematically in Fig. 8.6 for three different correlation times. An alternative description of the spectral density is that it represents the probability of finding a fluctuating magnetic component at any given frequency as a result of the motion, and as such, the area under each of the curves of Fig. 8.6 must then be equal. Thus, for a molecule with a short τ_c (rapid tumbling), there exists an almost equal but comparatively small chance of finding components at both high and low frequencies, up to about $1/\tau_c$ at which point the probability falls away rapidly. Conversely, there is only a very small probability that molecules that tumble slowly (*on average*) will generate rapidly oscillating fields, so the corresponding spectral density is concentrated into a smaller frequency window. These curves therefore predict how the relaxation rates will vary with correlation time. For a transition of frequency ω_i , the spectral density will be

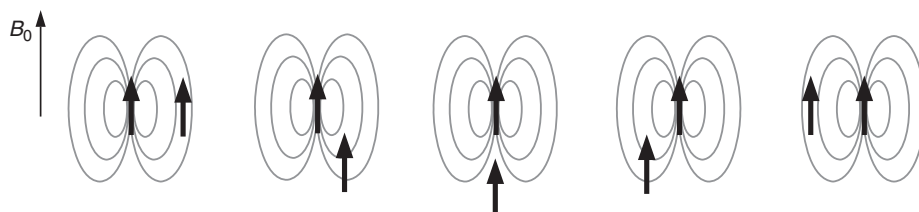
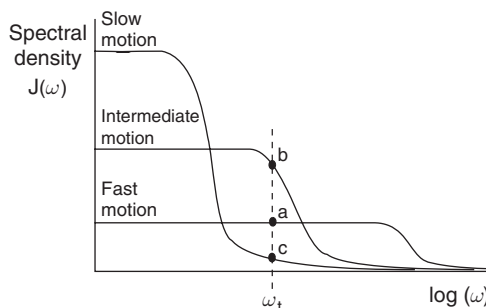


Figure 8.5. The direct, through-space interaction between two near spin- $\frac{1}{2}$ nuclei (the dipolar interaction). This fluctuates as the molecule tumbles in solution and can provide a time-dependent field capable of inducing spin transitions.

Figure 8.6. Schematic spectral densities for molecules tumbling in three motional regimes as a function of frequency, ω , where ω_t represent the frequency of the spin transition.



rather low if the molecular tumbling rate is far greater than this (point a) and thus the relaxation rates will also be low. As the rate of tumbling slows and approaches ω_t , the spectral density and hence relaxation rates increases (point b), only to decrease once more as the tumbling rate falls below ω_t . Thus, the dependence of relaxation rates on τ_c may be represented as in Fig. 8.7, with the fastest relaxation occurring when the correlation rate $1/\tau_c$ matches the frequency of the transition. The point of fastest W_1 relaxation also corresponds to the T_1 minimum and *vice versa*, so T_1 values themselves are also dependent on the rates of molecular motion.

Using these arguments it is possible to predict when the W_0 or W_2 processes will be dominant. For the zero-quantum W_0 processes, the energy differences involved are rather small, being the differences between the I and S frequencies, so $\omega_t = |(\omega_I - \omega_S)|$. These transitions will therefore be strongly favoured for a molecule that tumbles slowly in solution. The double-quantum W_2 transitions correspond to the sum of the I and S frequencies, so $\omega_t = (\omega_I + \omega_S)$, and these will be stimulated by rapidly tumbling molecules. As an example of the transition frequencies involved, consider a homonuclear proton system at an observation frequency of 400 MHz. The single-quantum W_{1I} (and W_{1S}) transition frequencies will correspond to approximately 400 MHz. The W_0 transition frequencies are given by the frequency differences of I and S, that is, their chemical shift differences, which will be in the Hz or kHz region. The W_2 transition frequencies are the sums of ω_I and ω_S , which will equate to around 800 MHz. Clearly, the frequency spread of molecular motions to which the NOE is sensitive is extremely large.

Qualitatively then, one can predict from the previous arguments that molecules that tumble rapidly in solution are likely to favour the higher energy W_2 process and hence exhibit positive NOEs whilst those that tumble slowly will favour the W_0 process and thus display negative NOEs; indeed, this is what is observed in practice.

Quantitative expressions for the relaxation rates in a dipolar coupled two-spin system have been derived, thus:

$$W_{1I} \propto \gamma_I^2 \gamma_S^2 \left[\frac{3\tau_c}{r^6 (1 + \omega_I^2 \tau_c^2)} \right] \quad (8.4a)$$

$$W_0 \propto \gamma_I^2 \gamma_S^2 \left[\frac{2\tau_c}{r^6 (1 + (\omega_I - \omega_S)^2 \tau_c^2)} \right] \quad (8.4b)$$

$$W_2 \propto \gamma_I^2 \gamma_S^2 \left[\frac{12\tau_c}{r^6 (1 + (\omega_I + \omega_S)^2 \tau_c^2)} \right] \quad (8.4c)$$

where the constant of proportionality is the same for each.

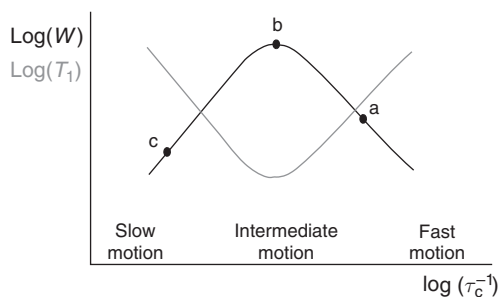


Figure 8.7. The schematic variation in relaxation rates, and hence relaxation times (shown greyed), as a function of molecular tumbling rates.

Notice that, in addition to the dependence on correlation times, these expressions contain a term for the internuclear separation, r , between spins S and I. Here, at last, one starts to see the origins of the famed ' r^{-6} ' distance dependence widely, and sometimes dangerously, associated with NOE interpretations [2]. This distance term is manifested in the degree of dipolar coupling between the two spins. An important point to note at this stage is that this distance dependence actually lies in the relaxation *rates* and, as we shall see, this can have enormous implications for the way in which NOE data are interpreted. The inverse-sixth relationship also means the NOE falls away very rapidly with distance, so in practice significant NOEs will only develop between protons that are within ca. 0.5 nm of each other (naturally, this will be influenced by how sensitive and stable the spectrometer is and hence how small an enhancement one is able to 'see'). Note also the dependence of Eq. (8.4) on the square of the magnetogyric ratios of the two spins, so very different rates may occur in heteronuclear systems, depending on the participating spins.

When a molecule tumbles so rapidly in solution such that $\omega\tau_c \ll 1$, all terms in these expressions containing ω become negligible and the rates simplify to:

$$W_{II} \propto \gamma_I^2 \gamma_S^2 \frac{3\tau_c}{r^6} \quad (8.5a)$$

$$W_0 \propto \gamma_I^2 \gamma_S^2 \frac{2\tau_c}{r^6} \quad (8.5b)$$

$$W_2 \propto \gamma_I^2 \gamma_S^2 \frac{12\tau_c}{r^6} \quad (8.5c)$$

This condition is referred to as the *extreme narrowing limit* since all broadening effects attributable to dipolar interactions are fully averaged to zero under these conditions. This regime typically applies only to small molecules in low-viscosity solvents, and the point at which this condition breaks down depends on the correlation time of the molecule as well as the field strength of the spectrometer (through ω).

NOEs and molecular motion

Having taken the trouble to see how the relaxation rates in a two-spin system depend on molecular motion, we are now in a position to predict the behaviour of the NOE itself as a function of this motion and of internuclear separation. Taking the rate constant Eq. (8.4) and substituting these into that for the NOE Eq. ((8.2)) produces the curve presented in Fig. 8.8 for the theoretical variation of the *homonuclear* NOE as a function of molecular tumbling rates as defined by $\omega_0\tau_c$, (where ω_0 is the spectrometer observation frequency, approximately equal to ω_I and ω_S). Note this is for a two-spin system, which relaxes solely by the dipole-dipole mechanism and as such represents the theoretically maximum possible NOE. The curve has three distinct regions in it, which we shall loosely refer to as the fast, intermediate and slow motion regimes. For those molecules that tumble rapidly in solution (short τ_c , those in the extreme narrowing limit), the NOE has a maximum possible value of +0.5 or 50%. Smaller organic molecules in low-viscosity solvents typically fall within this fast motion regime, which is traditionally the home ground of steady-state NOE measurements. At the other extreme, molecules that tumble very slowly in solution experience negative NOEs, as shown above. The maximum enhancement in this motion regime is obtained when W_2 and W_{II} are both zero, which

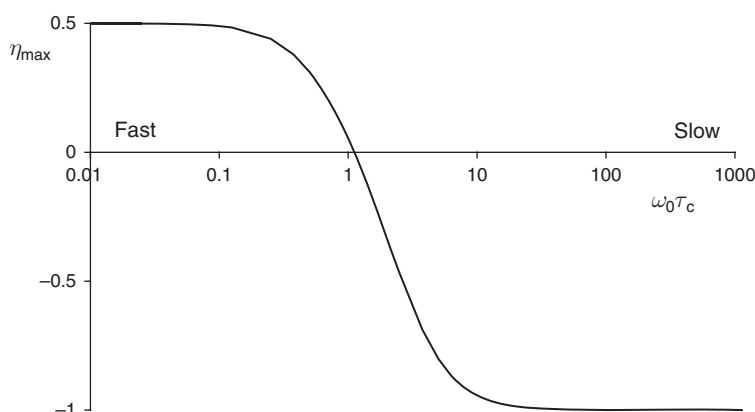


Figure 8.8. The variation in the maximum theoretical homonuclear steady-state NOE in a two-spin system as a function of molecular tumbling rates (defined by the dimensionless parameter $\omega_0\tau_c$). The region of fast motion is the extreme narrowing limit and that of slow motion is the spin-diffusion limit.

from Eq. (8.2) can be seen to be -1 or -100% . For NOE measurements in this region, it becomes essential to use transient experiments since those based on steady-state measurements become uninformative, as explained below. This is the region inhabited by (biological) macromolecules and it is studies of these systems that have traditionally made widespread use of transient NOE measurements, principally through the 2D NOESY experiment. Between these two extremes is the intermediate region in which the NOE changes sign and even becomes zero when $W_2 = W_0$. Within this region, the magnitude and sign of the NOE is highly sensitive to the rate of molecular motions and can be rather weak, possibly too weak to be observed, clearly a major hindrance to structural studies. The point at which this region is entered will be dependent on a number of factors – the size and shape of the molecule, solution conditions (viscosity, temperature, possibly pH, etc) and spectrometer field strength. As a rule of thumb, molecules with a mass of 1000–2000 daltons are likely to fall within this intermediate regime. The increasing interest in larger synthetic molecules in many areas, for example, supramolecular chemistry, is likely to mean that more molecules routinely handled by the research chemist will fall into this potentially troublesome region. The actual zero cross-over point occurs when:

$$\omega\tau_c = \sqrt{5/4} = 1.12 \quad (8.6)$$

or in other words, when the molecular tumbling rate approximately matches the spectrometer observation frequency. This is therefore field dependent, as illustrated in Fig. 8.9, which now shows the variation of the NOE as a function of τ_c itself for three different field strengths. The use of a higher field strength increases the likelihood of a relatively ‘small’ molecule falling within the intermediate regime or perhaps a mid-sized molecule passing from this into the slow motion regime. In some cases, therefore, the use of a higher field instrument, often regarded as a panacea for all chemists’ woes, may even prove detrimental to the NOE experiment as the ‘zero NOE’ condition is approached.

The field dependence of the intermediate regime suggests one solution to the problem of ‘zero’ NOEs – trying a different field strength. This is usually an impossible or impractical answer, so an alternative approach is to alter the solution conditions and hence the rate of molecular tumbling, one of the simplest approaches being to vary the sample temperature. A technically different experimental approach to the problem is to measure NOEs in the *rotating frame* instead. This is described further below, suffice it to say here that these NOEs remain positive for all molecular tumbling rates one is likely to encounter, so avoids the ‘zero cross-over’ problem altogether.

NOEs and internuclear separation

It is now possible to consider how the separation between two spins influences the steady-state NOE enhancement. Assuming that the molecule exists in the extreme narrow-line regime, then substituting the simplified rate Eq. (8.5) into Eq. (8.2), we obtain:

$$\eta_{\text{H}}\{\text{S}\} = \left[\frac{\left(\frac{12\tau_c}{r^{-6}}\right) - \left(\frac{2\tau_c}{r^{-6}}\right)}{\left(\frac{2\tau_c}{r^{-6}}\right) + 2\left(\frac{3\tau_c}{r^{-6}}\right) + \left(\frac{12\tau_c}{r^{-6}}\right)} \right] = \left[\frac{12 - 2}{2 + 6 + 12} \right] = \frac{1}{2} \quad (8.7)$$

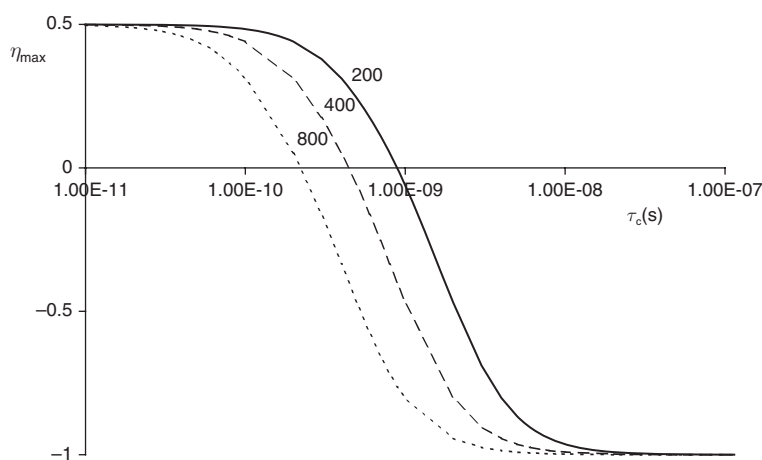


Figure 8.9. The variation in the maximum theoretical homonuclear steady-state NOE in a two-spin system as a function of molecular tumbling rates shown for three spectrometer observation frequencies (MHz).

As in Fig. 8.8 for the maximum NOE above, the enhancement is predicted to be 50%. However, it is also predicted to be *independent* of the internuclear distance. Thus, at least for the hypothetical isolated two-spin system considered here, the magnitude of the steady-state enhancement provides no distance information whatsoever. The important point here is that one cannot simply state that NOE enhancements within a molecule scale directly with r^{-6} . In realistic chemical systems, various ‘other’ factors must also be taken into account before any distance dependence is reintroduced; this is further pursued below. Although the *magnitude* of the steady-state enhancement is predicted to be independent of distance in this system, the *rate* at which this is reached is not because of the dependence of relaxation rates on distance (as expressed in Eq. (8.4)), meaning NOEs between closer spins develop more rapidly; this is the basis of the transient NOE measurements described in Section 8.4. This also implies that longer range NOEs will only have significant intensities when long presaturation periods are employed, a point of considerable practical importance.

Heteronuclear NOEs

The equivalent of Eq. (8.7) for a heteronuclear pair experiencing extreme narrowing is the more general expression:

$$\eta_{\text{I}}\{\text{S}\} = \frac{\gamma_{\text{S}}}{2\gamma_{\text{I}}} \quad (8.8)$$

For the common situation of carbon-13 observation in the presence of proton saturation (broadband decoupling), $\gamma_{\text{H}}/\gamma_{\text{C}} \approx 4$ and NOE enhancements can be as much as 200%, equating to a three-fold intensity increase. Since the relaxation of carbon nuclei is largely dominated by proton dipolar interactions, this maximum is almost met in practice. This is clearly a valuable route to sensitivity enhancement and, at least for the case of ^{13}C , compares favourably for routine acquisitions with the factor of four attainable with the ^1H to ^{13}C polarisation transfer sequences, given in Chapter 4. The maximum NOE enhancements for a variety of nuclei in the presence of proton saturation, denoted $\text{X}\{^1\text{H}\}$, are summarised in Table 8.2.

In heteronuclear systems, the observed NOE also depends on the signs of the magnetogyric ratios of the cross-relaxing spins, so NOEs from protons to nuclei with negative γ s will display negative NOEs even if the molecule is within the extreme narrowing regime. The most common examples of this are for ^{15}N and ^{29}Si for which a *reduction* of signal intensity occurs on proton saturation, so much that the observed resonance can itself become negative. If less than the full (negative) NOE is generated, the resonance may disappear altogether as the NOE cancels the natural signal, and because of this, it is usual to record the spectra of negative γ species in the absence of the NOE, either by use of the inverse-gated decoupling scheme (Section 4.2.3), by polarisation transfer methods (Section 4.4) or, less commonly, by the addition of a paramagnetic relaxation reagent to quench the NOE. In some instances, the heteronuclear NOE may be used more specifically for structural assignment also; see Section 8.9.

Beyond the extreme narrowing condition, the absolute magnitudes of heteronuclear $\text{X}\{\text{H}\}$ NOEs decrease and, in the case of ^{13}C , ^{15}N and ^{29}Si , closely approach zero, although, with the exception of ^{19}F , do not change sign. For very large molecules, there is then little sensitivity gain, or loss, arising from the heteronuclear NOE.

8.3.2. NOEs in a multispin system

The previous section considered the NOE for the hypothetical case of a two-spin system in which the spins relax exclusively via mutual dipole–dipole relaxation. In progressing to consider more realistic multispin systems, two key issues will be

Table 8.2. Theoretical maximum steady-state heteronuclear NOE enhancements in the presence of proton saturation. These numbers assume relaxation exclusively via dipole–dipole interactions, although for the metals in particular chemical shift anisotropy may also be a significant mechanism. The lithium isotopes are somewhat anomalous in that they are quadrupolar yet can still demonstrate NOEs. ^6Li in particular has the smallest quadrupole of all such nuclei, so the dipole mechanism still makes a significant contribution to relaxation

X	^6Li	^7Li	^{13}C	^{15}N	^{19}F	^{29}Si	^{31}P	^{57}Fe	^{103}Rh	^{109}Ag	^{119}Sn	^{183}W	^{195}Pt	^{207}Pb
$\eta_{\text{X}}\{^1\text{H}\} \%$	339	129	199	−494	53	−252	124	1548	−1589	−1075	−141	1202	233	239

addressed – how the presence of other spins affects the magnitudes of steady-state NOEs and how these reintroduce distance dependence to the NOE. These considerations lead to the conclusion that steady-state NOE measurements must be used in a comparative way to provide structural data and that they do not generally provide estimates of internuclear distances *per se*.

Additional relaxation pathways

The NOE arises as a result of dipolar cross-relaxation between two nuclei, and hence only the dipole–dipole relaxation mechanism is able to generate the NOE. All other competing mechanisms (with the subtle exception of scalar relaxation in a strongly coupled system, which is rarely of significance) serve to dilute the overall influence of the W_2 and W_0 pathways by stimulating the W_1 relaxation pathway only, and hence reduce the magnitude of the NOE. These various other contributions to spin relaxation may be grouped together and represented by the term ρ_I^* , the external relaxation rate for spin I. Adding this to Eq. (8.2) gives:

$$\eta_I\{S\} = \frac{\gamma_S}{\gamma_I} \left[\frac{\sigma_{IS}}{\rho_{IS} + \rho_I^*} \right] \quad (8.9)$$

This illustrates the diluting effect of ρ_I^* on the magnitude of the NOE, which is sometimes referred to as the ‘leakage’ term. The effect of this turns out to be of somewhat greater significance for molecules in the extreme narrowing limit than those in the negative NOE regime. For small, rapidly tumbling molecules, the absolute magnitude of σ_{IS} is small when compared with that of slowly tumbling molecules, so the relative contribution to Eq. (8.9) of ρ_I^* has greater significance. The effect of this is schematically illustrated in Fig. 8.10, which shows that leakage becomes more relevant as molecular tumbling rates increase, meaning very small NOEs may be observed for small molecules. In contrast, leakage effects are less problematic for molecules in the negative NOE regime.

To maximise the size of the NOE for molecules in the extreme narrowing condition, it is necessary to minimise ρ_I^* . The most significant contribution to this in routinely prepared solutions is from the paramagnetic oxygen dissolved in solvents. The unpaired spin- $\frac{1}{2}$ electron has a magnetic moment that is over 600 times that of the proton, so is able to provide an intense magnetic interaction capable of causing efficient relaxation. Degassing of solutions prior to NOE studies may become necessary when seeking longer range interactions in small molecules but is otherwise unnecessary for the majority of routine studies (see Section 8.10). Similarly, other paramagnetic impurities, for example, some metals, will quench the NOE and must be avoided. Samples to which relaxation agents have been deliberately added to promote relaxation are therefore not suitable candidates for NOE studies. *Intermolecular* dipolar interactions, arising from transient interactions with solute or solvent molecules, are another cause for potential interference. Solvent molecules are deuterated in most cases, so the relaxation arising from these nuclei is rather inefficient when compared to protons (due to the γ^2 dependence of longitudinal dipolar relaxation rates), and interactions with other (protonated) solute molecules are generally only likely to be a problem when very concentrated solutions are used, so these are thus best avoided for NOE studies. Other non-dipole relaxation mechanisms, such as those discussed in Chapter 2, also act to bypass the NOE. Of most significance is the quadrupolar relaxation associated with nuclei with spin $>\frac{1}{2}$. This is usually the dominant mechanism for such nuclei, so

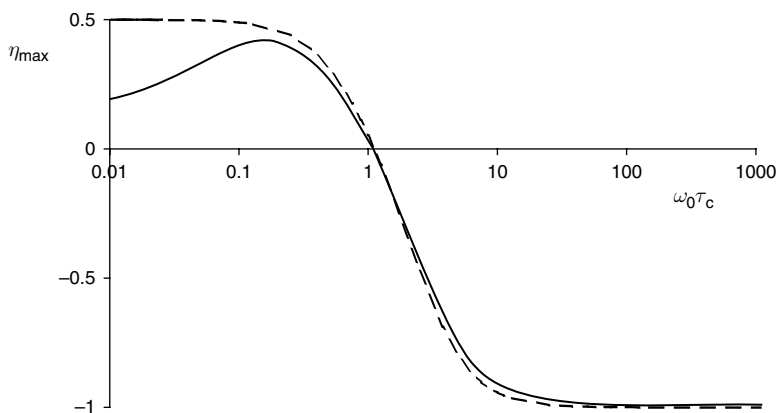


Figure 8.10. Schematic illustration of the maximum homonuclear steady-state NOE in the presence (solid line) and absence (dotted line) of external relaxation sources that compete with cross-relaxation.

NOEs onto quadrupolar nuclei are very rarely observed (the exception being ^6Li as mentioned in Table 8.2).

Internuclear separations (again)

Perhaps the most obvious 'other' contribution to the relaxation of spin I in a multi-spin system arises from neighbouring nuclei other than spin S within the same molecule. Dipolar interactions with these spins act to bring about longitudinal relaxation of spin I independent of cross-relaxation between I and S, so diluting the I-S NOE. To illustrate the influence of neighbouring spins, consider a hypothetical homonuclear three-spin system I, S and N (N, neighbour) in which relaxation arises solely from dipolar interactions ignoring, for convenience, all the other possible contributions to ρ_I^* described above (Fig. 8.11). Assuming, for simplicity, an NOE exists only between I and S, the steady-state NOE may be written:

$$\eta_I\{S\} = \eta_{\max} \left[\frac{r_{IS}^{-6}}{r_{IS}^{-6} + r_{IN}^{-6}} \right] \quad (8.10)$$

where $\eta_{\max}$ represents the maximum NOE possible in a homonuclear two-spin system, as previously. Notice that if spin N was not present, the NOE would simply be $\eta_{\max}$ and shows no distance dependence, exactly as predicted above for a two-spin system. The effect of introducing an additional spin is to reintroduce distance dependence, yet despite this, the magnitude of the steady-state NOE does not scale simply as r_{IS}^{-6} . In fact, it can be seen that the magnitude of the NOE will be dictated by a balance between the r_{IS} and r_{IN} distances. This is true for all steady-state NOE measurements; the result will always represent a balance between the I-S internuclear separation and all other I-N separations (and in a realistic chemical system, there may well be a large number of other neighbouring nuclei). To put it another way, the steady-state NOE arises from a competition between the I-S cross-relaxation and all other relaxation sources of spin I. Eq. (8.10) also shows that a reduction in the I-S internuclear distance now does indeed contribute to an increased NOE between I and S since the total contribution to the numerator will be relatively more than to the denominator. The arrival of a neighbouring spin has therefore reintroduced the idea that a smaller internuclear distance can be correlated to some degree with larger NOE enhancements.

This statement must still be treated with some caution, however, as illustrated in Fig. 8.12 in which $\eta_B\{A\}$ is considered for a three-spin system, A, B and C, where the B-C distance is varied (and ignoring any direct AC interaction). When C is distant from B it

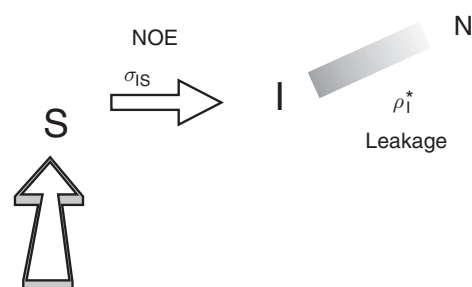


Figure 8.11. A three-spin system in which the I spin neighbour, N, acts as an external relaxation source for spin I through their mutual dipolar interaction.

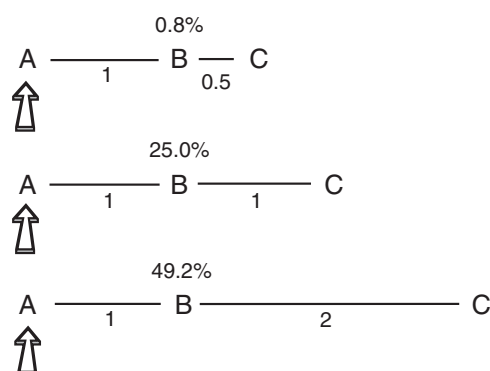


Figure 8.12. Calculated $A \Rightarrow B$ steady-state NOE enhancements for an isolated three spin system with the relative internuclear separations as shown. The arrow indicates the saturated spin. Any direct A-C interaction is ignored.

has little influence on its relaxation, allowing the AB cross-relaxation to dominate, producing close to the maximum NOE. When both A and C are equidistant from B, they play an equal role in relaxing B, and the NOE is thus half the maximum possible value. As C becomes very much closer to B than is A, it now dominates B spin relaxation, and A–B cross-relaxation loses out in the competition resulting in a small NOE. Thus, in general, despite two spins being ‘close’ to one another, in that they share a strong dipolar coupling, they still may not exhibit a large NOE if the enhanced spin has other near neighbours.

A further important feature emerges if we consider the results from saturating B and studying the effect on A, $\eta_A\{B\}$ (Fig. 8.13) and compare these with those of Fig. 8.12. With C distant from B, the relaxation of B is essentially completely dominated by A, so it experiences almost the maximum enhancement. As C approaches B, the enhancement on A is reduced only a little since its relaxation is still dominated by the much closer spin B. Thus, in general, steady-state NOEs between spins are not symmetrical, that is, $\eta_A\{B\} \neq \eta_B\{A\}$, because the neighbours surrounding A are unlikely to match those surrounding B in both number and proximity.

Indirect effects and spin diffusion

The examples discussed above have been restricted to discussing the direct NOE effects between A and B whilst, for convenience, ignoring effects that may be observed at C itself. If spin C is considered, it is apparent from Fig. 8.14 that it experiences a net decrease in signal intensity when A is saturated. This arises from a relay mechanism in which the population changes on B, brought about by the initial A–B NOE, subsequently alters the population of spin C when this also shares a dipolar coupling and hence also cross-relaxes with B. The *negative* NOE seen at C is a result of the *increase* in the B spin population differences generated by the A–B NOE. In the extreme narrowing limit, saturating a resonance (i.e. *decreasing* the population difference across the corresponding transition) causes a *positive* NOE, so by the same logic an *increase* in population differences for B (the A–B NOE) will in turn generate a *negative* NOE on its dipolar-coupled neighbours. This indirect effect, often referred to as the ‘three-spin effect’ should not be confused with direct negative NOEs observed for slowly tumbling molecules.

The magnitudes of negative three-spin enhancements are usually rather small since they rely on the build-up of a sizeable NOE on a neighbouring spin suitable for relaying. Similarly, they also tend to be slow to develop and show a characteristic lag period before appearing (Fig. 8.15), so tend to be observed only when longer presaturation periods are employed. The illustrations used above produce unrealistically high values for all enhancements since they assume pure dipolar relaxation throughout. Experimental NOEs are more often somewhat less than 20%, and three-spin effects are rarely more than a few percent at most. They appear most commonly when B and C are a diastereotopic geminal pair, since the B–C distance is then constrained to be rather short thus facilitating the relay. They are also favoured when the three spins have an approximately linear relationship, and their appearance can be diagnostically useful when observed. The reason for this geometry being particularly favourable arises from the balance between the negative indirect three-spin

Figure 8.13. Calculated $B \Rightarrow A$ steady-state NOE enhancements for an isolated three-spin system with the relative internuclear separations as shown. The arrow indicates the saturated spin. Any direct A–C interaction is ignored.

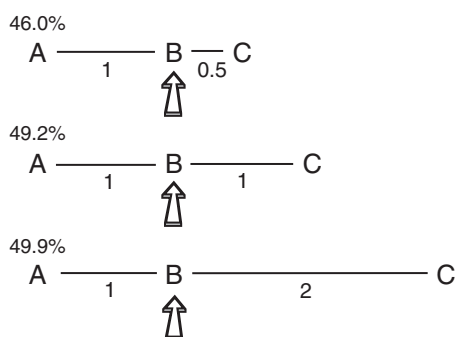
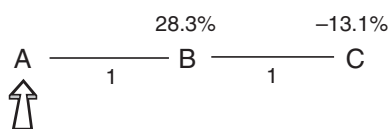


Figure 8.14. The three-spin steady-state effect. The negative enhancement at C arises from an indirect effect via spin B when spin A is saturated. The altered C spin populations also contribute to the enhanced NOE at B (cf Fig. 8.12).



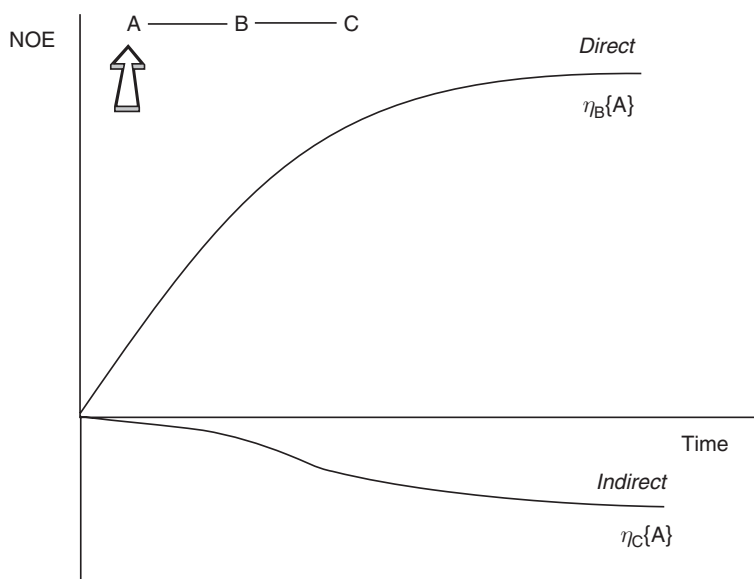


Figure 8.15. Schematic illustration of the build-up of direct and indirect NOEs in a rapidly tumbling three-spin system.

effect on C and the positive direct effect between A and C (Fig. 8.16). At small A–B–C angles the direct effect dominates that which is relayed via B, whilst when linear the opposite applies. In between these extremes, the two effects cancel so that even though A and C may be close in space, in that they share a strong dipolar coupling, an NOE between them may be rather small. This is another important point to be aware of (particularly when longer saturation times are employed) since in any realistic system there may well be a number of competing indirect pathways present. The curves of Fig. 8.16 demonstrate that the zero cross-over point varies with internuclear separations and that the slopes of the curves are large at this point, so although the NOE is unlikely to cancel to precisely zero, it may well be less than expected from simple geometrical considerations. Although in principle, four-spin relay effects can be predicted to give rise to positive NOEs, they are very rarely observed in practice simply because they are so very weak.

The influences of the various factors described above are further illustrated by reference to the four-spin system of Fig. 8.17, which lays testament to the need to consider all neighbouring spin interactions to correctly interpret steady-state NOE data. Clearly, the NOE enhancements between B and C differ dramatically despite these effects arising over

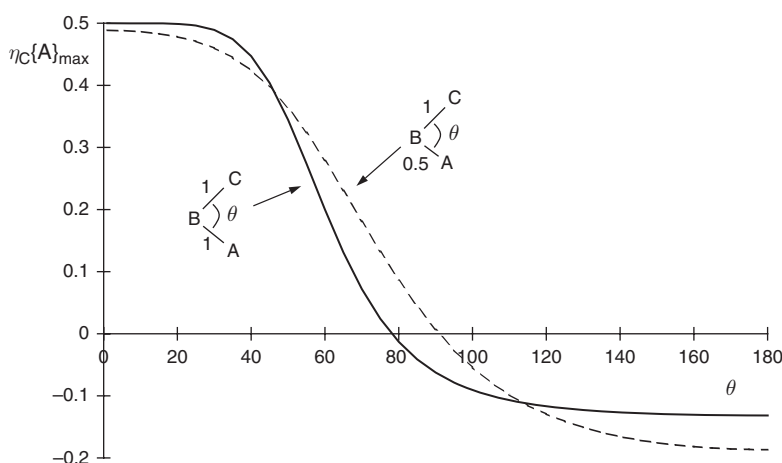


Figure 8.16. Calculated steady-state NOEs for spin C on saturation of spin A, as a function of the A–B–C angle in a rapidly tumbling isolated three-spin system. The resulting NOE is a balance between direct and indirect A–C effects and the two curves illustrate the dependence on relative internuclear separations.

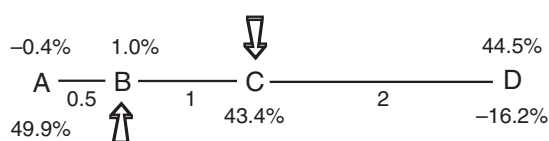
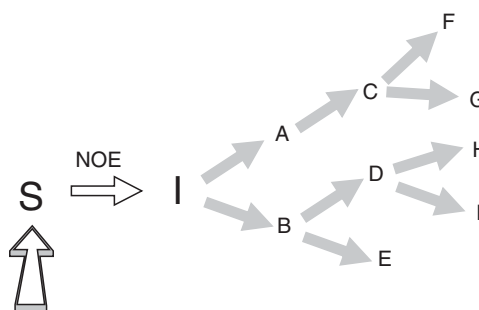


Figure 8.17. Calculated steady-state NOE enhancements in an isolated four-spin system with the relative internuclear separations as shown. These enhancements were derived from the equations presented in Table 3.1 of [1].

Figure 8.18. Schematic illustration of the spin-diffusion process in which the original S-I NOE is efficiently relayed onto neighbouring nuclei and propagated throughout the molecule.



identical internuclear separations. Furthermore, the enhancements $B \Rightarrow A$, $B \Rightarrow C$ and $C \Rightarrow D$ are all rather similar despite there being a factor of 4 difference in distance between the largest and smallest separations. The essence of correctly applying steady-state NOE data is to collect a number of measurements and check for self-consistency within the proposed geometrical arrangement, rather than relying on a single NOE enhancement to provide an answer. If only a single irradiation was performed from C in Fig. 8.17, one might be forced into the erroneous conclusion that D was its closest partner rather than B. A more cautious approach, also measuring the NOEs from both B and D, should lead one to question this conclusion. Again, such spectacular differences are unlikely to occur in reality largely because of the effects of the other numerous ρ_1^* relaxation sources we have chosen to ignore, but it should be clear that careful consideration is essential. Generally speaking, only in rather specific cases will a single NOE measurement lead to a definitive structural answer whereas a comparative study of many NOEs within the molecule is more likely to provide a conclusive and correct result.

When a molecule tumbles in solution so slowly that it exhibits negative NOEs, the consequences of indirect effects are more dramatic and far more problematic, so much that steady-state NOEs become largely useless. The problem is essentially two-fold; firstly these have the same sign as direct effects, so cannot be readily distinguished and secondly they grow rapidly and may attain very high intensities. The first of these is a consequence of the fact that a negative NOE (a population decrease) arises from saturation (a forced population decrease), so likewise the indirect effect on the third spin will also be negative, and so on. In the extreme case of extended saturation times, the NOE initially generated between two spins can spread throughout the whole molecule until all nuclei experience the same NOE enhancement (Fig. 8.18). This spreading of information throughout the molecule is often referred to as *spin diffusion* for fairly obvious reasons and may be likened to heat diffusing through a conductive solid. The limit of slow tumbling is also referred to as the *spin-diffusion limit*. Because of this, steady-state NOEs in the negative NOE regime fail to provide reliable distance or proximity information. Instead, it becomes necessary to consider the *rate* at which NOEs grow between spins to glean distance information, dictating the use of kinetic measurements in the form of transient NOE experiments (Section 8.4).

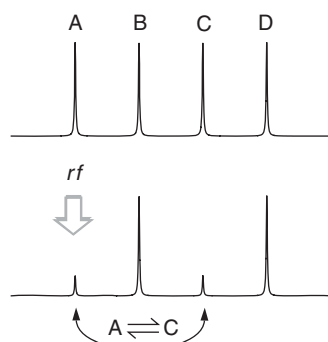


Figure 8.19. Schematic illustration of the saturation transfer process. Saturation of resonance A will lead to the simultaneous saturation of resonance C if nuclei A and C experience mutual chemical exchange during the saturation period.

Saturation transfer

Complications arising from the transfer of saturation from one resonance to another by means of chemical exchange (Fig. 8.19) also differ in the two extreme motional regimes. Exchange peaks always display the same sign behaviour as the originally saturated resonance, so have opposite sign to positive NOEs but the same as negative NOEs. The identification of responses arising from chemical exchange rather than the NOE is a further problem associated with the negative NOE regime; saturation transfer is considered further in the practical sections that follow.

8.3.3. Summary

The previous sections have covered the most important principles at the heart of the NOE. Despite having avoided much of the underlying mathematics, instead relying on pictorial models where possible, these sections are, necessarily, a little more technical than the other chapters in the book. The goal has been to present a reasonably thorough introduction to the NOE that is still compatible with the level of this text, rather than simply presenting the reader with statements of fact with little justification or support. This section reviews the key points that have been presented above, allowing the reader a more immediate reminder as to the most significant features of the NOE.

- The NOE is the change in intensity of the resonance of a nuclear spin, I ('interesting'), when the population differences across the transitions of a near neighbour, S ('source'), are perturbed from their equilibrium values, usually by *saturation* or by *population inversion*. It arises as the perturbed spin system alters its spin populations in an attempt to regain the equilibrium condition.
- *Steady-state* NOEs are those measured after a period of continuous S spin saturation during which a new 'steady-state' equilibrium condition has developed for the I spin populations. The NOE enhancement is usually denoted $\eta_{\text{I}\{\text{S}\}}$ and quoted as a percentage.
- Steady-state NOEs can provide information on relative internuclear distances only, not on absolute measurements of internuclear separation.
- The NOE only arises between nuclei that share a mutual *dipolar coupling* (a direct, magnetic through-space interaction) and thus relax each other via the dipole–dipole relaxation mechanism. Only this mechanism contributes to the nuclear spin population changes that produce the NOE, which is intimately related to longitudinal spin relaxation. The dependence on internuclear separation that makes the NOE so useful has its origins in the strength of dipolar coupling between two spins, this being inversely proportional to their separation, r_{IS} , (as r_{IS}^{-3}).
- Longitudinal spin relaxation requires a stimulus in the form of a magnetic field fluctuating at a frequency equal to the frequency (energy) of the transition. In the case of dipolar relaxation, this arises from the time-dependent field a spin experiences from its dipolar-coupled neighbour as the molecule rotates or tumbles in solution. The NOE in turn is dependent on the rates of molecular tumbling.
- The magnitude and sign of the NOE results from competition between various relaxation pathways. If the W_2 ($\alpha\alpha \leftrightarrow \beta\beta$) pathway dominates, positive NOEs are observed whereas a dominant W_0 ($\alpha\beta \leftrightarrow \beta\alpha$) pathway leads to the observation of negative NOEs. The W_2 and W_0 relaxation pathways (which involve the mutual flipping of the two spins) are collectively referred to as *cross-relaxation pathways*, and it is only these that contribute to the generation of the NOE. All other contributions to relaxation, in the form of W_1 processes ($\alpha\alpha \leftrightarrow \beta\alpha$ and $\alpha\beta \leftrightarrow \beta\beta$, involving the flip of only a single spin), serve to reduce the overall magnitude of the effect, and are thus referred to as *leakage* contributions.
- Rapid molecular tumbling, corresponding to a short correlation time, τ_c , favours the higher energy W_2 process, so small molecules in low viscosity solvents display positive homonuclear NOEs (the *extreme narrowing limit*). In contrast, slow molecular tumbling (long correlation times) favours the lower energy W_0 process meaning large molecules or smaller molecules in high viscosity solvents display negative homonuclear NOEs (the *spin-diffusion limit*).
- The maximum possible positive NOE is $\gamma_{\text{S}}/2\gamma_{\text{I}}$, in other words 50% in a *homonuclear* system. Enhancements in *heteronuclear* systems can be far larger, for example, 199% from ^1H to ^{13}C , and can serve as a useful source of sensitivity enhancement in the observation of the low- γ I spin. They also become negative if one of the γ s is negative, for example, -494% from ^1H to ^{15}N , and may in some cases lead to severe signal reduction or even disappearance.
- The maximum possible negative NOE in a homonuclear system is -100%.
- Between the extreme narrowing and spin-diffusion limits lies the difficult region in which NOEs can become zero (when $\omega_0\tau_c \approx 1$) or at least rather weak, often demanding a change in experimental conditions or the use of ROE measurements.
- In an isolated homonuclear two-spin system in the extreme narrowing limit relaxing exclusively via the dipole–dipole mechanism, the steady-state NOE is predicted to be +50% and *independent of internuclear separation*, r_{IS} . However, the initial *rate* at which the NOE grows is proportional to r_{IS}^{-6} .
- In a more realistic multispin system, neighbouring nuclei, N, that are close to I can also contribute to its W_1 relaxation pathway. The magnitude of the NOE then becomes dependent on the I–S internuclear separation (inversely as r_{IS}^{-6}), but also has a dependence on the distance(s) between I and its near neighbour(s) (inversely as r_{IN}^{-6}), amongst other factors.
- The direct consequence of this is that to correctly interpret steady-state NOE data, it becomes essential to consider not only the I–S internuclear separation, but also the proximity of all other nuclei (relaxation sources) surrounding I.
- This also means that steady-state NOEs are rarely symmetrical. That is, the NOE observed at spin A on saturating spin B, $\eta_{\text{A}\{\text{B}\}}$, is unlikely to equal the reverse measurement from saturating A and observing B, $\eta_{\text{B}\{\text{A}\}}$. This is a consequence of the fact that the neighbours surrounding A are unlikely to match those surrounding B in number and in distance.

- The relaxation of a spin with a very near neighbour will be dominated by this neighbour, and as a consequence, NOEs onto this spin from a more distant source spin will tend to be small. Conversely, nuclei that have no nearby neighbours will experience relaxation only from distant neighbours, and as a result, NOEs from these neighbours will be large despite the relatively large internuclear distance involved. Thus, it is to be expected that NOEs over similar distances onto a methylene and onto a methine proton will generally be somewhat smaller for the methylene proton since this will always have at least one near neighbour, its geminal partner.
- Longer range NOEs build up only slowly, due to the r^{-6} rate dependence, so require long saturation periods before becoming appreciable.
- NOE enhancements may also be *relayed* onto neighbouring spins. For example, an NOE from A to B may be further passed onto a spin C that also cross-relaxes with B, such events being referred to as *indirect effects*. The properties of indirect enhancements differ markedly in the positive and negative NOE regimes.
- When direct (A–B) NOEs are positive, indirect effects at C are weak and negative, are favoured when the three spins have an approximately linear relationship (so may provide useful geometrical information), develop only slowly with a characteristic lag time and are thus also favoured by long presaturation periods. These are referred to as *three-spin effects*. Although further relays are theoretically possible, they are generally too weak to be observed.
- It is possible that positive direct effects and negative indirect effects can cancel or act to reduce the magnitude of the NOE. Thus, despite two spins being close, NOEs between them may be rather small or even negligible (particularly when longer saturation periods are employed).
- When direct (A–B) NOEs are negative, indirect effects are also negative and so cannot be distinguished; they spread rapidly and have high intensities. This process is referred to as *spin diffusion* and is fatal for the steady-state NOE since it causes a loss of specificity and hence provides no information on molecular geometry. In this regime, it usually becomes necessary to use kinetic (transient) methods based on the measurement of NOE growth rates.
- Taking into account all the subtleties associated with the steady-state NOE presented above, it should be clear that it is unwise to place too much significance on the absolute magnitudes of steady-state NOE enhancements. In reality, differences of a few percent mean little when taken on their own, and it is generally necessary to consider a collection of enhancements when undertaking structural or conformational analysis to be certain of an unambiguous conclusion. A qualitative interpretation of many measurements is the most appropriate approach to interpreting steady-state NOE data.

8.3.4. Applications

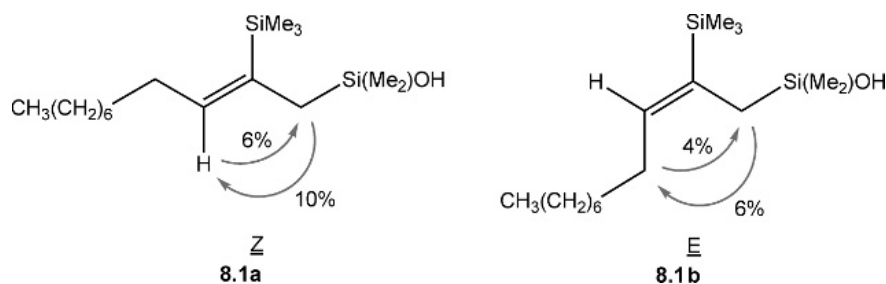
To illustrate the issues described above and how NOE measurements can be used to provide unique data in structural analysis, some specific examples are now presented. As stated in the introduction, the NOE is most often employed during the later stages of structural investigations when the gross structure of the molecule has, at least to a large extent, been defined. For rather small molecules, this may be possible from knowledge of the chemistry used and from 1D spectra, whilst larger or structurally complex molecules may demand the application of various correlation techniques before NOE experiments are considered. In either case, confidence in the accuracy of one's proton assignments is of paramount importance since errors in these are as likely (if not more likely) to lead to erroneous stereochemical conclusions being drawn than they are to being unmasked by the NOE studies themselves.

The examples presented here have all made use of the steady-state NOE difference method and are therefore restricted to 'small' molecules in which key resonances are sufficiently resolved for selective irradiation. This is often the case for many of the molecules routinely handled in the research laboratory when a judicious choice of solvent for optimum signal dispersion has been made. These also provide some indication of the magnitudes of NOE enhancements typically encountered in routine laboratory studies, which often fall short of the theoretical numbers discussed in the previous sections. Additional examples making use of other NOE techniques are presented in later sections.

E vs Z geometry

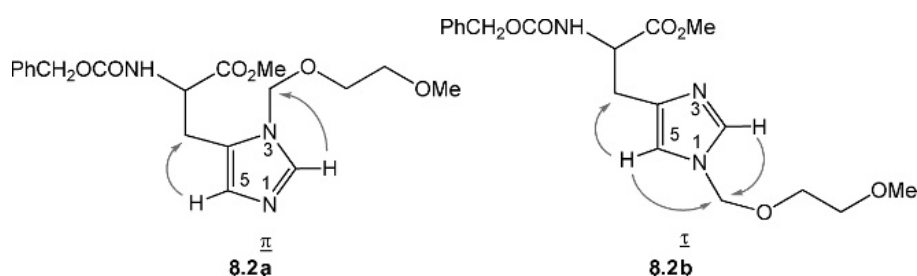
The differentiation of E and Z alkene isomers is often possible by direct measurement of vicinal proton–proton couplings across the unsaturation, whereby *cis* and *trans* couplings

are usually sufficiently different to allow a distinction to be made (typically, J_{cis} 7–11 Hz, J_{trans} 12–18 Hz). When only a single alkene proton exists, this method can no longer be used, and the NOE then offers an alternative approach provided a protonated group exists across the double bond. One such example is in the differentiation of the *E* and *Z* silanols **8.1a** and **8.1b** [3]. The *Z*-isomer was readily identified from the NOEs between the alkene proton and the CH_2Si group, and further confirmation was provided by the observation of contrasting NOEs for the other *E*-isomer. Asymmetry in the magnitudes of NOE enhancements is also clearly apparent in this example, and should come as no surprise following the preceding discussions.



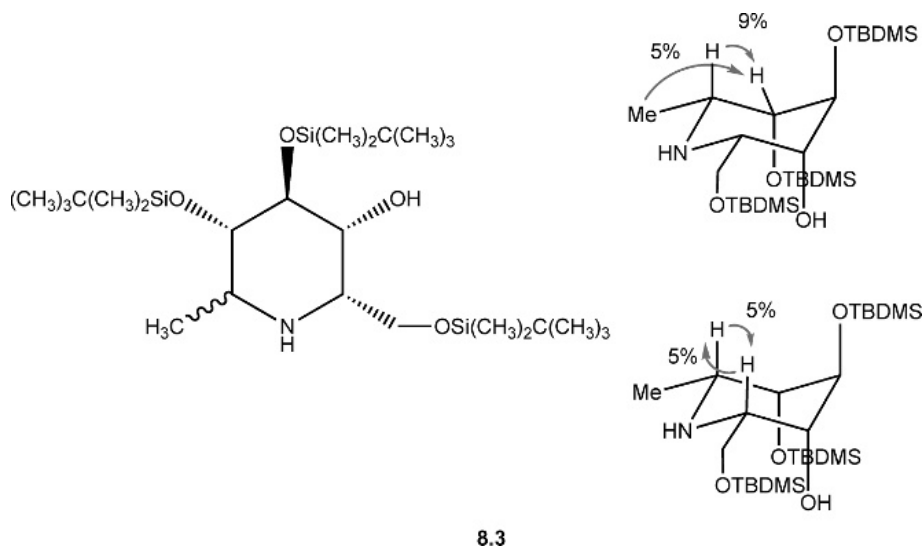
Aromatic substitution position

Determining the position of substitution within a molecule can also be problematic when no direct proton–proton couplings exist to link the new moiety, which is often the case when the substitution is made on a heteroatom. Particularly when dealing with aromatic systems, the NOE can often provide unambiguous solutions. These systems are particularly favourable because they are restricted to being planar and hence it is usually safe to assume that nearest neighbours will be those on adjacent positions in the ring. The differentiation of π - and τ -substituted histidines [4] **8.2a** and **8.2b** provides a simple illustration of this. In the π -substituted systems, irradiation of the H5 proton enhances only one of the CH_2 groups bound to the imidazole ring whereas in the τ -substituted isomers both were enhanced. Prior to the use of the NOE, differentiation was possible only through chemical degradation or through empirical rules based on differences in the small (<1.5 Hz) H2–H5 coupling constant, which could not always be resolved. An alternative approach to consider nowadays in such cases would be to establish connectivity by identifying long-range proton–carbon correlations across the heteroatom via the HMBC experiment (Section 6.4).

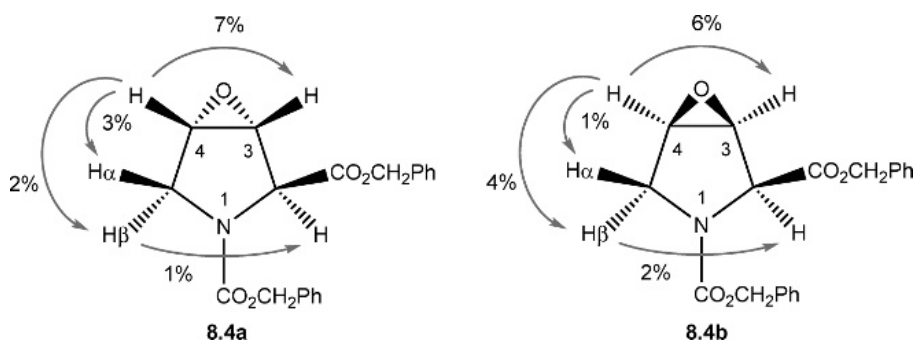


Substituent configuration

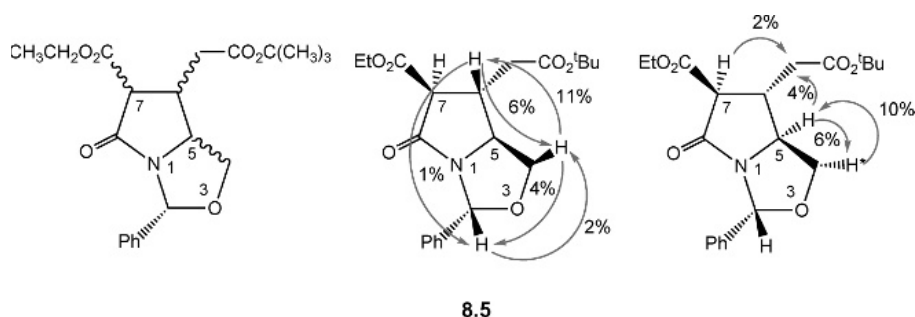
Another widely encountered question, and arguably the most common problem the NOE is used to address in synthetic chemistry, is the relative configuration of substituents on ring systems. This is most often applied to 5- and 6-membered rings, not only because of their ubiquity but also because larger rings tend to have far greater flexibility, making it more difficult to draw unambiguous conclusions. For six-membered rings in particular, direct analysis of proton coupling constants within the ring can in itself be informative because of the generally distinct differences in *axial–axial* versus *axial–equatorial*/*equatorial–equatorial*-coupling constants in chair conformations ($ax\text{--}ax \approx 10\text{--}12$ Hz, $ax\text{--}eq/eq\text{--}eq \approx 2\text{--}5$ Hz; see Section 5.6.2 and Fig. 5.51 for example). However, in the determination of the orientation of the methyl group in **8.3**, these would not have been informative, because of the similarity between $ax\text{--}eq$ and $eq\text{--}eq$ -coupling constants. A collection of NOE enhancements was able to identify the methyl as occupying the equatorial position, in particular the 1,3-diaxial methine proton NOEs.



Vicinal couplings in five-membered rings generally offer greater ambiguity in defining relative configurations and here careful NOE measurements are also required since *cis* and *trans* NOEs between adjacent protons are often of similar magnitude. In these cases, it is wise to collect as many NOE enhancements as is possible for the sample and to ensure self-consistency over all of these within the proposed stereochemistry. These points are exemplified by the configurational assignment of the synthesised epoxyprolines [5] **8.4a** and **8.4b**. The negligible *J*-coupling between H2 and H3 in **8.4a** tentatively suggested these protons to be *trans*, and the slightly greater 2.5 Hz coupling in **8.4b** suggested these share a *cis* relationship. Conclusions drawn from interpretation of the NOE data were consistent with this proposal, but were founded on the comparison of NOEs of rather similar magnitude between the H5 and H4 protons in each. The availability of both isomers allowed a comparison of their H4–H5 NOE patterns, based on identification of H5 β from its NOE with H2, and the differences observed between these provided further support for the assignments. Confirmation of these assignments was provided through additional synthetic structural correlations.

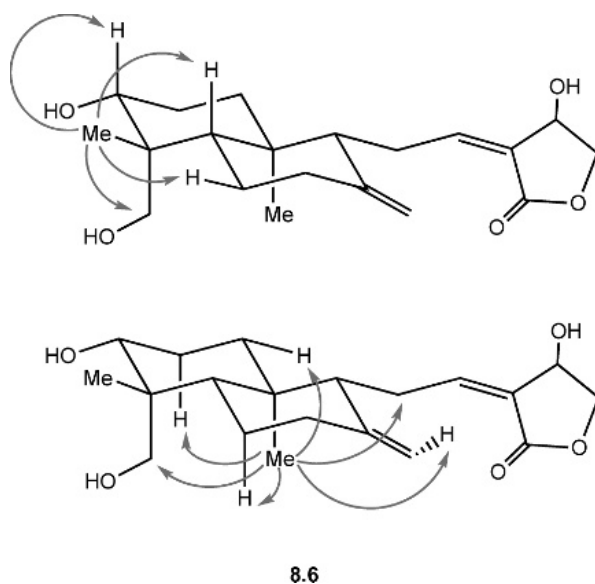


In the bicyclic lactam **8.5**, it was possible to determine the relative configuration at three stereo-centres based on the known stereochemistry at only one (C₂) through the sequential interpretation of the NOE data. The lack of NOEs between protons on adjacent carbons C5, C6 and C7 suggests that the neighbouring protons share *trans* relationships, although the absence of an NOE alone cannot always be considered definitive evidence, as has been stressed in previous discussions. More importantly, this stereochemistry is confirmed by the observation of the additional H6–H4, H6–H2 and H7–H5 NOEs. Again notice the asymmetry in the NOEs between H2–H4, H4–H6 and H4^{*}–H5, with the NOE onto the methylene proton being always less than the NOE from this onto the methine proton, owing to the close proximity of the geminal neighbours.



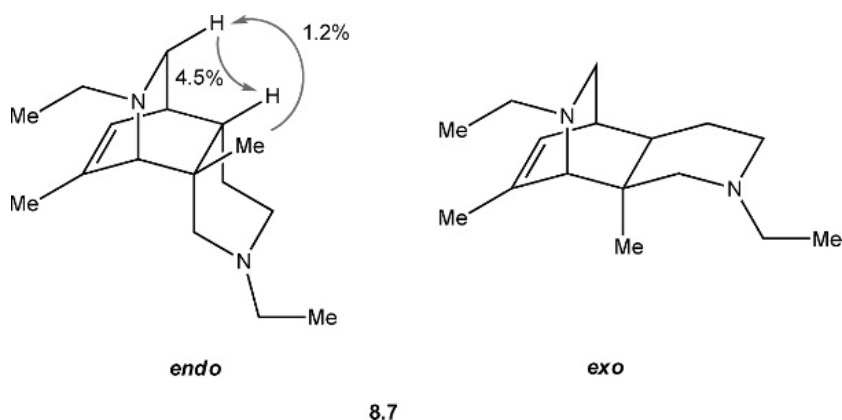
Resonance assignment

The prerequisite for most NOE studies of configuration or conformation is the assignment of the proton spectrum of the molecule, meaning each resonance can be associated with a unique proton within the gross structure. Such assignments are typically derived from 1D spectra and the various correlation methods described in previous chapters, although in some cases the NOE can itself be used for resonance assignment. It is most likely to be of use for assigning resonances of isolated groups that share no proton scalar couplings, although is by no means limited to this. An example is the assignment of the two methyl resonances in the hepato-protective agent andrographolide **8.6**, which are distant from one another in the molecule and show quite characteristic NOE patterns. The use of long-range heteronuclear correlation experiments should also be considered when addressing such problems, and indeed may be needed in the determination of the gross structure in the first place.



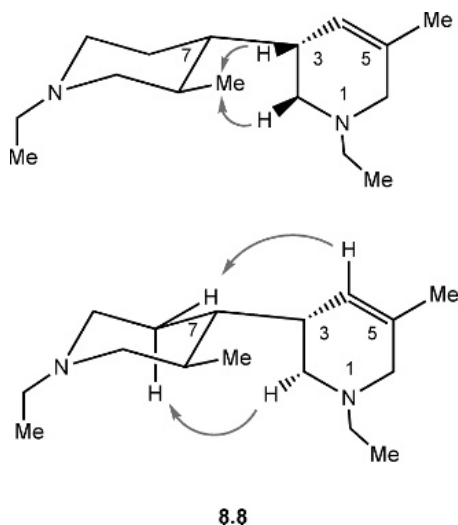
Endo vs exo adducts

The need to distinguish between *exo* and *endo* adducts in fused ring systems is another commonly encountered challenge that can often be addressed by the NOE, and the determination of the stereochemistry at C5 of the lactam **8.5** above can be viewed as one such example. In such cases, the NOE patterns observed between the proton(s) at the junction and those on the adjacent rings can often provide an unambiguous stereochemical assignment. This was the case for the identification of **8.7**, as the *endo* cycloadduct [6]. The material was synthesised as part of a model study towards the biomimetic synthesis of the manzamine alkaloids, a family of β -carboline alkaloids derived from marine sponges, which possess potent antileukaemic and cytotoxic properties. In this, it was important to confirm whether the product stereochemistry was consistent with the proposed biosynthetic hypothesis being investigated, as was shown to be the case. Here the bridgehead proton was in fact too heavily overlapped with an adjacent resonance to be selectively saturated, although NOEs onto this were quite distinct. When bridgehead protons cannot be used at all, or if non-existent, the assignment must then rely on the observation of NOEs between ring protons on either side of the junction.



Conformational preference

The definition of a favoured conformation in small, flexible molecules by use of the NOE represents a far greater challenge, notably because of the rapid interchange between many possible conformations and because the NOE itself will represent only a weighted average of internuclear separations present within the conformers. The detailed investigations required to extract meaningful data in these cases are therefore rarely undertaken. However, when restricted conformational processes lead to one conformation being strongly favoured, sufficient NOE data may be available, which allow this to be defined. Obvious examples might be the differentiation of chair and boat conformers of cyclohexanes or of slowly interconverting rotamers. Specific conformations may also be favoured in the presence of steric hindrances or strong hydrogen-bonding interactions, for example. An example of a favoured conformation defined by NOE measurements is structure **8.8** (also produced as part of the biomimetic synthesis of the manzamine alkaloids mentioned above [7]) in which the two heterocyclic rings are approximately orthogonal to one another. The limited and specific NOEs observed at the interface of the two rings are not consistent with free rotation about the single bond linking the rings, and the structure appears essentially locked, presumably by the presence of the C8 methyl group. This orthogonal relationship is further supported by the almost negligible coupling between the H7 and H3 protons, indicating that the dihedral angle between them is close to 90°. The combined use of NOEs and coupling constants often represents the optimum approach to questions of conformation.



8.4. TRANSIENT NOEs

It has been repeatedly stressed in the preceding sections that the steady-state NOEs measured between two nuclei cannot readily be translated into internuclear separations because they result from a balance between the influences of all neighbouring spins. At best, they provide information on *relative* internuclear distances only. It has also been

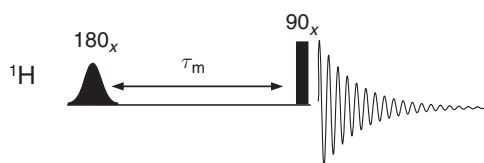


Figure 8.20. A general scheme for observing transient NOEs. Following inversion of a target (source) resonance, the NOE develops during the mixing time, τ_m , after which the system is sampled.

stressed, however, that the *rate* at which the NOE grows towards this steady-state can be directly related to these distances under appropriate conditions. It has also been shown that for molecules that exhibit negative enhancements, steady-state measurements may fail to provide any reliable information of spatial proximity, and here one is forced to consider the kinetics of the NOE. A logical approach to such measurements would be to follow that taken for the measurement of steady-state effects. Saturation of the target resonance for periods that are far less than those needed to reach the steady-state would allow some NOE to appear, which is then sampled. Repeating the experiment with progressively incremented saturation periods allows the build-up to be mapped. Owing to the use of shortened saturation periods, the enhancements observed with this method are termed *truncated driven NOEs* or *TOEs*.

Although once popular, this experimental approach is rather less used nowadays and as such shall be considered no further. The more common approach to obtaining kinetic data is to instantaneously perturb a spin system not by saturation but by inverting the target resonance(s) (i.e. inverting the population differences across the corresponding transitions) and then allowing the NOE to develop in the absence of further external interference. The new populations are then sampled with a 90° pulse as usual (Fig. 8.20). In this case, the NOE is seen initially to build for some time but ultimately fades away as spin relaxation restores the equilibrium condition; these enhancements are thus termed *transient NOEs*.

The measurement of transient NOEs gained widespread popularity, initially in the biochemical community, in the form of the 2D NOESY experiment, which remains an extremely important structural tool in this area and increasingly in the analysis of smaller molecules. The 1D transient NOE experiment, also referred to as 1D NOESY, is also used widely within the chemical community as a gradient-selected sequence capable of providing quite spectacular, high-quality NOE spectra. Transient experiments, whether 1D or 2D, are more commonly used qualitatively as ‘single-shot’ techniques, providing an overview of enhancements within a molecule rather than being employed to map the growth of the NOE, yet it is necessary to understand something of the kinetics of the NOE to correctly execute and interpret these methods.

8.4.1. NOE kinetics

Following the (assumed) instantaneous inversion of the S spin resonance, the *initial* growth rate of the NOE at I depends linearly on the cross-relaxation rate between these two spins *even in multispin systems*, such that:

$$\frac{dI_z}{dt} = 2 \sigma_{IS} S_z^0 \quad (8.11)$$

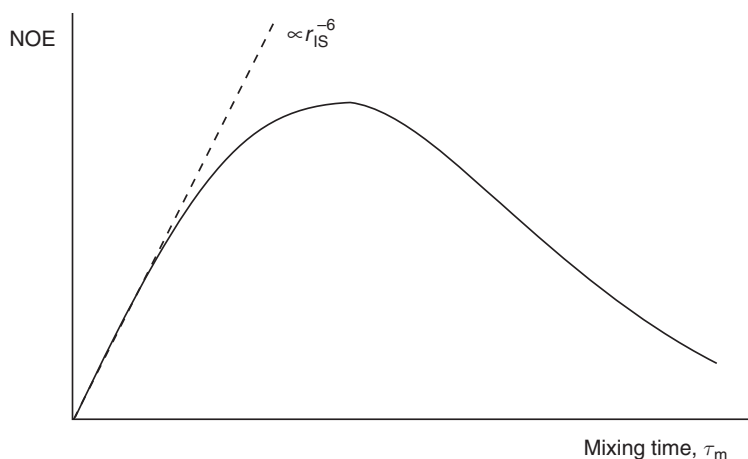
(here the factor of 2 arises simply from the use of inversion of populations in this case rather than saturation, as considered previously). Using Eq. (8.4) and the approximation $\omega_I = \omega_S$ for a homonuclear system, the cross-relaxation rate ($W_2 - W_0$) is given by:

$$\sigma_{IS} \propto \gamma^4 \left\{ \frac{6}{1 + 4 \omega_0^2 \tau_c^2} - 1 \right\} \frac{\tau_c}{r_{IS}^6} \quad (8.12)$$

Unlike the steady-state enhancements, the transient enhancements are influenced by only a single internuclear separation as r_{IS}^{-6} , whilst the so-called *initial rate approximation* is valid. In this, the two cross-relaxing spins initially behave as if they were an isolated spin pair and the growth of the NOE has a linear dependence on mixing time. As longer mixing periods are used, the relaxation of spin I begins to compete with cross-relaxation between I and S, so the build-up curve deviates from linearity and the NOE eventually decays to zero (Fig. 8.21). Thus, for the initial rate approximation to be valid, mixing times significantly shorter than the T_1 relaxation time of spin I must be used. Only under these conditions is meaningful distance measurement possible.

If, on the other hand, the goal is to qualitatively identify through-space correlations, as is more often the case in routine work, mixing periods comparable to T_1 provide maximum enhancements. Since transient NOEs develop in the absence of an external rf field, they tend

Figure 8.21. Schematic illustration of the development of the NOE between spins I and S as a function of mixing time.



to be weaker than steady-state effects. The maximum theoretical homonuclear enhancement is also reduced to 38% (from 50%) for positive NOEs, so careful choice of timing is crucial to the success of transient experiments. In all other respects, the dependence of transient NOEs on correlation times matches that of steady-state effects, with the maximum homonuclear enhancements for the negative NOE regime again being -100% owing to the domination of efficient cross-relaxation. When dealing with molecules that exist within this regime, it is also necessary to be aware of and if possible avoid potential complications arising from the dreaded spin diffusion (indeed, this is the primary reason why transient methods are used), which again demands the use of conservatively short mixing periods.

8.4.2. Measuring internuclear separations

Assuming the initial rate approximation to be valid (the NOE growth linear), the magnitude of an enhancement between two spins, A and B, after a period τ will be proportional to the cross-relaxation rate, which in turn depends on r_{AB}^{-6} :

$$\eta_A\{B\} = k \sigma_{AB} \tau = k' r_{AB}^{-6} \tau \quad (8.13)$$

The constants of proportionality here contain the molecular correlation time, τ_c , in addition to a number of known physical constants, and $\{B\}$ is now taken to signify inversion rather than saturation of B. In principle, if τ_c were known, this would directly provide a measure of r_{AB} . Whilst it is possible to determine this (such as from relaxation time measurements), this is rarely done in practice, and it is more common to use a known internal distance as a reference and avoid the need for such laborious measurements. If the NOE between reference nuclei X and Y of internuclear separation r_{XY} is also measured then:

$$\frac{\eta_A\{B\}}{\eta_X\{Y\}} = \frac{r_{AB}^{-6}}{r_{XY}^{-6}} \quad (8.14)$$

A direct comparison of the two NOE intensities thus provides the unknown internuclear distance. This simple relationship has been extensively used to provide measurements of internuclear separations, particularly in biological macromolecules. From a single experiment, distances can be estimated assuming the initial rate approximation is valid for all interactions. This relies on all internuclear vectors in question possessing the same correlation time, which may not be the case where internal motion is present. A consideration of these matter lies beyond the scope of this work; further details may be found in reference [1].

The significance of a single 'internuclear distance' must also be considered carefully. In reality, internuclear separations vary over time with conformational averaging, so the concept of a single distance is simply a convenient model of events. Furthermore, in cases of conformational exchange, the calculated distance tends to be heavily weighted towards shorter separations since the NOE is very much more intense for these because of the r^{-6} factor. Consider the case of rapid averaging on the NMR timescale between two equally populated conformers such that only a single resonance is observed for each chemically distinct site. In one conformer the separation between two spins is 0.25 nm whilst in the other it is 0.60 nm (Fig. 8.22). The NOE under conditions of fast conformational exchange averages as $\langle r^{-6} \rangle^{-1/6}$ (where $\langle \dots \rangle$ indicates the mean value) so that the *apparent* separation as would be calculated from observed NOE intensities is 0.28 nm rather than the mean 0.42 nm one may anticipate.

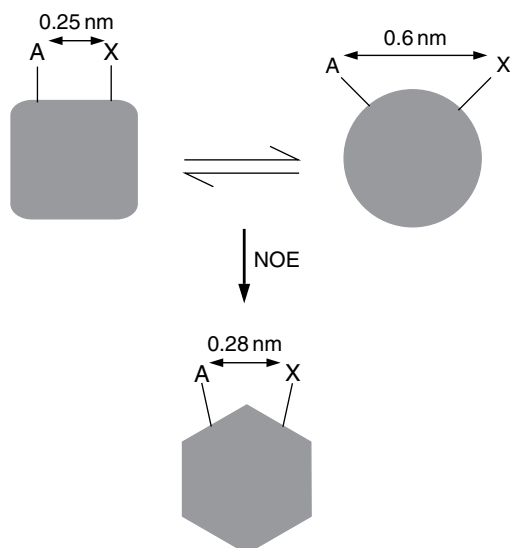


Figure 8.22. The influence of rapid conformational exchange on estimated internuclear separations based on NOE measurements.

In general then, NOE measurements will tend to underestimate rather than overestimate distances in such cases, which can be problematic for structure calculations. These problems are most severe in the case of small molecules, where extensive conformational averaging is to be expected, and detailed structure calculations based on *quantitative* distance measurements for flexible small molecules are rather rare. However, this approach has proved enormously successful in the structure calculations of biological macromolecules where, to avoid problems of averaging and errors in intensity measures, semi-quantitative measurements are usually employed (Section 8.7).

8.5. ROTATING-FRAME NOEs

The greatest problem associated with the methods described so far is clearly the ‘zero-crossing’ region around $\omega_0\tau_c \approx 1$ where the conventional (laboratory-frame) NOE observed via steady-state or transient techniques becomes vanishingly small. This typically occurs for mid-sized molecules with masses of around 1000–2000 daltons, depending on solution conditions and spectrometer frequency. With the increasing interest in larger molecules in many areas of organic chemistry research coupled with the wider availability of higher field instruments, this is likely to be a region visited ever more frequently by the research chemists’ molecules. Other than altering solution conditions in an attempt to escape from this, the measurement of NOEs in the *rotating-frame* provides an alternative solution, albeit an experimentally challenging one. These effects are the rotating-frame analogues of the transient NOEs described above, and many of the discussions in Section 8.4 relating to their application are relevant here also. In this case, however, the cross-relaxation rate between homonuclear spins is given by:

$$\sigma_{\text{IS}} \propto \gamma^4 \left\{ \frac{3}{1 + \omega_0^2 \tau_c^2} + 2 \right\} \frac{\tau_c}{r_{\text{IS}}^6} \quad (8.15)$$

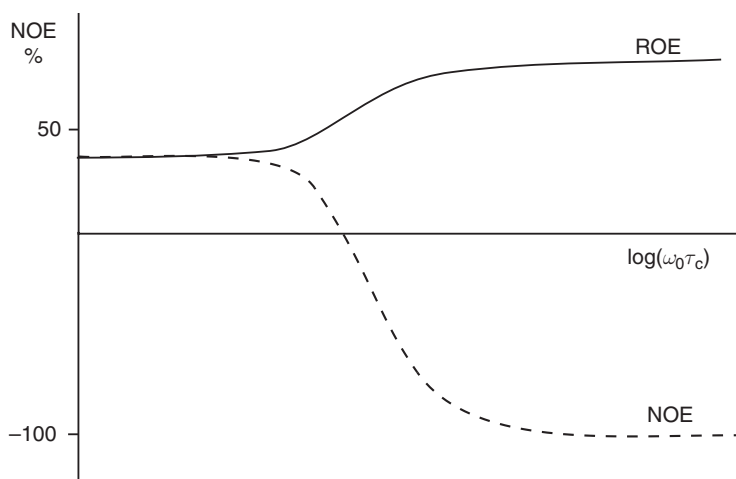
Unlike the corresponding equation for transient NOEs (Eq. (8.12)), this expression remains positive for all values of τ_c , and the undeniable benefit of ROEs is, quite simply, that they remain positive for all realistic molecular tumbling rates. For small molecules, the magnitude of the ROE matches that of the transient NOE, whilst for larger molecules it reaches a maximum for homonuclear spins of 68%, but under no circumstances does it become zero (Fig. 8.23). Similarly, the NOE and ROE growth rates are identical for small molecules but differ for very large ones. For a small molecule that has $\omega_0\tau_c \ll 1$, both Eqs 8.12 and 8.15 simplify to:

$$\sigma_{\text{IS}} \propto \frac{5\gamma^4 \tau_c}{r_{\text{IS}}^6} \quad (8.16)$$

In contrast, for very large molecules which have $\omega_0\tau_c \gg 1$, the left hand expression within the brackets of both Eqs 8.12 and 8.15 becomes negligible, giving:

$$\sigma_{\text{IS}}^{\text{NOE}} \propto \frac{-\gamma^4 \tau_c}{r_{\text{IS}}^6} \quad \text{and} \quad \sigma_{\text{IS}}^{\text{ROE}} \propto \frac{2\gamma^4 \tau_c}{r_{\text{IS}}^6} \quad (8.17)$$

Figure 8.23. Schematic illustration of the dependence of the ROE and transient NOE for an isolated homonuclear two-spin system as a function of molecular tumbling rates.



and hence:

$$\sigma_{\text{IS}}^{\text{ROE}} = -2\sigma_{\text{IS}}^{\text{NOE}} \quad (8.18)$$

For very large molecules, the ROE therefore grows twice as fast as the NOE, and has opposite sign [8].

The measurement of ROEs requires a somewhat different experimental approach (Section 8.8). In essence, ROEs develop whilst magnetisation is held static in the *transverse* plane, rather than along the *longitudinal* axis (hence they are sometimes also referred to as transverse NOEs). To generate the required population disturbance of the source spins, the target resonance is subjected to a *selective* 180° pulse prior to the non-selective 90° pulse, such that it experiences a net 270° flip and is thus inverted relative to all others. Transverse magnetisation is then ‘frozen’ in the rotating frame by the application of a continuous, low-power *spin-lock* pulse. This is analogous to the spin-lock described in Section 5.7 for the TOCSY experiment and serves the same purpose, that is, to prevent evolution (in the rotating frame) of chemical shifts. The simplest scheme for a 1D sequence is therefore that of Fig. 8.24. The experiment is more frequently performed as the 2D experiment where it is usually termed ROESY (rotating-frame NOE spectroscopy).

The situation during the spin-lock may be viewed as the transverse equivalent of events during the transient NOE mixing time (Fig. 8.25). The action of the spin-lock is to maintain the opposing disposition of magnetisation vectors, which would otherwise be lost through differential chemical shift evolution, and so allows the ROE to develop through cross-relaxation in the transverse plane. Spin relaxation here is characterised by the time constant $T_{1\rho}$ ($\cong T_2$). In utilising the spin-lock, one has effectively replaced the static B_0 field of the conventional NOE with the far smaller rf B_1 field, and it is this that changes the dynamics of the NOE. Whereas γB_0 typically corresponds to frequencies of hundreds of megahertz, γB_1

Figure 8.24. A general scheme for observing rotating-frame NOEs. The ROE develops during the long spin-lock pulse that constitutes the mixing period, τ_m .

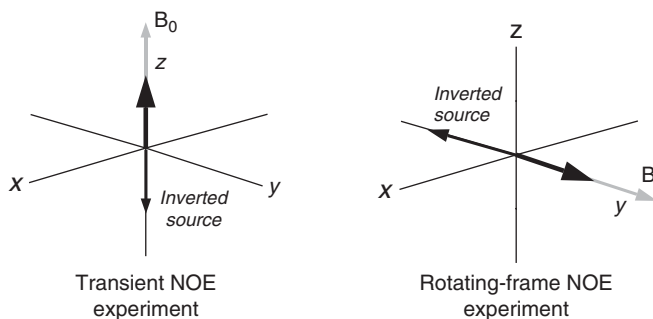
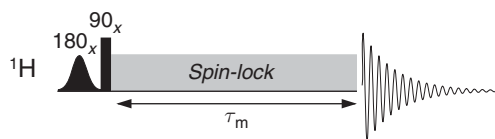


Figure 8.25. The rotating-frame NOE experiment can be viewed as the transverse equivalent of the transient NOE experiment.

is typically only a few kilohertz, meaning $\gamma B_1 \ll \gamma B_0$ and hence ω_1 (the rotating-frame frequencies) $\ll \omega_0$. The consequence of this is that $\omega_1 \tau_c \ll 1$ for all realistic values of τ_c , and all molecules behave as if they are within the extreme narrowing limit. Thus, ROEs are positive, any indirect effects have opposite sign to direct effects and tend to be weak, and saturation transfer can be distinguished by sign from ROEs, regardless of molecular size and dynamics.

Set against these obvious benefits are a number of experimental problems, principally TOCSY transfers also occurring during the spin-lock and signal attenuation from off-resonance effects. These issues are further addressed in the practical sections that follow, so it is sufficient to note here that even more care is required when acquiring and using ROEs than is needed for NOEs, so much so that some would regard this only as a specialist's technique.

Part II: Practical aspects

8.6. MEASURING STEADY-STATE NOEs: NOE DIFFERENCE

The basic requirement for the observation of steady-state NOEs is a suitable period of presaturation of the target resonance prior to acquisition of the spectrum, as discussed above and indicated in Fig. 8.1. This dictates that steady-state measurements are derived only from 1D spectra, in which spins experiencing the NOE will produce resonances with altered intensities relative to the conventional 1D spectrum. The question then remains as to how best to display and measure these changes. Although, in principle, this may be achieved by direct integration of resonances relative to a control spectrum, in reality this is a non-trivial exercise when enhancements may only be of a few percent. Instead, the universal approach is to use 'difference spectroscopy' and to subtract the control spectrum from the NOE spectrum, yielding a difference spectrum in which, ideally, the only remaining signals are the NOE enhancements and the saturated resonance (Fig. 8.26). This is the so-called NOE difference experiment that has played a pivotal role in structural organic chemistry for many years [9]. The purpose of the 'difference' approach is to make the *observation* of the enhancements *easier* and *more reliable* but imparts no new information to the difference spectrum that was not within the original NOE spectrum.

The NOE difference experiment is illustrated schematically in Fig. 8.27. The NOE spectrum is generated by applying presaturation to the target resonance for a period τ , after which the presaturating rf¹ is gated off and the 1D spectrum acquired with a 90° pulse. The control spectrum is acquired in an *identical* fashion except that presaturation is no longer required. To keep the acquisition conditions for both experiments as similar as possible, which is crucial for a successful difference experiment, the presaturation frequency is moved well away from all resonances (it is placed 'off-resonance') for the control rather than being turned off altogether, typically by placing it at the far edge of the

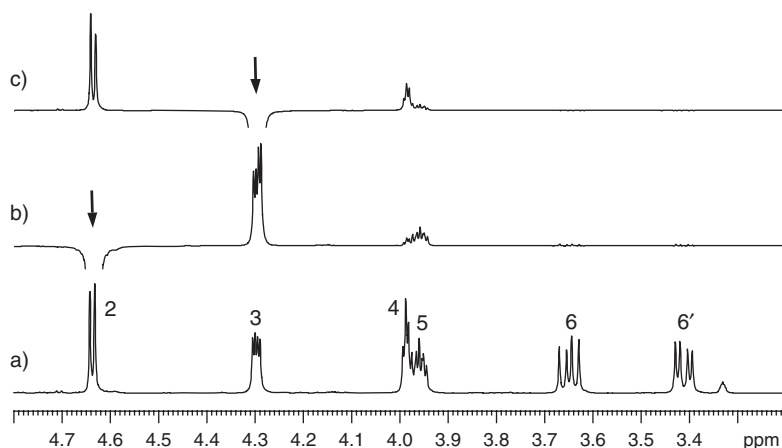
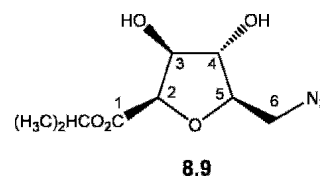
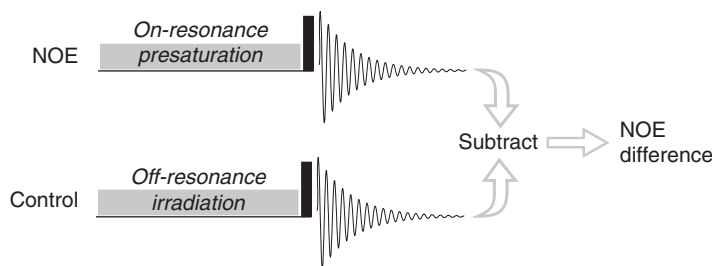


Figure 8.26. The control 1D spectrum (a) and NOE difference spectra (b and c) of **8.9** in MeOD. The difference spectra show only NOE enhancements and the truncated difference signal of the saturated resonance (arrowed). The observed enhancements are consistent with the indicated 2,5-*cis* geometry across the ring oxygen of **8.9**.



¹ Historically, the presaturating rf has been applied via the 'decoupler channel', traditionally, the second rf channel of the spectrometer. However, decoupling is a misleading term in the context of the NOE since spectra are acquired fully J-coupled, hence the term 'presaturating rf' is used throughout.

Figure 8.27. The procedure for generating the NOE difference spectrum.



spectrum. Subtracting the resulting spectra (or FIDs followed by FT) yields the difference spectrum. The success of this approach is critically dependent on the two spectra being identical in all respects other than those features introduced by the on-resonance presaturation; if this is not the case, spurious difference responses are introduced (see below). In practice, the perfect subtraction of resonances is experimentally very demanding, so various procedures have been developed to minimise undesirable variations and hence artefacts.

8.6.1. Optimising difference experiments

Minimising subtraction artefacts

NOE experiments typically require significant spectrometer time because the enhancements being sought are rather small. The NOE difference experiment represents a stringent test of both short- and long-term spectrometer stability, since changes in rf phase or frequency or in magnetic field contribute to *difference artefacts* (Fig. 8.28). These appear as dispersion-like signals, which may mask genuine NOEs, in addition to being unsightly. Improvements in spectrometer design have gone a long way to reducing these instabilities and make the observation of weaker NOEs ever more reliable as artefacts are reduced. Nevertheless, care is still required to achieve optimum results.

The first consideration is a stable sample environment, including sample temperature, a point of particular concern for aqueous solutions and for solutes with temperature-dependent shifts. Activity in the vicinity of the magnet has long been considered a contributing factor to poorer difference spectroscopy. How significant this is will depend on the physical location of the instrument, the field strength and on activities in adjacent labs or corridors (including those above and below) but in any case it is clearly wise to minimise disturbances of the field. Acquiring data overnight or at weekends may prove beneficial if such interferences prove to be detrimental.

Short-term random instrumental instabilities are suppressed by acquiring a large number of scans, just as random noise is similarly reduced by signal averaging, and the improved signal-to-noise in the resulting spectra also aids the reliable identification of enhancements. Longer term instabilities, such as from small field or temperature drifts, are addressed by interleaving acquisitions between the control and the NOE experiments so that over the course of the experiment all spectra experience the same net variation. A typical approach when multiple NOE measurements are required of a sample is to acquire 8 or 16 transients for each experiment and cycle round all irradiation frequencies (usually automatically under computer control). For further signal averaging, the appropriate data sets are co-added to acquire the desired total number of transients that must be identical for each data set collected. For multiple NOE experiments, a single control is usually sufficient for all difference calculations. Also included in each acquisition must be 2 or 4 ‘dummy scans’ (in which the recorded data are discarded) to ensure the complete decay of saturation effects

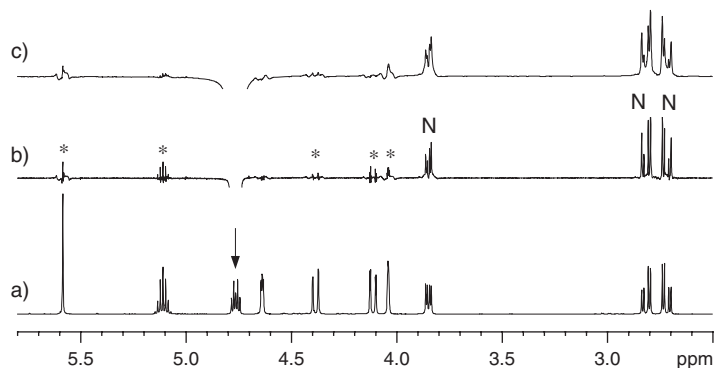


Figure 8.28. NOE difference artefacts shown by an asterisk in the difference spectrum (b), arise when two similar, but not quite identical, signals are subtracted in an attempt to reveal the genuine NOEs (N). The artefacts can be attenuated a little through the use of line-broadening window functions (c, lb = 1 Hz).

from the previous irradiation. Since presaturation is required for each acquisition, the usual relaxation delay between transients is not necessary and is wholly replaced by the presaturation period.

Subtraction artefacts can be further reduced by suitable processing of the data. Difference spectra can be generated by subtracting two FIDs (the NOE and the control) and Fourier transforming the resulting FID, or alternatively by directly subtracting the NOE and the control spectra, in which case they must both be processed with *digitally identical* phase corrections prior to the subtraction. The results of these methods are equivalent except when spectra exhibit a large dynamic range (i.e. where very large signals exist in the presence of very small ones) in which case subtracting FIDs may reduce spectrum noise [10]. In either approach, applying mild line broadening to the spectra, typically of a few Hz, helps reduce difference artefacts (Fig. 8.28c). The rationale behind this is that intensity of the residual signal obtained by subtracting closely overlapping Lorentzian lineshapes is inversely proportional to the resonance linewidths [11].

Optimising presaturation

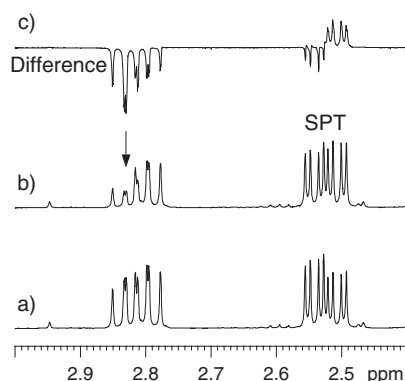
Selection of the optimum presaturation time τ is highly dependent on the information required of the molecule and on its size and structure, so general guidelines only can be presented here. Since steady-state measurements are only of use in the positive NOE regime, these considerations are limited to small- and medium-sized molecules, and, in fact, most of what we need to know has already been presented in the previous sections since the choice of τ is dictated by the kinetics of the NOE. Thus, if measurements of genuine quantitative steady-state enhancements are required, then τ must be greater than $5T_1$ of the slowest relaxing spins, which could make signal averaging very time consuming. However, most structural work does not require the full steady-state to be attained so long as a measurable NOE has been able to develop, allowing more transients to be collected in the time available, which itself facilitates the observation of the NOEs. The use of shorter presaturation times then favours the observation of short-range interactions since these are quickest to develop. In contrast, long-range interactions and indirect effects are slow to build and are enhanced with extended presaturation. For the majority of structural studies, information on immediate neighbours is sufficient, so relatively short presaturation times of around 4 or 5 s (about 3 times the longest T_1) tend to be used in routine NOE investigations of small organic molecules ($M_r < 500$). Under such conditions, one should not be surprised to find that longer-range interactions cannot be observed, and if these are expected to be informative, or if indirect effects may provide useful geometrical information, further experiments with extended presaturation ($>>5T_1$, possibly tens of seconds) will be required in order to detect these. Clearly, it is advantageous to have some prior knowledge of the approximate T_1 values of protons in the molecule, and these may be measured by the quick inversion-recovery method, given in Section 2.4. This additionally provides supporting data since remote protons that are isolated from dipolar relaxation sources will exhibit unusually long T_1 s as well as slow to develop NOEs.

Selective saturation and SPT

To ensure the integrity of NOE data, it is essential that only a single resonance is subject to presaturation at any one time. Even a small degree of saturation of a neighbouring resonance caused by 'spillover' of the presaturating rf can be detrimental for structural studies. Such spillover can usually be readily observed in the difference spectrum and should call for great caution when interpreting data. Naturally, it is more useful to spot this failing before the experiment is left running for many hours and to take measures to alleviate it. An effective approach to this is to directly overlay the NOE and control spectra obtained after the first experiment cycle (e.g. after 8 transients for each) using the spectrometer dual-display mode. Whilst NOE enhancements are unlikely to be apparent at this stage (unless very large), saturation effects close to the target resonance are usually clear and may suggest that changes to the experimental set-up are required.

Ideally, each target resonance should be well removed from all others, but this criterion cannot always be met. Changes of solvent can be useful here and can sometimes lead to dramatic shifts of resonances, which may fortuitously place those of interest in a more exposed position. Beyond this, a variety of experimental procedures can help. The most direct way to reduce the frequency window over which the rf is effective and hence improve selectivity is to attenuate its power. This also reduces the degree of saturation of the target resonance which in turn reduces the *absolute* magnitude of the NOE enhancement, so a compromise must be sought between the two, usually with the emphasis on selectivity. Additional artefacts may also arise when using low-presaturation powers if multiplets are subject to *unequal* perturbation. This arises from so-called selective population transfer

Figure 8.29. SPT artefacts. Unequal saturation of a resonance (b) can cause SPT intensity distortions to appear at its J -coupled neighbours. These appear as anti-phase patterns in the difference spectrum b-a (c), which have zero net integral but may mask genuine NOEs.



(SPT), which has already been described in Section 4.4. This is a manifestation of polarisation transfer between J -coupled spins and is related to the process by which crosspeaks are generated in COSY spectra. The responses appear as *anti-phase* multiplets for those spins J -coupled to the saturated spin (Fig. 8.29). The integrated intensity of such anti-phase lines is zero if correctly phased, so should not interfere with NOE quantification, although the potentially intense SPT responses could be distracting and may mask genuine NOE responses, so are well worth suppressing.

One approach to achieving even saturation of a multiplet when using weak rf powers to maintain selectivity is to cycle the presaturation frequency between individual lines *within* each multiplet [12]. The process involves irradiating each line for a short period in turn, then repeating the sequence a number of times to achieve the desired total presaturation period. For each irradiation, the aim is to saturate only a single line rather than the whole multiplet, so considerably lower rf powers may be used, whilst the cycling ensures approximately equal suppression across the whole multiplet. An important consideration here is the saturation period used for each line; if too short the saturation may be ineffectual (and unwelcome frequency modulation artefacts introduced) whilst too long a period will enable relaxation to become effective leading to uneven saturation. The relaxation behaviour of the spins is again important, and in general, periods of 50–300 ms work well for most small molecules, again trial-and-error is the best approach to optimisation (Fig. 8.30). Thus, a five-line multiplet with 200 ms saturation of each line requires five cycles to achieve a total presaturation period of 5 s. Such sequences can be readily programmed to operate automatically on modern spectrometers.

An additional approach to suppressing SPT distortions is to collect spectra with an *exact* 90° observation pulse. This effectively spreads saturation evenly throughout all multiplet components and so removes the source of these distortions. The accuracy of the 90° pulse can be improved by the use of a composite pulse (Section 10.1) for which the $270_x360_{-x}90_y$ sequence has been suggested [13]. The 90° acquisition pulse also leads to maximum signal, and since long presaturation periods are used between transients, this represents a suitable choice for optimum sensitivity and is standard for the NOE difference method. In situations where a multiplet is overlapped by another resonance, the frequency cycling method is unsuitable for uniform multiplet suppression, but the use of an accurate 90° pulse means only a single line from the multiplet need be available for saturation for the experiment to work, although the absolute magnitude of the NOE(s) will be small in such cases because of

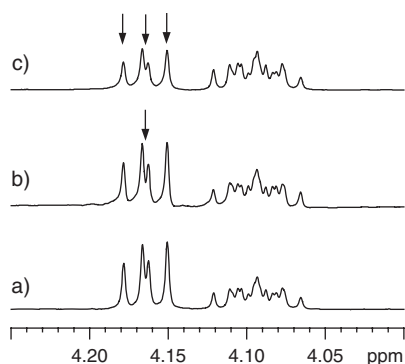


Figure 8.30. Selective multiplet saturation through frequency cycling. (a) Control spectrum, (b) single frequency, low-power presaturation applied to the centre of the multiplet and (c) presaturation using the same power but with frequency cycling over the four lines. The low power ensures the neighbouring multiplet is untouched.

the small degree of population perturbation. In cases of problematic overlap, the alternative is to employ the 2D NOESY experiment described below in which selective irradiation is avoided altogether.

Saturation transfer

An additional mechanism by which saturation of a resonance can occur, other than by direct irradiation, is *saturation transfer* brought about by chemical exchange processes that are slow on the NMR chemical shift timescale. This may be, for example, conformational exchange whereby saturation of a spin transition in one conformer will progressively lead to the saturation of the corresponding spin in another if exchange occurs during the presaturation period (see Fig. 8.19 above). This may be a bonus in some circumstances since it may allow the indirect saturation of an otherwise obscure resonance or it may be studied quite separately as a means of analysing exchange dynamics. In terms of NOE measurements, it is more often an additional complication to be aware of since the observed NOEs then result from the saturation of more than one resonance. Similarly, any NOEs that develop may also be transferred to other resonances by the exchange, further complicating matters. Exchange processes are generally more efficient than cross-relaxation, and signals arising from exchange are usually more intense than NOEs and exhibit the same sign behaviour as the originally saturated resonance. Hence, they appear with opposite sign to positive NOE enhancements, allowing them to be distinguished, but with the same sign as negative enhancements. This is a further pitfall of working in the negative NOE regime no matter which experimental approach is adopted.

A relatively common feature of NOE experiments performed in organic solvents containing traces of water is transfer of saturation between exchangeable protons and the water (Fig. 8.31). Often these types of resonances can be rather broad and may be unwittingly saturated if they happen to spread under neighbouring signals, which in turn may produce unexpected negative responses elsewhere in difference spectra as a result of exchange. This effect can also be used to identify the resonances of exchangeable protons in a molecule by saturation of the water resonance, as was used in the structure determination of the antibiotic Pulvomycin **8.11** [14] (Fig. 8.32).

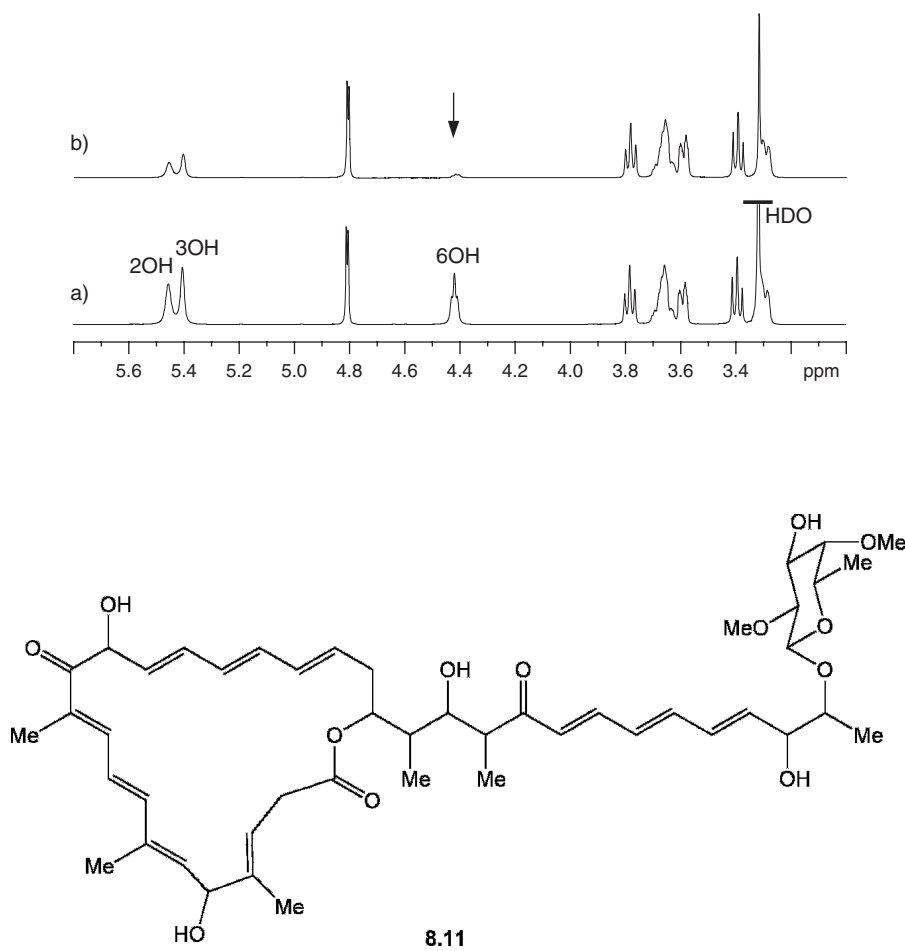
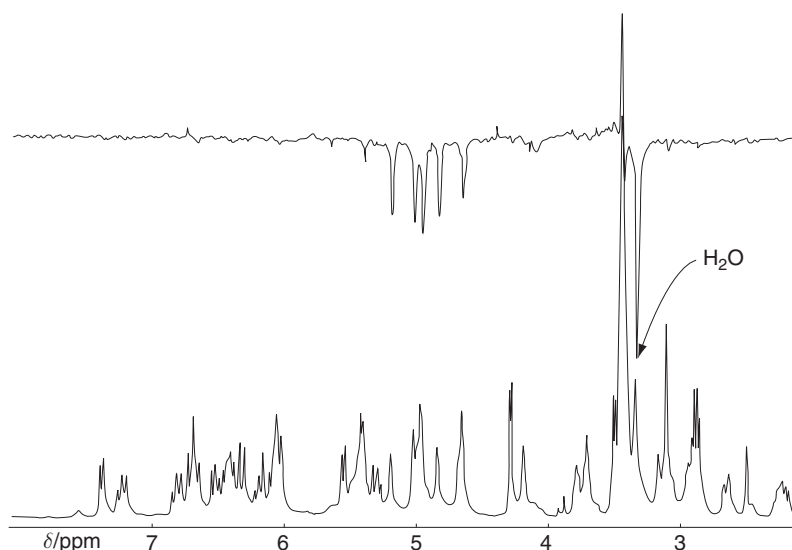


Figure 8.31. An experimental demonstration of saturation transfer. Direct saturation of the 6-OH resonance of α-cyclodextrin **8.10** in DMSO leads to the simultaneous *indirect* partial saturation of the 2- and 3-OH resonances as well as that of the water (shown truncated in (a)).

Figure 8.32. The identification of the resonances of exchangeable protons of pulvomycinin **8.11** in a saturation transfer difference experiment. The H₂O peak was selectively saturated, leading to the partial saturation of the five hydroxyl resonances through chemical exchange (reproduced with permission from [14], Copyright 1985, American Chemical Society).



Quantifying enhancements

The quantification of percentage NOE enhancements can be made most economically by direct analysis of the NOE difference spectrum alone, rather than the perhaps more obvious option of directly comparing integrals between the control and NOE spectra. The saturated peak may be used as an internal reference for these measurements with the peak of a single resonance referenced to -100% or that of a methyl group to -300% and so on (the minus signs arise from the *convention* of plotting enhancements as positive-going responses, which define the saturated peak as having negative intensity when NOEs themselves have positive sign). All NOE enhancements are then integrated relative to this reference and the percentages are obtained directly. In doing this, one is at the same time compensating for incomplete saturation that may have been brought about by using lower presaturation powers; by scaling the reference peak to assume complete saturation, we likewise scale all enhancements. This procedure is justifiable since absolute NOE enhancements scale in direct proportion to the degree of saturation. The careful use of baseline correction of the difference spectra prior to integration should be considered since baseline errors can make dramatic differences when measuring small enhancements. Even so, very accurate measurements of enhancements are usually of limited use in the final analysis since such small differences cannot be interpreted meaningfully. Quoting results to 1 or perhaps 0.5% is sufficient for most qualitative interpretations where the emphasis should be on interpreting a collection of enhancements rather than relying on a single percentage measurement.

8.7. MEASURING TRANSIENT NOEs: NOESY

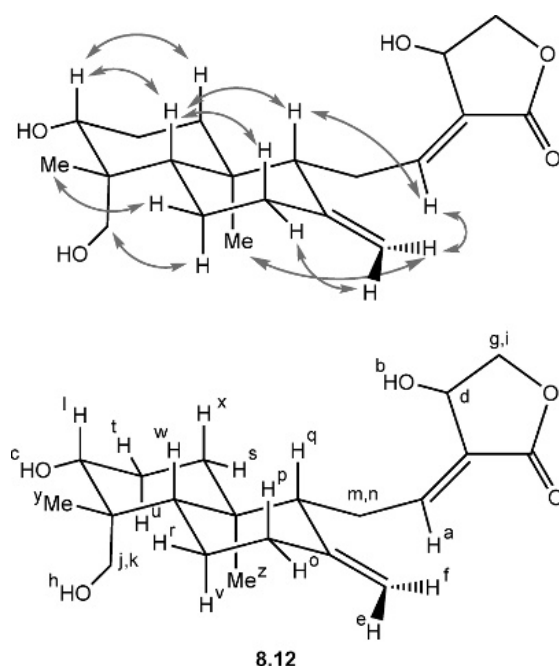
Nowadays, two approaches for observing transient NOEs find widespread use – the homonuclear two-dimensional NOE spectroscopy (NOESY) and its selective 1D analogue employing pulsed field gradients for signal selection. The popularity of the 2D NOESY was driven originally by interests in biological macromolecules, for which NOEs are negative, strong and fast growing, and this area remains the home territory of NOESY. Traditionally, this was less used for smaller molecules principally because the NOE in the extreme narrowing limit is weaker and tends to grow more slowly, and because the steady-state NOE difference experiment provided a viable, and very effective, alternative. However, with improved instrument sensitivity and stability, the NOESY experiment now finds widespread application in routine small-molecule studies since it may offer benefits over the NOE difference experiment. The most obvious advantage is the non-selective nature of the experiment, providing the opportunity, at least in principle, to observe all NOEs within a molecule in a single experiment (these factors parallel the benefits of 2D COSY over 1D selective spin-decoupling). Added to this is the simplicity of setting up NOESY relative to the more time-demanding NOE difference, which has advantages in an automated environment, for example.

Steady-state measurements are well suited to small-molecule configurational and conformational studies since the enhancements obtained will be intrinsically larger than those from transient measurements. Energy is continually fed into the spin system in the form of rf saturation, and this is able to drive the development of the NOE. Transient enhancements,

in contrast, develop solely from the population disturbances brought about by initial resonance inversion so tend to be of lower intensity and of fleeting existence, requiring even more care in their capture. Furthermore, very small molecules ($M_r < 200$) that tumble very rapidly in solution produce extremely weak NOEs because cross-relaxation tends to be inefficient, and the search for transient enhancements presents still greater challenges in such cases. With larger molecules, more success is to be expected. Nevertheless, the popular gradient-selected 1D transient NOE experiments described below have made a significant impact on small-molecule NOE studies and now enjoy widespread use, in particular because of the exceptional quality of spectra they are able to provide, typically generating cleaner spectra in shorter times than does NOE difference. Note, however, that their use requires a fundamentally different approach to the analysis of NOE data from that used when interpreting NOE difference spectra because one is measuring *transient* not *steady-state* enhancements, as discussed further below.

8.7.1. The 2D NOESY sequence

The NOESY experiment [15] again follows the principles presented in Chapter 5 for the generation of a 2D data set and has a similar appearance to the homonuclear COSY-based correlation spectra, although in this case the crosspeaks of the 2D spectrum indicate NOE interactions between the correlated spins. These peaks arise from the *incoherent* transfer of magnetisation between spins during the mixing time via the NOE, so allowing through-space proximities to be mapped directly. The NOESY spectrum of the naturally occurring terpene andrographolide **8.12** in DMSO is shown in Fig. 8.33 and displays a comprehensive map of close contacts within the molecule, some of which are illustrated on the structure. All NOE crosspeaks have opposite phase to the diagonal, indicating that these arise from positive NOE enhancements, as anticipated for a molecule of this size ($M_r = 350$) under ambient conditions. The few crosspeaks sharing the same phase as the diagonal in the 3–6 ppm region are attributable to hydroxyl protons and H₂O and arise from chemical exchange of the protons within these groups (Section 8.7.4).



The NOESY sequence (Fig. 8.34) is closely related to COSY, so here we shall consider only the features specific to the NOE experiment. The most significant of these is the mixing period, τ_m , during which the NOE develops, and to understand the role this plays in a 2D experiment, we return to the vector model (Fig. 8.35). Following initial excitation and t_1 evolution, the magnetisation vector exists in the transverse plane. The second 90° pulse places one component of this onto the $-z$ axis and has therefore generated the required population inversion that enables the transient NOEs to develop during the subsequent mixing period (the components that remain in the transverse plane are those detected in the COSY experiment). After a suitable period, τ_m , the new populations are sampled with a 90° pulse and the FID collected; one can readily see a parallel with the 1D ‘inversion-mixing-sampling’

Figure 8.33. The 2D NOESY spectrum of the terpene andrographolide **8.12** in DMSO at 25°C. The spectrum was recorded with a 600 ms mixing time, and a recovery delay of 1.5 s. 2 K data points were collected for 512 increments of 16 scans, using TPPI f_1 quadrature detection. Data were processed with a squared cosine-bell window in both dimensions with a single zero-fill in f_1 .

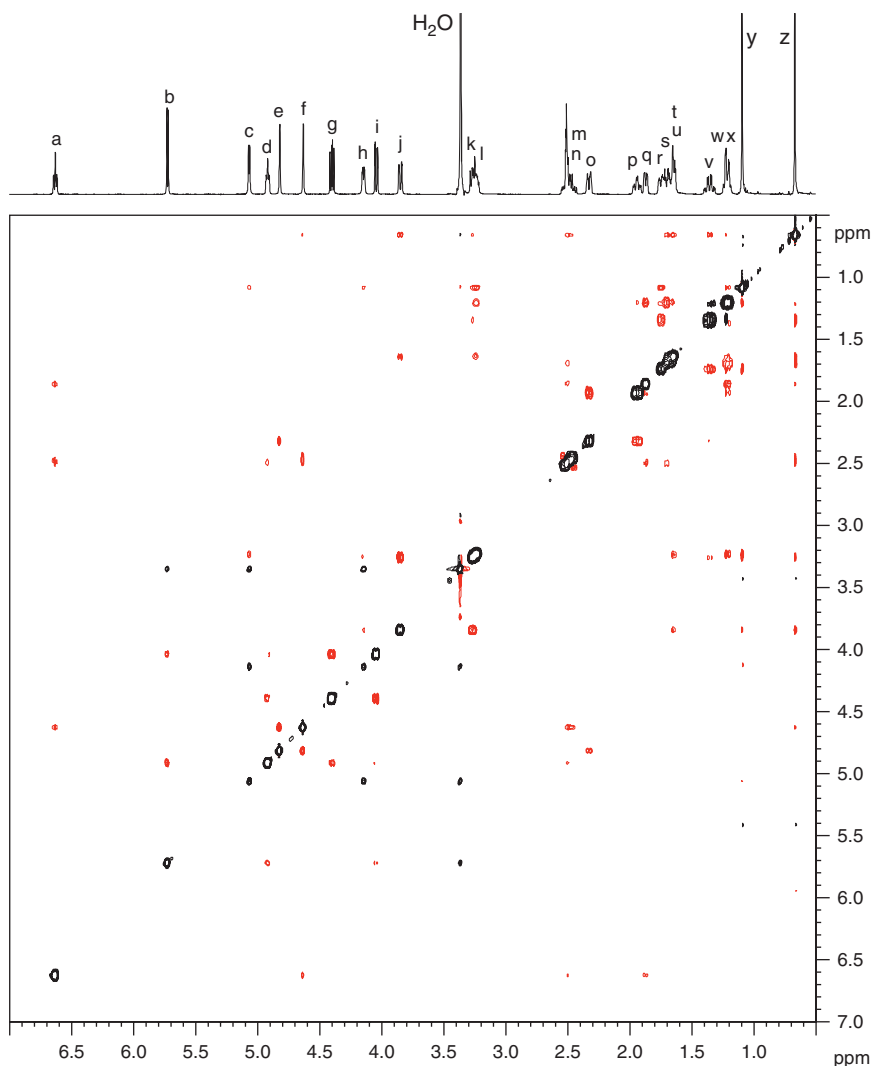


Figure 8.34. The 2D NOESY sequence and the associated coherence transfer pathway. The optional use of a pulsed field gradient during the mixing time (shown greyed) is described in the text.

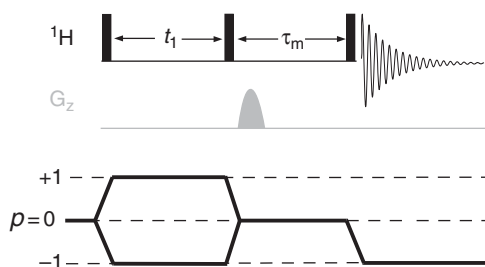
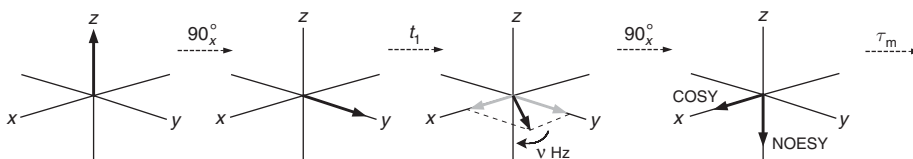


Figure 8.35. The evolution of magnetisation vectors during NOESY. The inverted magnetisation generates the NOE during the mixing time, whilst the remaining transverse components are removed by phase-cycling or pulsed field gradients.



sequence illustrated in Fig. 8.20. The sequence is repeated to collect sufficient transients for an acceptable signal-to-noise ratio. Whilst, in principle, it should be necessary to leave at least $5T_1$ between transients to allow populations to recover; this would lead to excessively long experiments, particularly in the case of small molecules. In practice, shorter recovery delays of typically 1–3 s for small molecules ($\approx 1-2T_1$ of the slowest relaxing spins) represent an acceptable compromise [16].

Table 8.3. The Basic NOESY phase-cycle for the three pulses (P_N) and the receiver (P_R), including the suppression of axial peaks

Transient	P_1	P_2	P_3	P_R
1	x	x	x	x
2	x	x	y	y
3	x	x	-x	-x
4	x	x	-y	-y
5	-x	x	x	-x
6	-x	x	y	-y
7	-x	x	-x	x
8	-x	x	-y	y

The NOESY sequence, aside from details of the timings used, is identical to the DQF-COSY sequence, so it remains to select the signals arising from NOEs and suppress all others by means of a suitable phase cycle (Table 8.3). Since NOEs are associated only with spin populations, that is, z -magnetisation of coherence order zero, they are insensitive to rf phase changes and a four-step cycle is sufficient to cancel signals originating from single- and double-quantum coherences present during τ_m . The cycle is repeated with additional phase inversion of the preparation pulse and receiver to cancel axial peaks and produces a final eight-step cycle. Since chemical exchange between sites is also associated with longitudinal magnetisation exchange between resonances, this experiment will also detect these processes, in an analogous fashion to the saturation transfer described for the 1D experiments above, and for this reason this exact sequence is also termed EXSY (exchange spectroscopy) when used to study dynamic exchange processes (see Section 8.7.3). The use of a phase-sensitive presentation is highly recommended for NOESY since this allows the discrimination of chemical exchange from positive NOEs on the basis of sign, aids the identification of some of the artefacts described below and allows quantitative measurements [17], in addition to the usual benefits of a higher resolution display. NOESY crosspeaks are then in-phase, have pure absorption lineshapes and appear symmetrically about the diagonal [18] (since transient enhancements are symmetrical in nature). Their sign with respect to the diagonal is dictated by the molecule's motional behaviour and hence the sign of the NOE itself (Table 8.4).

Selection of the desired coherences may also be performed via PFGs, so avoiding the need for phase cycling [19–21]. This may be achieved simply by inserting a single z -gradient within the mixing time such that all coherences are dephased and only the desired z -magnetisation remains (Fig. 8.34). The time-saving benefits of such an approach are likely to be significant only when large sample quantities are available and rather few scans per increment provides sufficient sensitivity. When working with aqueous solutions, additional solvent suppression schemes may be added to this basic sequence; these are described in Section 10.5.

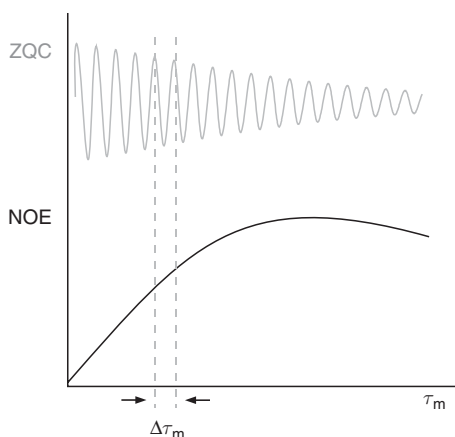
Zero-quantum interference in NOESY

The principal unwanted signals remaining from the NOESY sequence are those arising from zero-quantum coherences (ZQCs) that existed during the mixing time and which are subsequently transformed into observable signals by the last 90° pulse. Since these also possess coherence order zero they are not removed by the phase-cycling or gradient selection procedures and thus may contaminate the final spectrum. These coherences arise between J -coupled spins and so give rise to COSY-like peaks, which are the zero-quantum analogues of the signals detected in the DQF-COSY sequence and have a similar anti-phase multiplet appearance. Worse still, they appear with dispersive lineshapes if spectra are phased conventionally. If both a ZQC and NOE peak are coincident, which may occur when J -coupled spins also share an NOE, the NOE peak may appear somewhat distorted by this superposition. For large molecules with broad lines ZQC peaks, being anti-phase, tend to cancel, but in small or mid-size molecules, their active removal may be beneficial.

Table 8.4. Responses observed in the NOESY experiment. By convention the diagonal is phased to be positive and the sign of all other signals are given relative to this

Sign of diagonal	Origin of crosspeak	Sign of crosspeak
Positive	Positive NOE	Negative
	Negative NOE	Positive
	Chemical exchange	Positive

Figure 8.36. The variation of NOE and ZQC intensities during the NOESY mixing time. The ZQC contributions may be suppressed by making small random variations to τ_m , whereas this has negligible effect on the NOE intensities.



A number of approaches have been suggested for the suppression of ZQC peaks [15,22,23], the simplest being to introduce a small, random variation in the mixing time between transients or, more commonly, between one t_1 -increment and the next. Whilst during the mixing time ZQCs oscillate with a frequency equal to the chemical shift difference of the two J -coupled spins, $(\nu_A - \nu_X)$, NOEs grow progressively (Fig. 8.36). Random variation of τ_m will cause the ZQCs to average away whilst NOE intensities are little affected (in fact, this randomisation of the ZQC signals merely makes them appear as t_1 -noise rather than discrete crosspeaks). The degree of variation required must be at least comparable to the inverse of the smallest shift differences, $|(\nu_A - \nu_X)|^{-1}$, so assuming this to be, say, 50 Hz, a random fluctuation of ± 10 ms is required. Even with such an approach, it is not uncommon to observe some residual COSY-like structure within crosspeaks very close to the diagonal. Additional artefact peaks can also arise between J -coupled spins when pulse widths deviate from 90° [24], so careful calibration of these is required.

A more direct approach to the elimination of zero-quantum interference is via the use of swept-frequency inversion pulse applied in the presence of a field gradient, which destroys the zero-quantum contributions in a *single transient*. The application of this has already been described in Section 5.7.3 with a view to obtaining pure lineshapes in TOCSY spectra, and the operation of the filter is itself described in Section 10.6 and so will not be explored here. The implementation of this in the 2D NOESY sequence is illustrated in Fig. 8.37 and requires the incorporation of the filter within the usual mixing time, followed by a purging gradient. The effective suppression of zero-quantum interference is illustrated in the high-resolution NOESY spectra of Fig. 8.38 in which spectrum (a) shows clear anti-phase

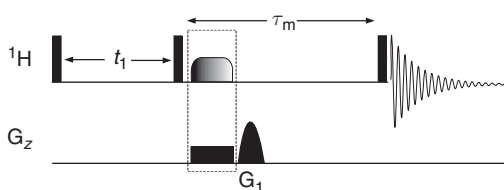


Figure 8.37. The NOESY sequence incorporating the frequency sweep/gradient zero-quantum filter.

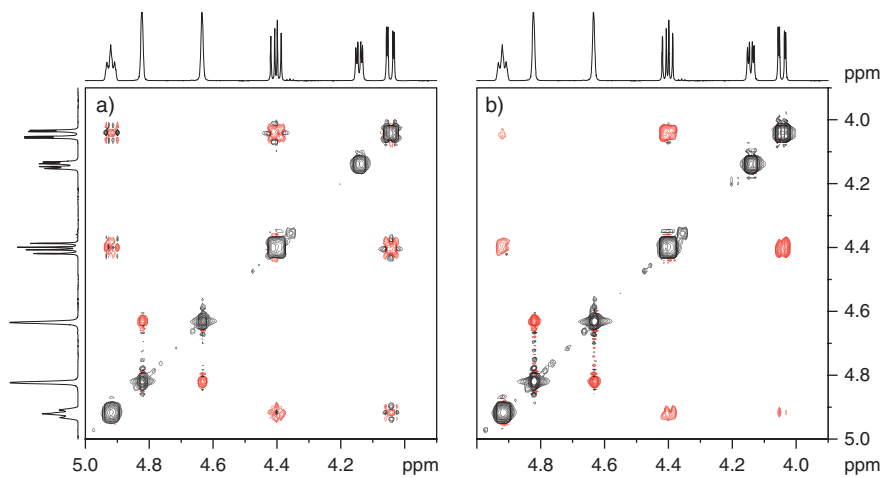


Figure 8.38. Regions of 400 ms NOESY spectra recorded (a) without and (b) with the inclusion of the zero-quantum filter shown in Fig. 8.37. The ZQC suppression employed a 20 ms adiabatic smoothed CHIRP pulse with a 40 kHz frequency sweep.

dispersive contributions to crosspeaks between J -coupled spins whereas these cancel to reveal the NOEs more clearly in (b).

Optimum choice of mixing time

The appropriate choice of the mixing time, τ_m , is critical to the success of transient NOE experiments. Incorrect choice of τ_m can cause complete absence of observable signals since too short a value means the enhancements have yet to grow to a detectable level whilst an excessively long τ_m can mean the enhancements have decayed through relaxation. The motional properties of the molecule and hence the longitudinal relaxation times of the nuclei play the most significant role in dictating NOE growth and in turn dictate the selection of τ_m (Fig. 8.39). This choice is also very dependent on the type of information required of the spectrum. If the desire is to establish a qualitative map of NOEs within a molecule, as is often the case in small-molecule work, then the optimum mixing time will be where the NOEs have their maximum intensities. If the data were to be used for quantitative or semi-quantitative distance measurements for use in structural calculations, then it is necessary to ensure that one is working within the linear growth region of the NOE development, and mixing times well short of the NOE maxima will be required.

Selection of τ_m for qualitative studies can be estimated from knowledge of longitudinal relaxation times, T_1 . Often these are not known prior to running the experiment, so a quick measurement may assist here, or failing that an estimate based on previous knowledge. Precise measurements are of little benefit since there will inevitably exist a spread of values within the molecule and a compromise value for τ_m is required in any case. For small molecules, a mixing time of around T_1 will produce near maximum intensities, and for many routine organic molecules with masses of <500 daltons in low-viscosity solvents, T_1 values typically range from around 0.5 to 2 s. As molecular tumbling rates decrease (owing to molecular mass or solution viscosity increases, for example), T_1 s likewise decrease and correspondingly shorter mixing times are, in principle, desirable. In such cases, one should be aware of approaching the dreaded $\omega_0\tau_c \approx 1$ region where efficacy of the experiment will diminish. For molecules in the negative NOE regime shorter values of around 0.5 T_1 are more appropriate to avoid complications arising from spin diffusion.

Distance measurement

Whilst a comprehensive discussion of this matter lies beyond the scope of this book, the principles involved are very briefly introduced here, the idea being that the reader should at least be able to follow discussions on structure calculations based on NOE measurements found in the chemical literature. The vast majority of work in this area has been applied to biomolecular structures and the protocols developed with macromolecules in mind, which will not translate directly to quantitative measurements in small molecules [25], although the general principles remain the same.

The most used approach to distance measurements stems from Eqs 8.13 and 8.14 above and relies on a known reference distance, r_{XY} , from which others may be calculated [26], and the assumption of uniform isotropic molecular tumbling. The basic equation was:

$$\frac{\eta_A\{B\}}{\eta_X\{Y\}} = \frac{r_{AB}^{-6}}{r_{XY}^{-6}} \quad (8.19)$$

The ratio of the NOEs is determined by one of two general approaches. The first (Fig. 8.40a) involves determining the NOE growth rate for both the reference and the unknown distances, by recording a series of NOESY spectra over a range of τ_m values and monitoring the crosspeak build-up intensities through their volume integrals [27]. The second, simpler approach (Fig. 8.40b) directly compares crosspeak intensities I_{XY} and I_{AB} measured at a single mixing time that is known to lie within the linear growth regime

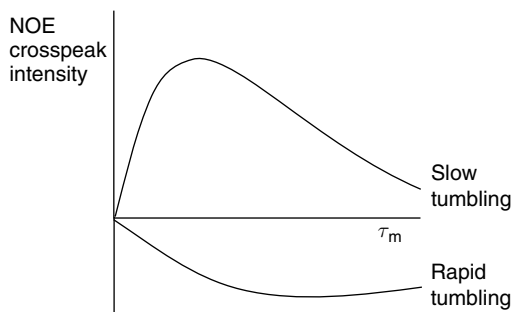
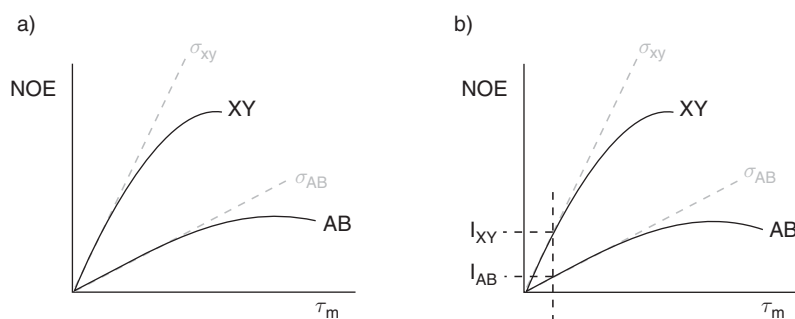


Figure 8.39. Schematic illustration of NOE crosspeak growth for large, slowly tumbling and small, rapidly tumbling molecules. The diagonal peak is assumed to be positive.

Figure 8.40. Schematic illustration of two approaches for estimating internuclear separations from NOE measurements, in which a reference spin pair, XY, provides the internal calibration for the unknown pair, AB (see text).

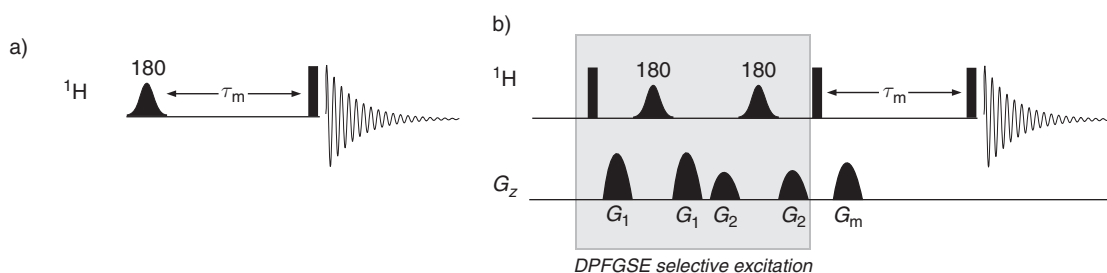


for both spin pairs. The success of these approaches relies, in part, on the insensitivity of the calculated distance on the accuracy of the experimentally measured NOEs, due to the r^{-6} dependence. Thus, a two-fold error in the growth rate corresponds to only $\approx 10\%$ error in the final distance estimate. Nevertheless, the measurement of a single 'accurate distance' does not allow for internal flexibility, so has little meaning in a solution-state structure, and a more general and widely used approach has been to make use of semi-quantitative distance measurements in structure calculations. This involves categorising peak intensities as strong, medium and weak relative to the reference peak, which in turn are taken to indicate upper (and perhaps lower) distance bounds on internuclear separations of, typically, ≤ 0.25 , ≤ 0.35 and ≤ 0.5 nm respectively. The internal reference distance measurement for proton NOEs is typically that between diastereotopic geminal protons (0.175 nm) or between *ortho* aromatic protons (0.28 nm). Although these constraints are lax, the combined application of many of them is able to produce well-defined solution conformations, as has been amply demonstrated for numerous protein structures [28,29]. An alternative approach that makes use of a single NOESY spectrum utilises the ratio of crosspeak and diagonal peak intensities in place of growth rates [30]; both methods have been applied to the determination of the solution conformation and configuration of a small molecule and shown to produce similar results [31].

8.7.2. 1D NOESY sequences

Traditionally 1D transient NOE experiments, often referred to as 1D NOESY, enjoyed limited use in routine structural work, finding application principally in situations where quantitative distance measurements are sought, a potentially time-consuming process because of the need to collect experiments over a range of τ_m values. The simplest 1D scheme (Fig. 8.41a) requires the use of difference spectroscopy to reveal NOE enhancements, thus suffering from the dreaded difference artefacts and so finds limited use. However, there can be no doubt that the introduction of 1D gradient-selected experiments [32–34] has revolutionised the use of 1D NOESY in small-molecule applications and are therefore considered here. These experiments do not depend on difference spectroscopy to reveal the NOE enhancements, and cleanly remove those signals not arising from the NOE, so providing very high-quality spectra in shorter times. These are most useful when a collection of enhancements are used qualitatively to provide structural answers and present a more rapid alternative to the NOE difference experiment. They may also be employed for quantitative measurements through the analysis of NOE build-up rates [35]. For many routine laboratory applications, the 1D gradient NOESY experiments have largely superseded the traditional 1D NOE difference experiment for initial stereochemical investigations. The steady-state measurements still fulfil a supporting role when the quantitative enhancements provide useful comparative data, a situation most often encountered with stereochemical assignments in small ring systems; see results for isomers 8.4a and b presented above for example.

Figure 8.41. 1D NOESY schemes. The basic scheme (a) requires difference spectroscopy to reveal the NOEs whereas the gradient-selected DPGSE-NOE (b) reveals NOEs without interference from difference artefacts.



1D gradient NOESY

The most robust experiment to date [33] (Fig. 8.41b) is based on the ‘double pulsed field gradient spin-echo’ (DPFGSE, also referred to as ‘excitation sculpting’) sequence as a means of selecting a target resonance from which the NOEs ultimately develop. This approach to selective excitation is described in Section 10.4.2, so here it is sufficient to note that it serves to selectively refocus only the target spin magnetisation in the transverse plane, leaving that of all other spins dephased and thus unobservable. The subsequent 90° pulse places the target magnetisation along the $-z$ axis, after which the NOE enhancements grow, with a purge gradient (G_m) applied to remove any residual transverse components that remain. Finally, the magnetisation is sampled in the usual way. The recorded spectrum contains only the inverted target resonance and the NOEs that have developed from it, without the need for a difference spectrum to be calculated. High-quality spectra can therefore be collected that are free from difference artefacts; this is the principle benefit of the gradient-selected schemes. The experiments also tend to be quick to perform since extensive signal averaging is no longer required for artefact reduction, and because optimum use may be made of the receiver dynamic range since only the desired signals are ever acquired.

In practice, relaxation processes operating during τ_m will cause the unwanted dephased magnetisation components to recover and produce small observable signals in the final spectrum for all resonances. These can be kept to close to zero intensity by the insertion of typically 1 or 2 non-selective 180° pulses (bracketed by opposing purging gradients) spaced judiciously within the mixing period [33] with any small signals that may remain cancelled by a 4-step difference phase cycle (EXORCYCLE). Thus, although difference spectroscopy is being used, in the form of the phase cycle, the signals to be cancelled have close to zero intensity and are readily removed, in contrast to conventional difference spectroscopy where the signals have essentially maximum intensity. For small and mid-sized molecules, experience suggests, as a rule of thumb, that one inversion pulse during τ_m is sufficient to reduce the background signals to undetectable levels with mixing times of up to 500 ms, whereas 2 are preferred for a τ_m up to 1.5 s. A typical result using the sequence of Fig. 8.41b is shown in Fig. 8.42 for the small bicyclic lactam **8.13**. Each resonance was selected with a 40 ms Gaussian 180° pulse with two non-selective 180° pulses applied during the 800 ms mixing time. Each spectrum was the result of only 12 min data collection on a 10 mg sample, yet are perfectly acceptable for qualitative stereochemical assignments. The observed signals may be attributed to NOE enhancements or to SPT-type artefacts, with an otherwise featureless baseline.

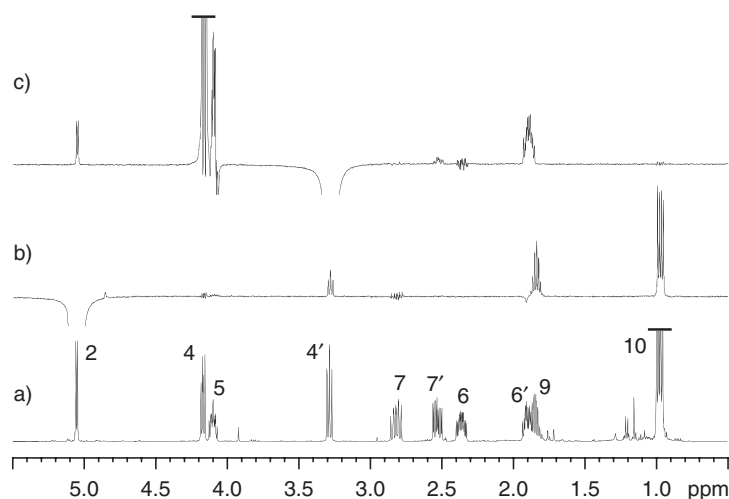
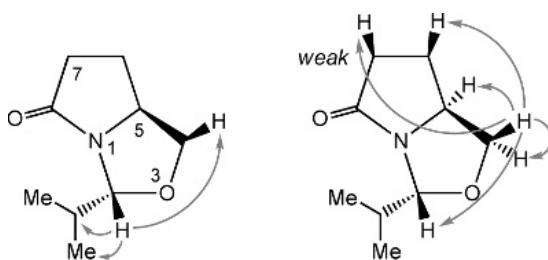


Figure 8.42. Selected 1D gradient NOESY spectra of the bicyclic lactam **8.13** recorded with a mixing time of 800 ms. Spectrum (a) is the conventional 1D spectrum and the strong geminal H4-H4 NOE in (c) has been truncated.



8.13

As for the 2D NOESY data described above, spectra are vulnerable to interference from undesirable zero-quantum contributions between coupled spins; this is apparent, for example, in trace (c) of Fig. 8.42 between H4' and H5. This can be especially problematic when shorter mixing times are employed, as in the generation of NOE build-up curves [35], and again the inclusion of the swept-frequency/gradient zero-quantum filter should prove beneficial. The complete selective 1D NOESY sequence incorporating this and employing the optimised DPGSE selection procedure is presented in Fig. 8.43, and an illustration of the improvements provided by the filter may be seen in Fig. 8.44 where the removal of unwelcome anti-phase dispersive contributions between coupled spins is apparent in (b).

Doubly selective 1D gradient TOCSY–NOESY

One of the principle limitations to the use of the 1D selective NOESY experiments is the reliance on the presence of a well-resolved peak for selective inversion meaning any that are hidden are not amenable to investigation by these methods. One may have the option to change the solvent, which may advantageously alter chemical shifts and reveal the desired target resonance (see the example in Section 3.3.1), but when such means are ineffective or inappropriate, an alternative approach is to employ a *selective* transfer preparation step from a well-resolved resonance *onto* the overlapped target resonance so as to reveal this. This leads to a concatenated or doubly selective experiment for which a selective TOCSY preparation element is well suited to generating the initial transfer [36], this having been referred to in this context as the STEP (selective TOCSY edited preparation) function [37]. Thus, the TOCSY stage carries excited magnetisation onto the overlapped resonance (with which it shares a spin-coupled network), which is then itself laid bare for a further selective NOESY step, leading to the generation of NOEs from only this buried resonance, despite its apparent inaccessibility. A general scheme illustrating this concatenation based on the excitation sculpting method is shown in Fig. 8.45 in which the selective TOCSY experiment (Section 5.7.4) precedes the

Figure 8.43. The 1D DPGSE-NOESY sequence incorporating zero-quantum suppression. The mixing time contains non-selective 180° nulling pulses bracketed by opposing purging gradients spaced throughout as shown, followed by the ZQ filter.

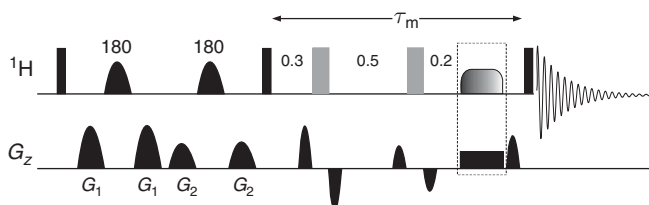


Figure 8.44. 1D DPGSE-NOESY spectra recorded (a) without and (b) with the zero-quantum suppression scheme of Fig. 8.43. The ZQC suppression employed a 20 ms adiabatic smoothed CHIRP pulse with a 40 kHz frequency sweep.

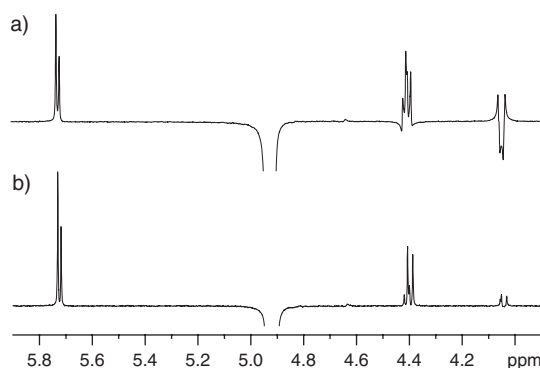
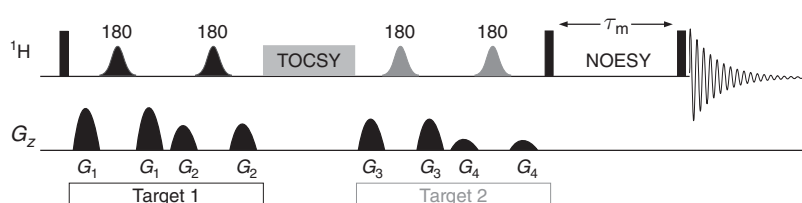


Figure 8.45. A general scheme for implementing the doubly selective 1D TOCSY-NOESY experiment for observing NOEs originating from hidden resonances. The selectivity of the 180° pulses may be optimised independently for the two target resonances 1 and 2.



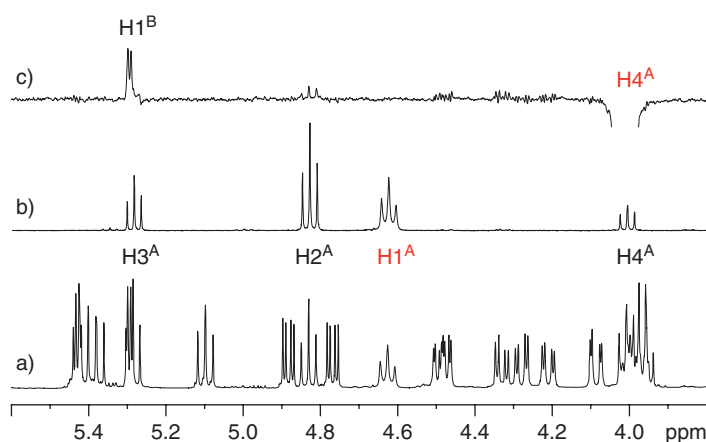
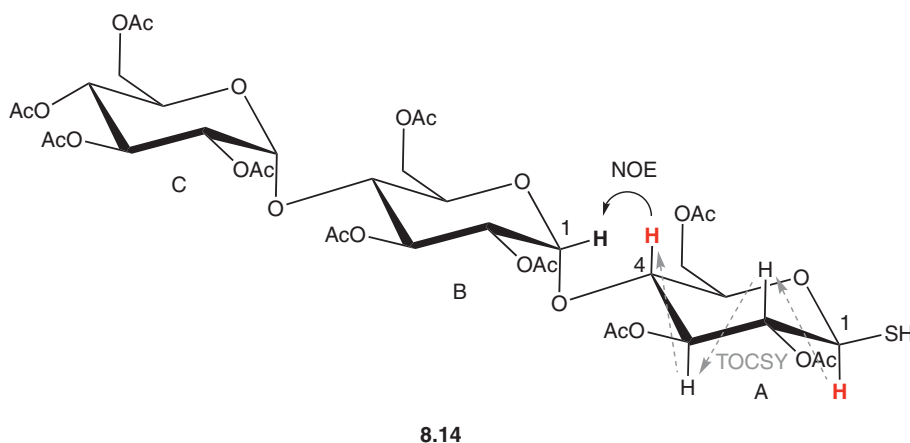


Figure 8.46. The application of a doubly selective 1D TOCSY-NOESY experiment to reveal NOEs from the buried $H4^A$ resonance of **8.14**. Trace (b) shows the 1D-TOCSY from the resolved $H1^A$ used in preparation to reveal the shift of $H4^A$ ($\tau_m = 80$ ms) and trace (c) shows the full TOCSY-NOESY combination selecting the target resonances (labelled in red) of $H1^A$ and $H4^A$ respectively ($\tau_m = 80$ ms TOCSY, 400 ms NOESY). Each transfer step included zero-quantum filtration as described above.

selective NOESY element. A more refined and fully optimised sequence incorporates the zero-quantum filter described above into both the TOCSY and NOESY mixing elements and includes 180° nulling pulses during the NOE mixing period, such a scheme being referred to as 1D STEP-NOESY [37]. Figure 8.46 illustrates the application of this method in allowing the selective observation of NOEs from the $H4$ proton of ring A across the glycosidic linkage to $H1$ of ring B in the trisaccharide **8.14**. Although the $H4^A$ proton lies hidden in a cluster of four resonances, its location is revealed clearly in a 1D TOCSY experiment (Fig. 8.46b). This resonance is then available for clean excitation in the second selective step of the 1D TOCSY-NOESY experiment (Fig. 8.46c).



Both the 1D gradient NOESY and the doubly selective counterpart may be modified through the use of *single* PFG spin-echoes in the selection step(s) to reduce the total sequence duration (Section 10.4.2), which may prove advantageous when dealing with larger molecules that have faster transverse relaxation rates. The doubly selective method may also substitute a ROESY mixing element in place of the NOESY mixing when such a scheme is to be preferred; see Section 8.8.

Interpreting 1D NOESY

The clear advantages of the gradient-selected NOE experiments over the conventional steady-state NOE difference means these have become a popular tools in small-molecule structural studies. However, despite their similar appearances, there are fundamental differences between the data presented by the two experimental protocols, with *steady-state* experiments observing *equilibrium* NOEs and *transient* experiments observing *kinetic* NOEs. As a consequence, 1D NOESY experiments demand a somewhat different approach to data interpretation over that adopted for steady-state NOE difference measurements, some of the key considerations include:

- The NOE enhancement will be acutely sensitive to the choice of mixing time and may vary markedly with changes in this.
- The NOE enhancement will also depend on the degree of target inversion, which may well be less than complete owing to pulse imperfections and other experimental shortcomings.

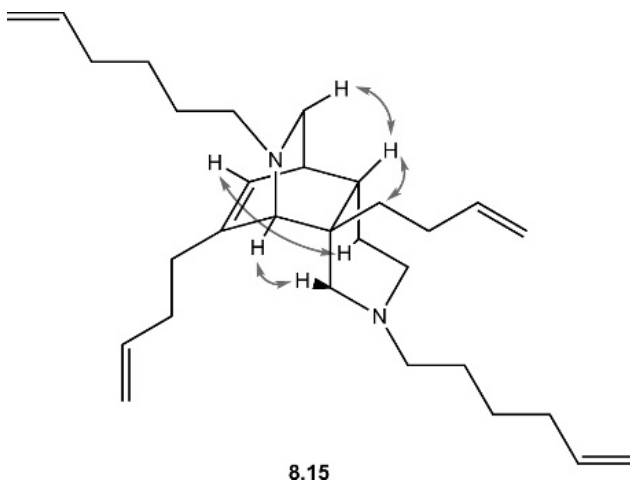
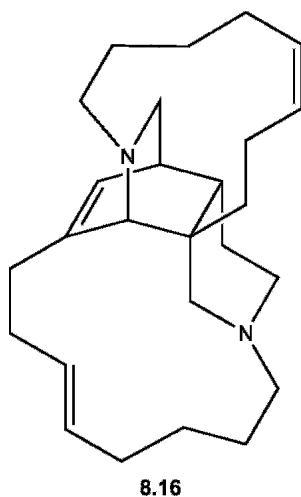
- Experimentally, the measurement of percentage enhancements is more complex. The target resonance cannot be used as a reference, as it conveniently is in steady-state difference spectra, since it is reduced by relaxation during τ_m . A reference spectrum with $\tau_m \approx 0$ s is required from which enhancements can be quantified and the degree of inversion of *each* target resonance must also be determined. The experimentally measured NOE enhancements must then be scaled accordingly for '100% inversion'.
- The *absolute* percentage enhancements from transient experiments will be smaller than steady-state enhancements, even though they may be clearer to see. The perception of what is considered a 'reliable' or 'measurable' enhancement must therefore be adjusted if percentages are reported.

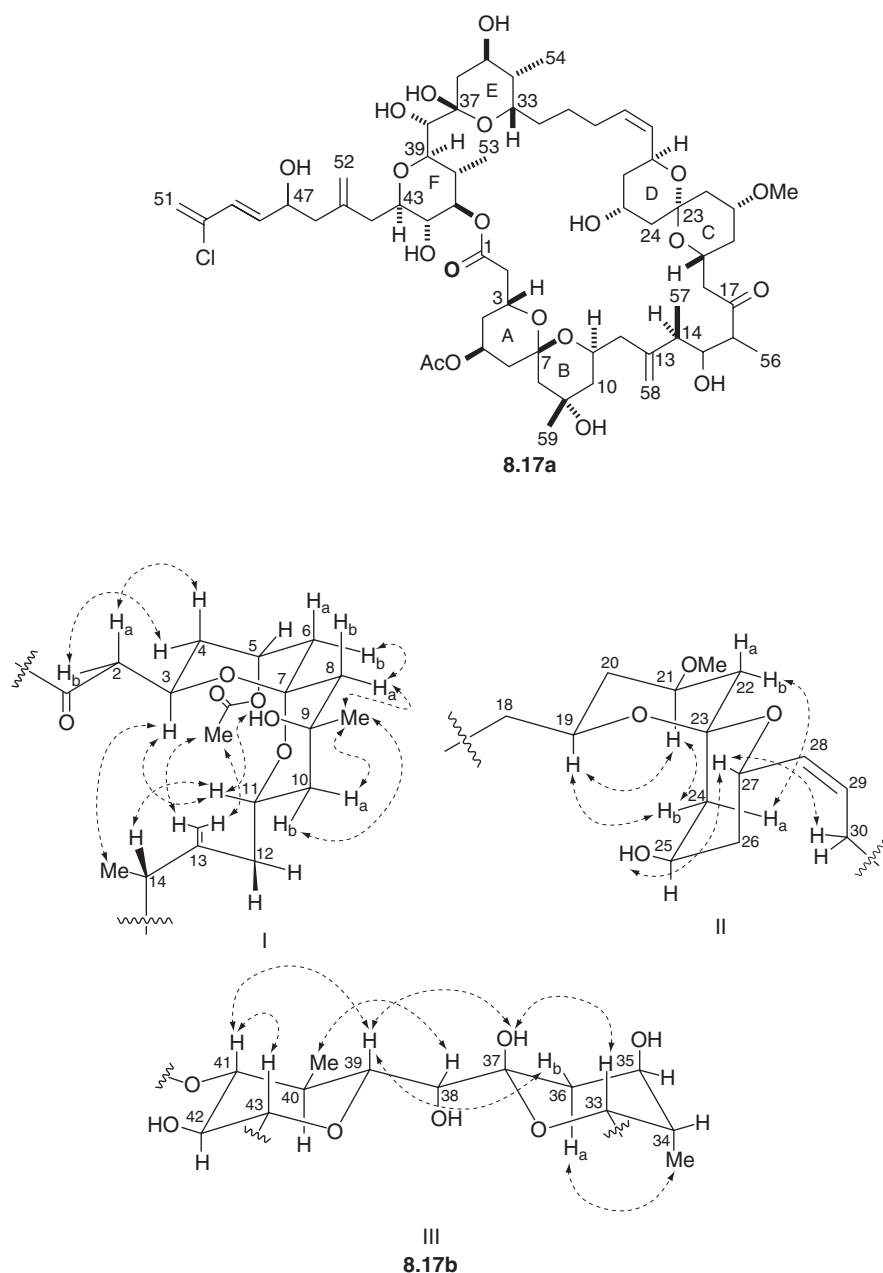
The experimental complexities associated with the correct measurements of percentage enhancements are likely to mean that these figures will not be routinely determined for the qualitative structural studies commonly undertaken (and anyone purporting to have measured these should give details of the approach taken). A more realistic approach to reporting results would seem to be that adopted in macromolecular NOE studies using semi-quantitative classification of enhancements (small, medium and weak). Assuming mixing times are kept short such that enhancements lie within, or at least close to, the linear growth regime, a comparison *within* each trace provides approximate relative distance relationships between the target spin and those exhibiting the enhancements. Piecing together data from a number of experiments to produce a self-consistent argument is again advisable.

8.7.3. Applications

Despite finding its origins largely in the hands (or labs) of biological spectroscopists, there is nowadays a widespread use of NOESY methods for structural and conformational analysis of the small to mid-sized molecules encountered in the chemical laboratory. The examples presented here demonstrate a variety of systems to which these experiments have been applied and also serve to illustrate some the benefits of using these in preference to the steady-state experiment previously encountered.

In the first example, 2D NOESY spectra were used to define the stereochemistry in the synthetic cycloadduct **8.15** [7], a potential biomimetic precursor to the naturally occurring marine-sponge alkaloid Keramaphidine B, **8.16**. This problem is essentially the same as that addressed for **8.7** above using the NOE difference experiment, but in this case, the additional unsaturated side chains caused extensive overlap in the proton spectrum and precluded the use of selective presaturation. Sufficient characteristic NOEs present in a 600 ms NOESY spectrum gave conclusive proof of the *endo* stereochemistry, as shown. Only positive NOEs were observed, consistent with a molecule of mass 436 daltons in chloroform. NOESY spectra have also been successfully applied to the structure elucidation of molecules for considerably greater mass and complexity, as illustrated by the cytotoxic macrolide cinachyrolide A, **8.17** [38], also from a marine sponge. The structure of the molecule was determined through extensive 600 MHz 2D NMR experiments, of which NOESY played a crucial role in defining the relative stereochemistry of the six oxane rings, as shown (reproduced with permission from reference [38], Copyright 1993, American Chemical Society). Mixing times of up to 700 ms were employed, and the relatively large mass of the molecule, approximately 1200 Daltons, may have been close to the cut-off for the successful observation of NOEs. Probably for this reason, supporting evidence also came from 200 ms ROESY spectra.





A common area in which 2D methods have been employed to help provide resonance assignments is in the study of peptides or small proteins, specifically when the same amino acids occur more than once in the peptide sequence. The so-called *sequential assignment* process is used to define the position of a specific amino acid residue within a peptide and relies upon the observation of NOEs between protons in adjacent residues (Fig. 8.47). Typically, these will be between an alpha proton and the amide NH of the following residue. The identification of neighbouring amino acids in this way can be used to string these units together, which may then be mapped onto the usually known peptide sequence. Once all residues have been sequentially identified, this can provide the basis for

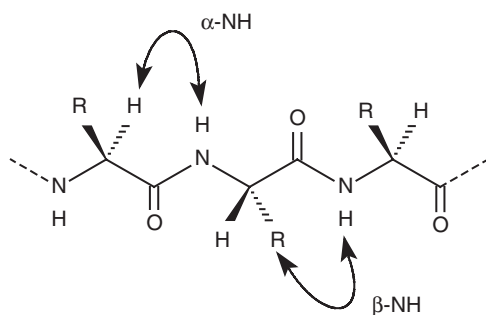
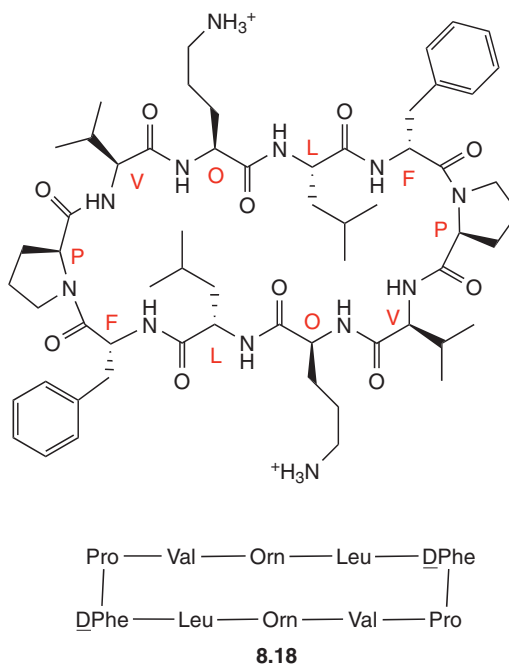


Figure 8.47. The intramolecular NOEs used to identify neighbouring residues in a peptide sequence.

conformational studies through longer range NOE contacts [39–41]]. As an illustration, the stepwise identification of neighbouring resonances is mapped in a NOESY spectrum for the cyclic decapeptide gramicidin-S **8.18** in Fig. 8.48.



Whilst most studies rely on the observation of intramolecular NOEs, intermolecular NOEs can also be used to define relationships between molecules that share close proximity. This can be of particular relevance in the study of ‘host–guest’ complexes where one wishes to determine how the guest sits within the host, and here scalar coupling information can play no direct part. One example of this is the characterisation of rotaxanes, extended conjugated systems sitting within protective encapsulating molecular jackets. The azo-dye rotaxane **8.19** uses α -cyclodextrin as the sheath molecule, which is inherently asymmetric with a smaller cavity entrance at the 5–6 rim of the molecule. The 1D gradient NOESY experiment described above was employed to map long-range host–guest NOEs [42], which differentiated the two ends of the complex and presented a picture of how the α -CD sat. NOEs from both HG’ and HI to the 3 and 5 protons on the inner face of the α -CD confirmed

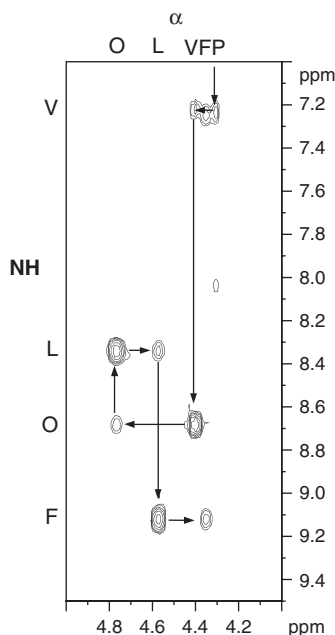
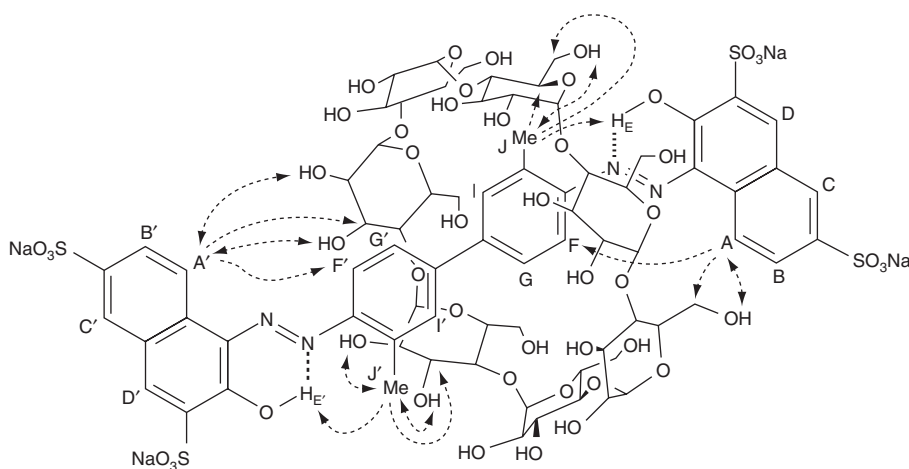


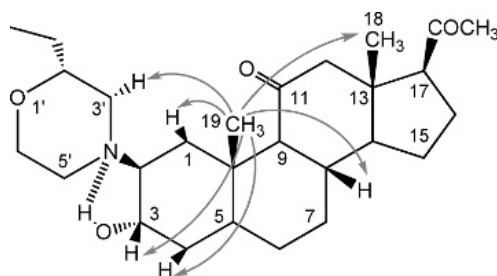
Figure 8.48. The sequential assignment process illustrated for the 300 ms NOESY spectrum of gramicidin-S **8.18** in DMSO, starting from the α -proton of proline. Inter-residue α H–NH NOES are often stronger than the intra-residue NOEs in an extended backbone conformation.

that the central portion of the guest was encapsulated within the hydrophobic cavity (structure reproduced with permission from reference [42], Copyright Wiley-VCH Verlag GmbH & Co. KGaA.). All NOEs for these molecules in DMSO were negative due to the limited mobility of these bulky complexes.



8.19

Both 1D gradient and 2D NOESY experiments have also been used to assess the conformations of anaesthetic steroids [43]. These possess crowded proton spectra requiring the use of 2D experiments, and mean 1D methods can only be applied to a limited set of resonances, which nevertheless prove significant. In **8.20** in particular, long-range NOEs in the 1D experiment were observed from methyl 19 specifically to the H3' equatorial proton of the attached morpholine ring but not to other protons within this moiety. This indicated free rotation of the ring was restricted (by an intermolecular hydrogen bond) and that it occupied a fixed position relative to the steroid skeleton. Knowledge of such conformations proves useful in elucidating the mechanisms by which these molecules cause anaesthesia.



8.20

The final example specifically illustrates some advantages of the 1D gradient NOESY experiment over the conventional NOE difference method. One goal of this work was to compare the solution structure of the diphenylallyl palladium complex **8.21** with the crystal structure and from this derive mechanistic insights [44]. The proton spectrum was assigned through combined analysis of phosphorus-decoupled proton, DQF-COSY and ROESY spectra recorded with a 500 ms mixing time. Specific close proximities were also probed through 1D NOE methods where suitably resolved resonances were available, selected examples of which are shown in Fig. 8.49. Some specific points are worthy of comment, the first of which is the clarity in the NOE spectra that are not confused by difference artefacts, thus giving one confidence that the observed enhancements are genuine. Secondly, these spectra were collected in only 14 min each, whilst the equivalent difference experiments required data collection over many hours to adequately suppress resonances in the cluttered aromatic region. Finally, notice the enhancements of the broad resonance at 7.4 ppm, associated with the *ortho* protons of the dynamically restricted phosphine-Ph¹ group. These were distinctive and highly informative but could not be reliably distinguished in either the 1D difference or the 2D experiments. The ability to extract data of this sort with confidence makes the 1D gradient NOESY experiment an extremely powerful tool in modern structure elucidation.

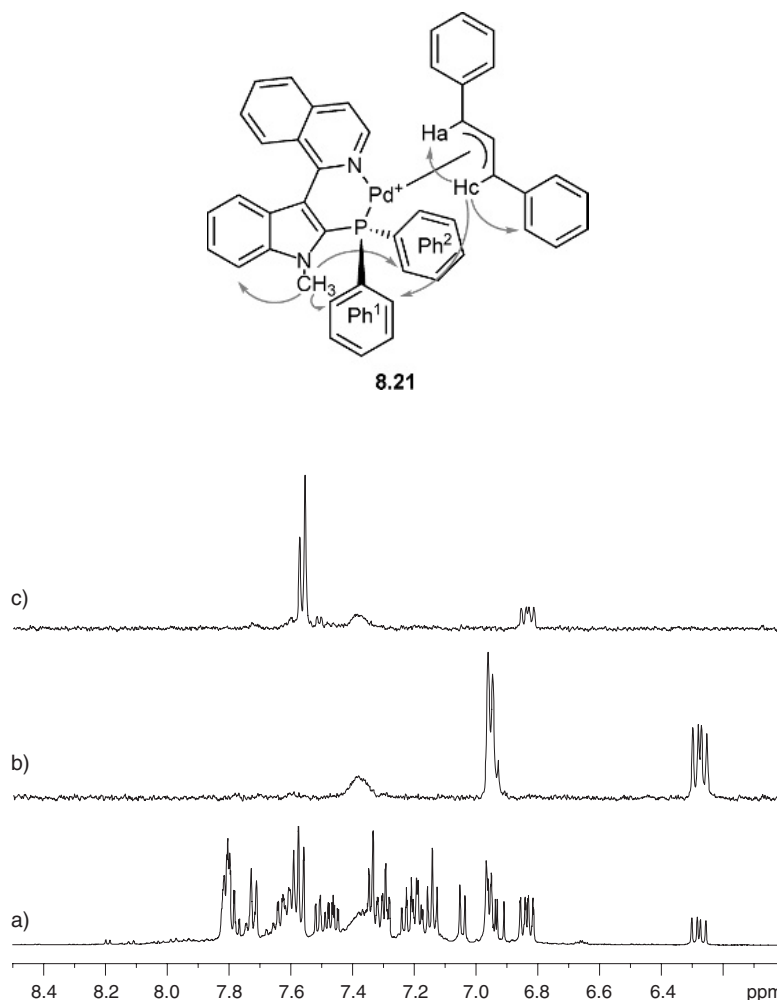


Figure 8.49. Selected 1D gradient NOESY spectra of the palladium complex **8.21** recorded with a mixing time of 800 ms in each case. (a) Parent 1D spectrum, (b) Hc selected (not shown) and (c) N-Me selected (not shown).

8.7.4. Measuring chemical exchange: EXSY

Although the mechanisms of chemical exchange and the NOE are quite unrelated, they share in common the transfer of longitudinal magnetisation and as such can be detected with the same 1D or 2D NMR experiments. In fact, the NOESY and EXSY [45] pulse sequences are one and the same and 1D transient NOE sequences can equally well be applied to the measurement of rate constants [46–48]. Furthermore, the presaturation sequence used for the 1D steady-state experiment also serves as a simple method to provide evidence of slow conformational exchange processes, particularly in proton spectra, by virtue of saturation transfer effects, provided the exchange is fast relative to the spin relaxation rates. The practical difference lies in the EXSY dependence on exchange rates rather than spin relaxation rates. Before proceeding, these experiments are now considered in the context of observing exchange processes. Some basic understanding of the exchange phenomenon is assumed here; see, for example, Hore [49] Chapter 4, or Gunther [50] Chapter 9. More extensive discussions of 1D and 2D methods for studying dynamic processes may be found in reviews [51–54] and in dedicated texts [55,56].

In parallel with studies of the NOE, exchange experiments can be used both qualitatively, to map exchange pathways, and quantitatively to determine rate constants. In systems of multisite exchange, the 2D EXSY experiment proves particularly powerful in the measurement of these for all pathways. However, for either application, the exchange processes studied must be slow on the NMR chemical shift timescale since the exchanging resonances must be resolved in order to observe transfer between them. Furthermore, this exchange must not be too slow, otherwise relaxation processes occurring during τ_m will remove all memory of the exchange process. Thus, magnetisation transfer experiments are suitable for only a limited range of exchange rates and as a rule of thumb, these must be at least comparable to the longitudinal relaxation rates ($k_{ex} \geq 1/T_1$). In practice, this means these experiments are sensitive to exchange process with $k_{ex} \approx 10^2 - 10^{-2} \text{ s}^{-1}$. The study of

different nuclei provides a window on different rates within this range due to differences in relaxation times, with slower relaxing spins able to probe slower exchange processes. Fast exchange processes that lead to resonance coalescence can be studied by lineshape analysis (often referred to as 'bandshape analysis') [48,52,55], which generally requires the use of computer simulation [57]. As an illustration of the 2D method, Fig. 8.50 shows the boron-11 EXSY spectrum of a 1:1 mix of BCl_3 and BBr_3 . The exchange crosspeaks demonstrate the presence of ligand scrambling processes and indicates the mechanism involving the exchange of a single halogen atom only [58]. A quantitative analysis of these data also provided (pseudo) first-order rate constants for each exchange process (see below).

The correct choice of the mixing time, τ_m , is again important for the successful application of EXSY and is dependent on the exchange rate constants. Since these are unlikely to be known with any accuracy (since there would be little point in performing the experiment if they were) and as there may exist a spread of these within the kinetic system being studied, some compromise is again required. In the absence of known rates, an upper limit can be defined (at least for the case of symmetrical two-site exchange) from the knowledge that the rate constant at coalescence, k_c , is given by $\pi\delta\nu/\sqrt{2}$, where $\delta\nu$ is the shift difference (in Hz) in the slow exchange limit, and hence $k_{\text{ex}} < k_c$. In qualitative work, the aim is to achieve maximum crosspeak intensity and setting $\tau_m \approx 1/k_{\text{ex}}$ is recommended. Under such compromise conditions, multistep transfers may operate when suitable pathways are possible, producing 'indirect' exchange peaks, which may be confused with direct peaks from relatively slow processes. For example, this has been observed for the ligand scrambling reactions of tin halides [59,60], boron halides [58] and of lead(IV) tetracarboxylates [61]. Often one has some element of control over the exchange rates through the use of temperature variation, and optimisation of this can also assist in the collection of exchange spectra.

The choice of τ_m for quantitative work will be dictated by the method used to calculate k_{ex} , of which there are essentially two widely used approaches [58]. The simplest involves collecting EXSY spectra over a range of τ_m values and following the initial linear growth of exchange crosspeaks (the initial rate approximation again, by exact analogy with the approach taken for NOESY analysis). The great disadvantage to this is clearly the need to collect many 2D spectra, which may place unreasonable

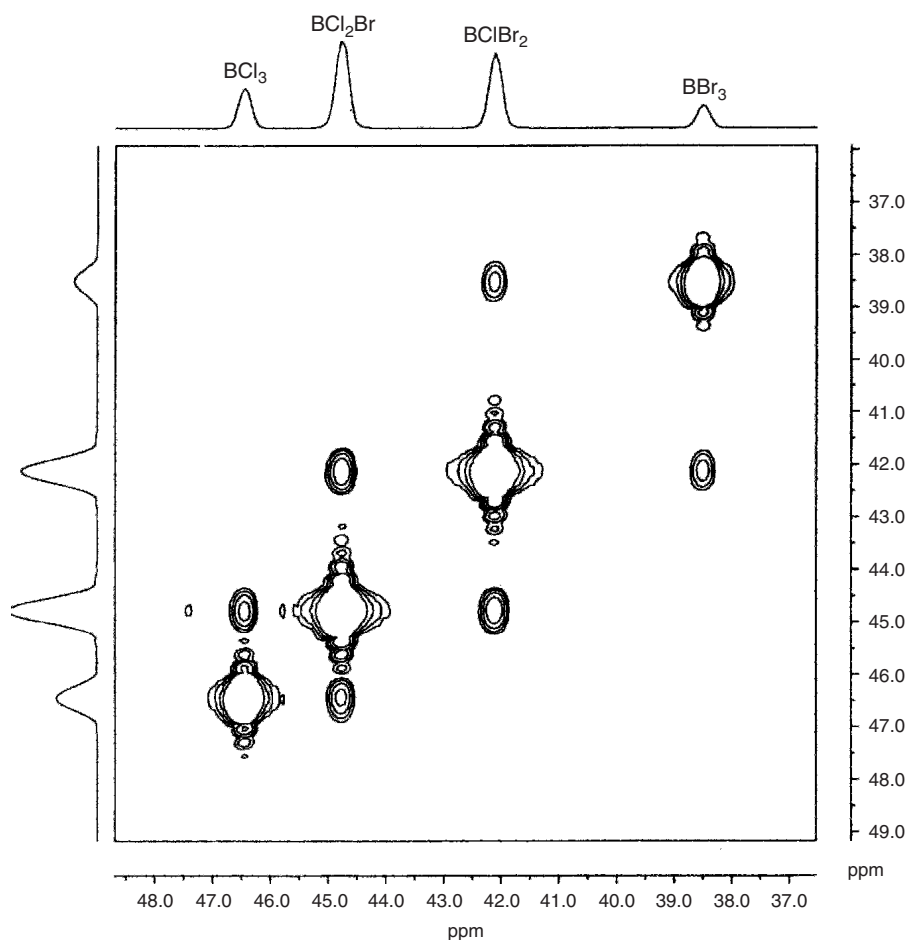


Figure 8.50. The 128 MHz boron-11 2D EXSY spectrum of a 1:1 mix of BCl_3 and BBr_3 at 400 K. The spectrum was recorded with a mixing time of 50 ms (reproduced with permission from [58], Copyright 1991, Elsevier).

demands on instrument time. The second approach is to determine rate constants from a single EXSY spectrum and for this the appropriate choice of τ_m is absolutely critical; too small and crosspeaks will have weak intensities, which are subject to significant error, too long and the intensities become insensitive to the kinetic parameters. Methods for determining the optimum τ_m in such cases have been described [62,63]. Having obtained suitable data, computational analysis (e.g. with the program D2DNMR [64]) may be employed to calculate exchange rates for multisite systems. For the simpler case of equally populated 2-site exchange, explicit equations have been presented for the estimation of rate constants from diagonal and crosspeak intensities [65]. Studies involving low natural abundance nuclei such as ^{13}C or those that are present only sparingly in a molecule (e.g. ^{31}P) have the significant benefit of avoiding potential complications from ^1H - ^1H NOE effects that may be operative. Dynamic proton studies may be subject to such interferences when exchange rates and NOE build-up rates are comparable and in situations where molecular correlation times are such that negative NOEs are produced, the differentiation of exchange and NOE peaks may be problematic owing to them displaying the same sign. In this case, the use of the ROESY experiment described below is advantageous since NOEs will appear with opposite sign to both the diagonal and the exchange peaks, although quantification of kinetic parameters can be more complex due to interfering effects from TOCSY transfers and from offset-dependent crosspeak intensities.

1D and 2D magnetisation exchange experiments have found greatest use in the study of inorganic and organometallic systems, for which a high degree of fluxionality often exists, and extensive reviews of these areas have been presented in which a wide variety of example applications may be seen [52,54,66,67]. These methods have also found application to the study of supramolecular complexes [68].

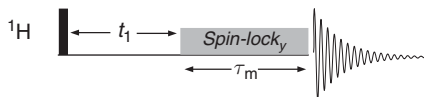
8.8. MEASURING ROTATING-FRAME NOEs: ROESY

The benefits of recording ROEs have been described in earlier sections, and stem from the fact that they are positive for all molecular tumbling rates and hence all molecular sizes, and prove particularly advantageous in the study of mid-sized molecules that exhibit very small conventional NOEs owing to their motional properties ($\omega_0\tau_c \approx 1$). Since the observation of an ROE involves the measurement of a transient enhancement, their application and interpretation largely parallels that for NOESY and need not be repeated here. The principal concern with ROESY experiments (originally termed CAMELSPIN (cross-relaxation appropriate for minimolecules emulated by locked spins) [69] owing to the similarity of the motion of a figure skater during this manoeuvre and the behaviour of magnetisation vectors during the experiment) is the fact that they are susceptible to a number of processes other than cross-relaxation and hence may contain a variety of interfering and potentially confusing responses, in addition to the desired ROEs. An appreciation of these effects, and how to deal with them, is therefore mandatory for anyone wishing to employ these methods, and some caution is required in the application of ROE experiments.

8.8.1. The 2D ROESY sequence

The original and simplest ROESY sequence (Fig. 8.51) has the mixing period defined by the duration of a continuous, low-power rf spin-locking pulse during which the ROE develops. Magnetisation not parallel to the spin-lock axis is dephased by rf field inhomogeneity (Fig. 8.52), so the only phase cycling required is that for axial peak suppression and f_1 quadrature detection. Gradient-selected variants have also been presented [20]. For small and mid-sized molecules, the selection of τ_m follows that presented for NOESY with, typically, $\tau_m \leq 600$ ms, whereas for large molecules in the spin-diffusion limit, shorter values are more appropriate since the ROE growth rate is twice that of the NOE (Section 8.5). The 2D spectrum maps through-space interactions through crosspeaks that have *opposite* phase to the diagonal.

Figure 8.51. The 2D ROESY sequence. The mixing time, τ_m , is defined by the duration of the low-power spin-lock pulse.



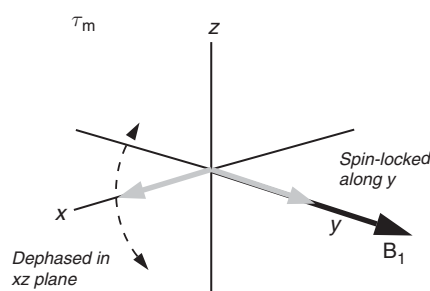


Figure 8.52. The ROESY spin-lock. During this, magnetisation parallel to the B_1 field remains spin-locked whereas orthogonal components are driven about this (here in the xz plane) and eventually dephase through B_1 field inhomogeneity.

Complications with ROESY

As stated previously, crosspeaks may arise in ROESY spectra as a result of processes that occur during the spin-lock other than cross-relaxation between spins. The principal complications that can arise are [70]

- COSY-type crosspeaks between J -coupled spins
- TOCSY transfers within J -coupled spin systems, and
- crosspeak attenuation from rf off-resonance effects.

The first of these arises when the long spin-lock pulse acts in an analogous fashion to the last 90° pulse of the COSY experiment, so causing coherence transfer between J -coupled spins. The resulting peaks display the usual anti-phase COSY peak structure and tend to be weak and so are of least concern. A far greater problem arises from TOCSY transfers that arise because the spin-lock period in ROESY is similar to that used in the TOCSY experiment (Section 5.7). This may, therefore, also induce coherent transfers between J -coupled spins when these experience similar rf fields, that is, when the Hartmann–Hahn matching condition is satisfied. Since the ROESY spin-lock is not modulated (i.e. not a composite pulse sequence), this match is restricted to mutually coupled spins with similar chemical shift offsets or to those with equal but opposite offsets from the transmitter frequency. These conditions are often met in natural products such as carbohydrates, nuclei acids, peptides, alkaloids and steroids for which resonances cluster about the centre of the spectrum. In addition to unwanted direct TOCSY effects, multistep transfers involving both TOCSY and ROE stages can lead to ‘false’ ROEs, crosspeaks that, *a priori*, appear to arise from a ROE between two correlated spins but in fact do not [71]. Thus, for example, a TOCSY transfer from spin A to B followed by ROE transfer from B to C in the system of Fig. 8.53 would produce an apparent crosspeak suggesting a ROE between A and C.

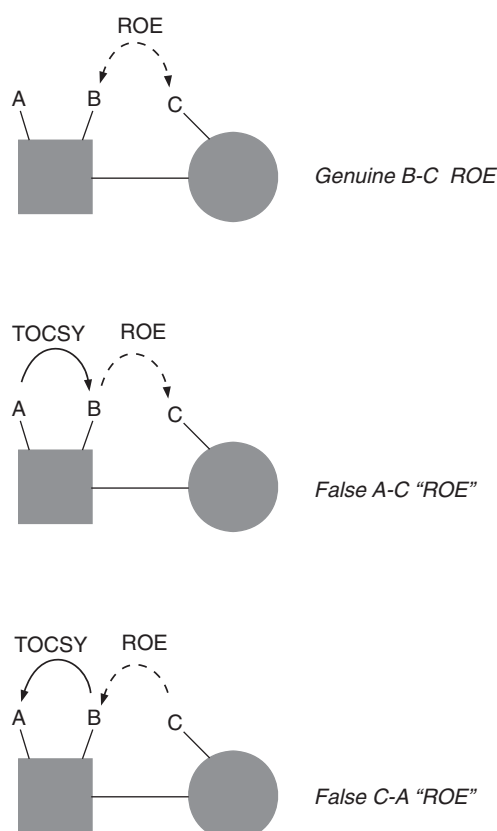


Figure 8.53. The generation of ‘false’ ROE peaks in ROESY spectra may arise from combined ROE and TOCSY mechanisms.

Table 8.5. Responses observed in the ROESY experiment. By convention the diagonal is phased to be positive and the signs of all other signals are given relative to this

Sign of diagonal	Origin of crosspeak	Sign of crosspeak
Positive	direct ROE	Negative
	indirect ROE (3-spin effect)	Positive (weak)
	TOCSY	Positive
	TOCSY-ROE	Negative (false ROE)
	ROE-TOCSY	Negative (false ROE)
	Chemical exchange	Positive
	COSY-type	Anti-phase/mixed phase

ROE, rotating-frame nuclear Overhauser effect.

Likewise, the reverse ROE-TOCSY steps would produce a similar signal, but the conclusion would be wrong in either case. Since TOCSY transfer retains signal phase, its involvement in crosspeak generation is not obvious and can lead to drastically incorrect conclusions. Furthermore, if TOCSY and ROE transfers occur simultaneously between two spins, the ROE peak may be reduced in magnitude or even cancelled owing to opposite peak phases. The various transfer pathways that may occur during ROESY are summarised in Table 8.5.

Considerable attention has been given to ways of avoiding TOCSY transfer during ROESY, the simplest of which is through limiting the Hartmann–Hahn match with judicious positioning of the transmitter frequency to ensure coupled spins either side of this are not symmetrically disposed [72]. Alternatively, recording two spectra with differing transmitter offsets leaves genuine ROE peaks little changed but should significantly alter the intensities of those involving a TOCSY step and so facilitate their identification [71]. Modification of the spin-lock itself also leads to a reduction in TOCSY efficiency (Fig. 8.54). Using low-power rf (Fig. 8.54a) with γB_1 comparable to the maximum resonance frequency offset [70] (typically 2–3 kHz) or a pulsed spin-lock [73] (Fig. 8.54b) offer similar attenuation [74], neither of which is complete in reality.² More effectively, an alternating-phase spin-lock (Fig. 8.54c) has been shown to be effective at reducing TOCSY transfer [75–77] and has been termed Tr-ROESY (transverse ROESY). This approach destroys the Hartmann–Hahn match between coupled spins, so suppressing TOCSY transfer, and measures an average of the ROE and NOE since the magnetisation vectors spend time in both the transverse plane and along the longitudinal axis as they follow a swinging ‘tic-toc’ trajectory (Fig. 8.55). The drawback is a potential reduction in crosspeak intensity relative to conventional ROESY due to this averaging process. In small molecules ($\omega_0\tau_c \ll 1$) there is no theoretical loss, this becomes a factor of 2 for mid-sized molecules ($\omega_0\tau_c \approx 1$) and a factor of 4 for very large molecules ($\omega_0\tau_c \gg 1$). Hence there is something of a compromise for molecules in the $\omega_0\tau_c \approx 1$ region for which rotating-frame measurements prove most beneficial. This method generally requires γB_1 to be twice the maximum resonance frequency offset (γB_1 typically 4–6 kHz), and its success is highly dependent on the use of accurately calibrated 180° pulses. The ability of this scheme to suppress interference from the TOCSY mechanism is illustrated in Fig. 8.56 for a tetrameric carbopeptoid **8.22** in which the disappearance of both TOCSY and false-ROE peaks alongside the revelation of genuine ROEs in (b) is clear.

The third complicating factor specific to ROESY is the attenuation of crosspeak intensities as a function of resonance offset from the transmitter frequency [78]. Off-resonance spins experience a spin-lock axis that is tipped out of the x – y plane (Section 3.2.1) resulting in a reduction in observable transverse signal in addition to a reduction in cross-relaxation rates. This is more of a problem for quantitative measurements, although fortunately mid-sized molecules show the weakest dependence of ROE cross-relaxation rates on offset. The so-called compensated ROESY sequence [78] eliminates these frequency-dependent losses should quantitative data be required.

8.8.2. 1D ROESY sequences

In parallel with the 1D NOESY sequences above, the 2D ROESY experiment also has its 1D equivalent (in fact, this was the original ROE experiment [69]) and gradient-selected analogues [36,79,80] all of which incorporate selective excitation of the target spin such as via the DPFGE selection described for 1D NOESY above [81]. These can be derived from 1D NOESY sequences by incorporation of a suitable spin-lock in place of the 90° – τ_m – 90° segment of NOESY, and thus require no further elaboration.

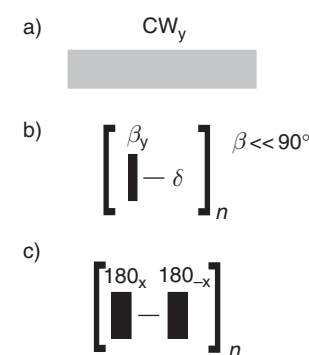


Figure 8.54. Practical mixing schemes for the ROESY experiment. (a) A single, low-power pulse, (b) a pulsed spin-lock comprising a repeated sequence of a small tip angle pulse followed by a short delay, and (c) the Tr-ROESY alternating-phase spin-lock.

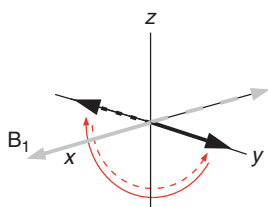


Figure 8.55. The swinging ‘tic-toc’ motion of spin-locked vectors during the Tr-ROESY mixing sequence.

² Considerable care should be taken in the rf power used for the spin-lock as damage to transmitters and/or probes may result if high powers are applied for excessively long periods.

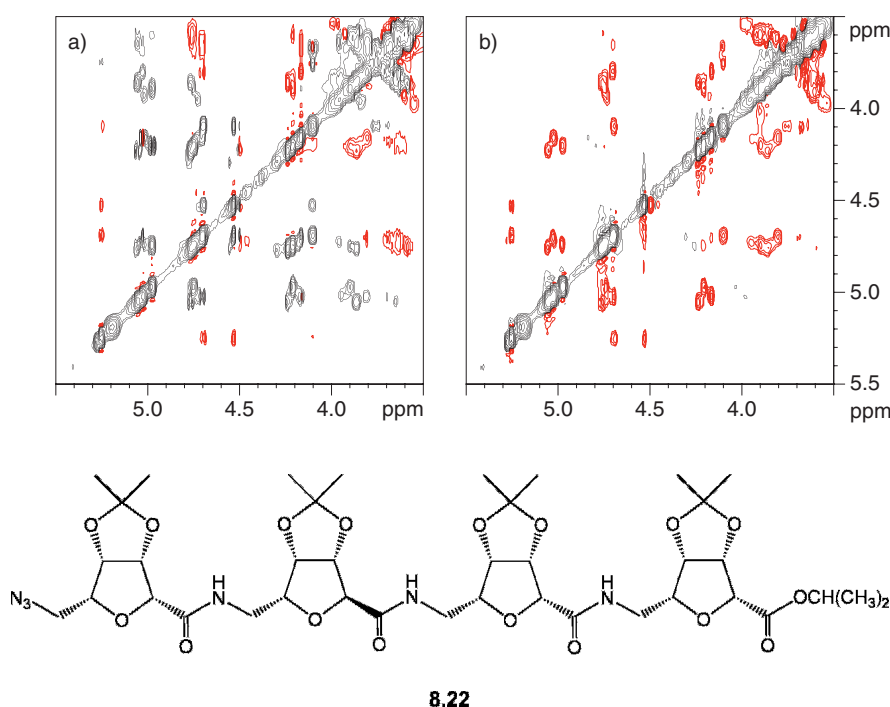


Figure 8.56. ROESY spectra of a tetrameric carbopeptoid **8.22** recorded with (a) a single 2.6 kHz continuous spin-lock pulse and (b) a 3.7 kHz phase-alternating Tr-ROESY spin-lock at 4.5 ppm. Spectrum (a) is dominated by TOCSY peaks that share the same phase as the diagonal, whereas these have been largely suppressed in (b), so revealing the genuine ROE peaks.

8.8.3. Applications

In view of the various complicating factors associated with the ROESY experiment, it is perhaps prudent to avoid using the technique whenever possible, and instead select a steady-state or conventional transient experiment as first choice. Certainly for small molecules within the extreme narrowing limit, there is no theoretical difference between NOEs and ROEs, so there seems little to be gained from undertaking potentially more complex ROE investigations. Nevertheless, situations arise when the use of rotating-frame measurements are unavoidable because conventional experiments yield negligible enhancements ($\omega_0\tau_c \approx 1$), and it is not surprising that most applications of ROESY have involved larger molecules, notably macrocyclic natural products, peptides, oligosaccharides and host–guest complexes, as illustrated by the selected examples below.

In the structure elucidation of the cytotoxic dimeric steroid crellastatin A [82] **8.23**, the basic skeleton of the molecules was derived principally from numerous long-range proton–carbon correlations observed in HMBC spectra. The relative stereochemistry of the bicyclic system at the junction of the two steroid units was derived from analysis of ROESY spectra recorded at 600 MHz with a 400 ms mixing time, some key enhancements being illustrated. The final configuration at C22' was determined by comparison of the lowest energy molecular mechanics conformers of the two possible stereoisomers with opposite configurations at this position. Only the C22'-R configuration placed the 21' and 26' methyl groups in the proximity required for the observation of an ROE between them. The large mass of the molecule, 933 daltons, no doubt precluded the use of NOESY measurements, and the literature contains many examples of this sort where rotating-frame measurements have played a crucial role in characterising novel natural products. Conformational studies of peptides have also benefited from the ROESY experiment since it is usually larger peptides that possess defined conformations and hence potentially interesting pharmacological properties. Studies of oligomers of synthetic β -peptides have now identified various sequences with helical structures in solution that have been extensively characterised by NMR. In the sequence **8.24**, distinctive ROEs were observed between the amide proton of residue i and the H^β protons of residues $i+2$ and $i+3$ along the sequence and were characteristic of the structure adopted by the peptide [83]. These and additional ROEs were used to define 18 distance constraints for use within molecular dynamics protocols by classifying their intensities as weak, medium and strong (Section 8.7.1), enabling the conformation of the hexapeptide to be calculated. The 14 lowest energy structures derived from this are shown in Fig. 8.57 and clearly illustrate the well-defined helical structure adopted in pyridine. In a second example, ROEs were used in a similar manner to define the structure of a hexameric *cis*-oxetane-derived β -peptide **8.25** as a left-handed helix stabilised by 10-membered intramolecular hydrogen bonds [84] (Fig. 8.58). Characteristic $i-i+2$ ROEs were observed along the sequence (only one set is illustrated on the structure), and the hydrogen bonds suggested by these patterns were consistent with the amide NH stretches observed in infra-red spectra.

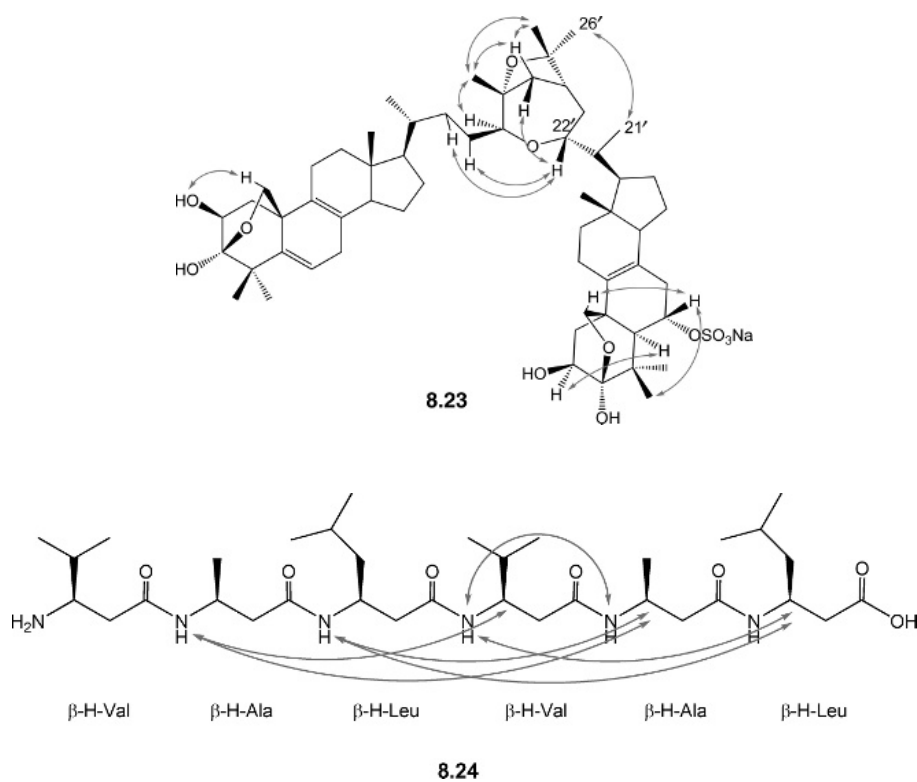


Figure 8.57. Stereo representations of the 14 lowest energy calculated structures of the hexameric β -peptide **8.24** based on distance restraints derived from ROESY spectra. Side and top views are shown in (a) and (b) respectively, with the side chains of the β -amino acids omitted for clarity (reproduced with permission from [83], Copyright Wiley-VCH Verlag GmbH & Co. KGaA.).

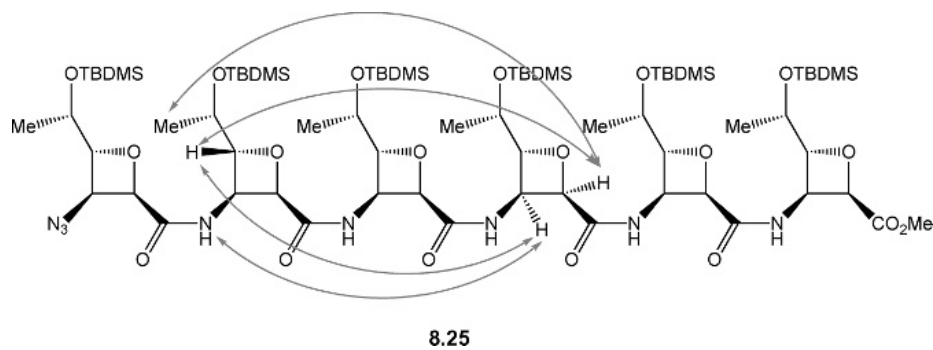
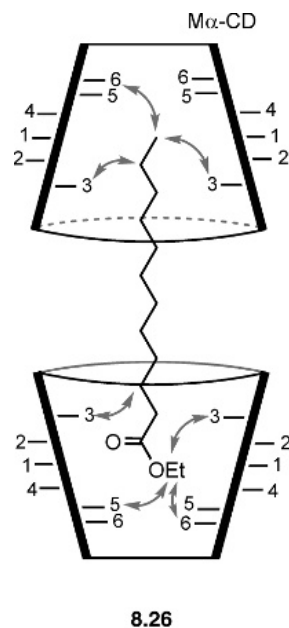
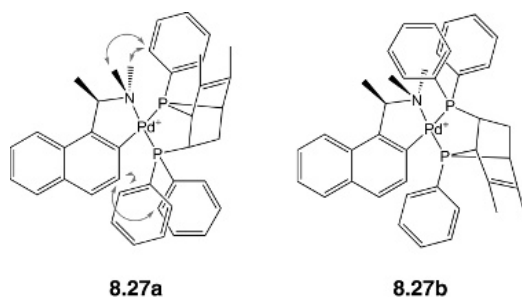


Figure 8.58. Helical structures of 10 overlaid low-energy conformers derived for the hexameric β -peptide **8.25** shown as side-on (left) and top-down (right) views. The *t*-butyldimethylsilyl (TBDMS) protecting groups are not shown beyond the silicon atom. These figures appear in colour on the back cover.

Host–guest inclusion complexes are also frequently restricted to study by rotating-frame measurements because of the requirement for a sufficiently large host to be able to accommodate the guest, and one of the best studied host systems appears to be the cyclodextrins, CD (see structure **8.10** above). One such investigation was into the inclusion of constituents of the natural sex pheromone mix of the olive fruit fly as a means of controlling the release of these volatile substances [85]. Characterisation of the complexes so formed, such as ethyl dodecanoate encapsulated within permethylated α -CD ‘cups’ **8.26**, was necessarily made from intermolecular ROEs since NOESY enhancements were negligible. Interference from TOCSY transfer was also evident in this work in the form of false ROEs from the guest molecules to the H4 protons of the cyclodextrin. Since these protons sit on the outer face of the CD cup, the ROEs are unlikely to arise from direct effects but instead are produced by transfer of the genuine ROEs between the guest and the inner H3 and H5 protons. Rotating-frame measurements have also been used extensively in the characterisation of relatively bulky organometallic complexes, such as **8.21** in section 8.7.3 above. A second example is the definition of the absolute configuration of the P-chiral diphosphine, [86] **8.27**. One aspect of this was the identification of the complex as either regio-isomer **8.27a** or **8.27b** prior to further investigation, and this was shown to be **8.27a** through the observation of ROEs from the phenyl rings, as shown. Analysis of the ROESY data further distinguished between two possible diastereomers and so defined the absolute stereochemistry.



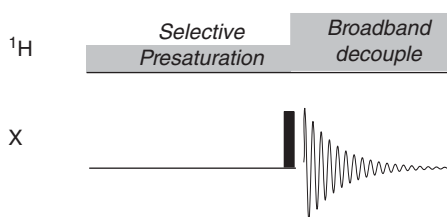
8.9. MEASURING HETERONUCLEAR NOEs

Beyond its general use as a means of sensitivity enhancement of spin- $\frac{1}{2}$ nuclei with low magnetogyric ratios, the specific heteronuclear NOE has occasionally been used as a tool in structural studies [87] and is capable of providing a unique source of structural information in favourable circumstances. Techniques for its observation largely parallel those for homonuclear experiments in the form of 1D steady-state difference and 2D transient experiments, so there is nothing fundamentally new to understand here. The main limitation with these approaches is often the low sensitivity associated with the observation of the low- γ spin, meaning heteronuclear NOEs tend to be far less used than their homonuclear proton counterparts. The most widespread applications have involved the ^1H – ^{13}C NOE, in which the proton is saturated and the heteroatom observed, the ^1H – ^6Li and ^1H – ^7Li NOE and increasingly the ^1H – ^{19}F NOE, notably in the context of ion-pair associations. In the case of ^{13}C , the relaxation of a proton bearing carbon is dominated by its dipolar interactions with this proton, meaning only quaternary carbons tend to show useful specific long-range NOEs. Furthermore, the selective irradiation of a proton resonance is often restricted to the parent ^{12}C line leaving the ^{13}C satellites in the spectrum unaffected, which means the ^{13}C centre associated with the target proton usually does not show an enhancement under these conditions. The lithium isotopes are somewhat exceptional in that they are both quadrupolar, yet their small quadrupole moments allow dipolar relaxation and hence the NOE to remain operative. Lithium-6 in particular exhibits the lowest quadrupole moment of any quadrupolar nucleus, with caesium-133 being the only other quadrupolar nucleus to give measurable NOEs [88].

8.9.1. 1D Heteronuclear NOEs

The 1D NOE difference sequence (Fig. 8.59) is essentially identical to the homonuclear equivalent, except for the addition of broadband proton decoupling during the acquisition. Since this generates non-specific NOE enhancements, a sufficient recovery period must be left between transients to allow this to decay. The presaturation period is now dictated by the potentially long relaxation times of the heteroatom, but beyond this consideration,

Figure 8.59. The classic sequence for measuring 1D steady-state heteronuclear NOE enhancements.



similar steps are required for optimisation as for the homonuclear experiment. Problems associated with selective presaturation of the target proton resonance may be handled in a similar fashion, although here SPT interferences are suppressed by the use of broadband decoupling. A 1D gradient-selected transient heteronuclear NOE experiment has also been presented that makes use of selective excitation in the generally better dispersed carbon dimension and which uses proton detection for enhanced sensitivity [89]. This also demonstrates superior signal suppression through gradient selection and provides a useful route to the detection of these transient NOEs. In this approach, it is NOEs from the ^{13}C centres onto the observed protons that is employed and thus does not exactly parallel the 1D steady-state experiment for which the protons are the source of the NOE.

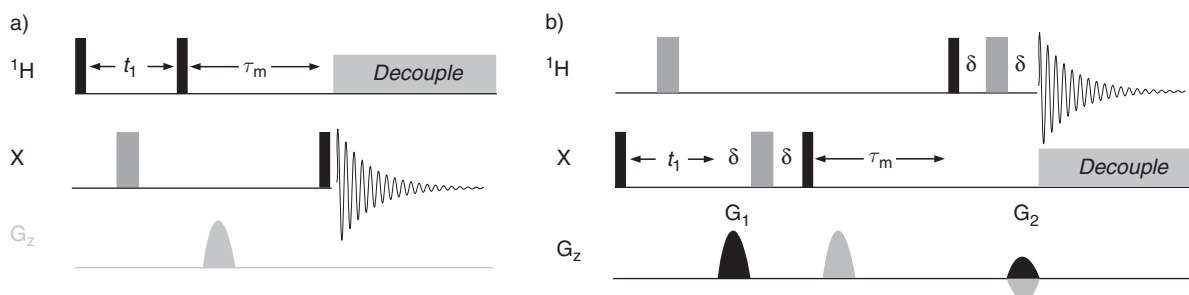
8.9.2. 2D Heteronuclear NOEs

Increasingly when heteronuclear NOEs are employed, their observation is through the use of 2D experiments for the detection of transient enhancements, avoiding the need for any form of selective irradiation. The traditional approach utilised the observation of the lower- γ heteroatom (X) in detecting NOEs from the proton onto X, denoted $\text{X}\{^1\text{H}\}$, whereas more recent sequences have adopted the 'inverse' approach and have made use of proton detection in particular, $^1\text{H}\{\text{X}\}$. This latter approach offers two possible benefits. Firstly there is the potential for enhanced sensitivity by observing the proton. However, in this it is the $\text{X} \rightarrow ^1\text{H}$ NOE that is detected which is intrinsically weaker than the equivalent $^1\text{H} \rightarrow \text{X}$ transfer and the actual sensitivity gain expected from proton detection arises from a balance between these effects (see discussions in reference [89]) although some sensitivity advantage is expected in most cases. Secondly, and often more importantly, there is the ability to acquire the typically more crowded proton dimension at higher resolution by observing this directly and frequency labelling the heteroatom in the indirect dimension.

The classic 2D sequence [90,91], referred to as HOESY (heteronuclear Overhauser spectroscopy, Fig. 8.60a,) parallels that of homonuclear NOESY, with the additional 180° pulse during t_1 serving to refocus the $^1J_{\text{XH}}$ coupling evolution and hence provide X-nucleus decoupling in f_1 . The use of an optional purging gradient during the mixing time τ_m serves to eliminate residual transverse components during this period. Equations have been presented to allow an estimation of the optimum τ_m for observing structurally informative long-range NOEs to non-protonated nuclei [92]. For the case of the ^1H - ^{13}C NOE where the carbon relaxation times are considerably longer than those of the proton, maximum cross-peak intensity is achieved when $\tau_m \approx 2T_1$ of the proton. Negative three-spin $^1\text{H} \rightarrow ^1\text{H} \rightarrow ^{13}\text{C}$ effects may also become apparent [93].

The proton-detected sequences, loosely referred to as inverse HOESY, depend critically for their success on the incorporation of PFGs. In experiments involving nuclei of low natural abundance, the gradients are required to suppress the intense background resonances arising from those protons in molecules that do not contain the heteroatom at the site of

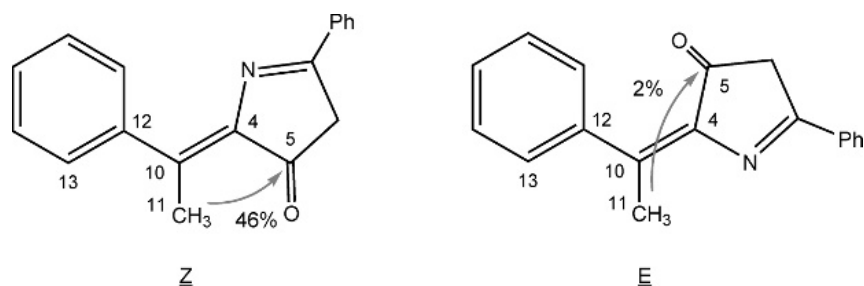
Figure 8.60. 2D sequences for measuring transient heteronuclear NOEs. (a) The classic HOESY and (b) the gradient selected inverse-HOESY employing the echo-antiecho scheme. Both sequences may employ the optional purging gradient during the mixing period (shown greyed) and for (b) the gradient ratio $G_1:G_2$ is set according to $\gamma_{\text{H}}:\gamma_{\text{X}}$.



interest and so prevent the required NOEs being masked by intense t_1 artefacts [94,95]. A general scheme employing gradient selection is illustrated in Fig. 8.60b [96] and again includes the option of a purging gradient during τ_m . The gradient ratios $G_1:G_2$ are set according to the magnetogyric ratios of the spins $\gamma_H:\gamma_X$ and G_2 alternated in sign according to the echo–antiecho protocol for f_1 quadrature detection. One potential weakness with all gradient-selected sequences proposed to date is the potential for signal attenuation arising from molecular diffusion during the NOE mixing period (See Chapter 9), a particular problem if NOEs are slow to develop, requiring τ_m to be long, and where molecular diffusion is rapid, such as in the case of smaller molecules.

8.9.3. Applications

Heteronuclear experiments have found use in ^{13}C NMR spectroscopy for the assignment of quaternary carbons and for the determination of stereochemistry in situations where the ^1H – ^1H NOE cannot be applied. Quaternary assignments are nowadays more often made via long-range correlation techniques such as HMBC, although the inability to distinguish *a priori* 2- and 3-bond correlations may lead to ambiguity. The short-range nature of the NOE means it can prove useful in distinguishing nearby non-protonated centres (i.e. those two bonds away [97]) from more distant ones [98,99]. The use of heteronuclear NOEs in the determination of stereochemistry is illustrated by the example of compound **8.28** [100]. Homonuclear proton NOEs could not be used to distinguish *E*- and *Z*-isomers of the oxazolones shown, although this was possible from the heteronuclear NOEs generated at the carbonyl carbon following saturation of the methyl protons

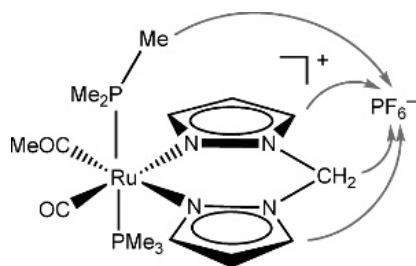


$\eta\{\text{Me}^{11}\}/\%$

	4	5	10	12	13
<i>Z</i> :	25	46	62	15	–6
<i>E</i> :	10	2	40	21	–5

8.28

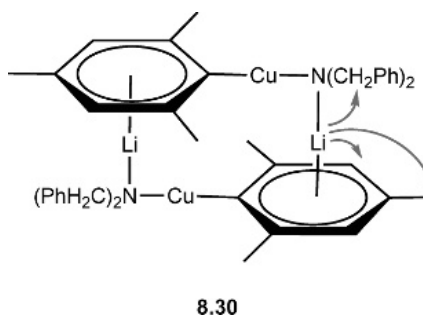
In recent years, $^{19}\text{F}\{^1\text{H}\}$ NOEs have found widespread use in studies of ion-pair interactions and have frequently been employed alongside ^1H and ^{19}F diffusion measurements (Chapter 9) [101,102]. Typically, the classic HOESY experiment has been employed with ^{19}F observation when small counterions such as BF_4^- , PF_6^- and CF_3SO_3^- are being considered. For example, in the complex **8.29**, $^{19}\text{F}\{^1\text{H}\}$ NOEs located the relative orientation of the ion-pair [103], which was subsequently rationalised from computation to arise from an accumulation of positive charge at the CH_2 group.



8.29

Another area in which heteronuclear NOEs have proved effective is that of organolithium chemistry, where both ^6Li and ^7Li have been exploited. ^6Li has been investigated by the traditional $^6\text{Li}\{^1\text{H}\}$ HOESY experiment [104–106] but has been less amenable to the inverse approach [94]. This is most likely due to the longer relaxation times of this nucleus requiring

longer mixing times and so leading to unacceptable signal attenuation arising from diffusion processes. In contrast, ^7Li appears well suited to inverse detection [94,95,107], and $^1\text{H}\{^7\text{Li}\}$ NOEs have for example been employed to aid in the identification of the solution species arising from the dynamic equilibrium observed for the lithium organo-amidocuprate $\text{CuLiMes}(\text{N}(\text{CH}_2\text{Ph})_2)$ **8.30** (where Mes = 2,4,6-trimethylphenyl) [107].



8.10. EXPERIMENTAL CONSIDERATIONS

The prerequisite for any NMR experiment is, of course, a well-prepared sample (Section 3.3). In addition to this, there are a number of experimental factors that have particular significance regarding NOE measurements, especially for the NOE difference method that is quite intolerant of poor experimental set-up. A stable magnetic field throughout the course of the experiment is essential, which means the lock system that is responsible for the field-frequency regulation must do its job well. Field drift is especially noticeable and detrimental in the NOE difference method, and optimal performance can be achieved by selecting solvents with a strong and sharp lock signal where possible, for example, acetone. Solvents with weak deuterium signals such as CDCl_3 , or broad deuterium resonances such as D_2O , are likely to perform less well in this respect. The choice of solvent and sample temperature can also be used to good effect to control the molecular tumbling rates (correlation times), which can be particularly useful for mid-sized molecules close to the $\omega_0\tau_c \approx 1$ zero-NOE condition. Use of a low-viscosity solvent and/or higher temperatures will increase tumbling rates and move motion towards the extreme narrowing limit whilst high-viscosity solvents and/or lower temperatures will encourage a move towards the spin-diffusion limit. Lock solvents with temperature-dependent shifts, notably D_2O , call for particular attention regarding temperature stability and will almost certainly require active temperature regulation. Drift in the lock resonance frequency arising from sample temperature changes will cause a shift of all resonance frequencies in the spectrum as the field follows this drift, disastrous for difference experiments. If no temperature regulation is available or if it performs inadequately, then a small quantity of an additional deuterated solvent (5–10% d_6 -acetone is a good choice) may be used for locking and usually provides superior results (for the same reason, one should always lock on the CD_3 resonance rather than the OD resonance of d_4 -methanol).

It is also worth avoiding very high solute concentrations in NOE samples since transient interactions between protonated molecules promote *intermolecular* dipole–dipole relaxation, which competes with the generation of the NOE itself. Another external relaxation source that can quench the NOE is paramagnetic impurities, notably certain metal ions and molecular oxygen. Whilst these are an unwelcome addition to any NMR sample, their presence can be particularly detrimental to NOE studies since paramagnetic relaxation dominates cross-relaxation. Metal ions can be removed by filtration through suitable chelating resins whereas oxygen may be removed with the freeze-pump-thaw method described in Section 3.3. Whether it is worth the effort to remove oxygen traces depends very much on the sample being studied. For very large molecules, efficient cross-relaxation means the NOE builds rapidly to large values, and as such, oxygen-induced paramagnetic relaxation has relatively little significance and degassing little effect. In contrast, external relaxation for smaller molecules that tumble rapidly causes the NOE to be drastically reduced (see Fig. 8.10), and degassing is likely to be of greater benefit. Likewise weak, slowly developing NOEs arising from long-range enhancements or indirect effects will also be enhanced if external relaxation is minimised. In general, the smaller the molecule and the further the internuclear separations being investigated, the greater the gain from solvent degassing. In practice, however, *routine* NOE experiments are successful in non-degassed solutions for all but the smallest molecules and, with the above caveats in mind, it is not essential to degas for the majority of organic molecules.

Finally, sample spinning can also be detrimental to NOE experiments (or indeed any NMR experiment when small responses are sought) particularly through the process known as ‘Q-modulation’, the variation in coupling between the rf coil and the sample caused by a slight wobbling of the sample as it rotates in the probe³. With the improved non-spinning lineshapes attainable with modern shim assemblies, sample spinning can no longer be recommended for either 1D or 2D NOE experiments.

REFERENCES

- [1] D. Neuhaus and M.P. Williamson, *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, 2nd ed., Wiley-VCH, New York, 2000.
- [2] R.A. Bell and J.K. Saunders, *Can. J. Chem.*, 1970, **48**, 1114–1122.
- [3] D.M. Hodgson and P.J. Comina, *Tetrahedron Lett.*, 1996, **37**, 5613–5614.
- [4] R. Colombo, F. Colombo, A.E. Derome, J.H. Jones, D.L. Rathbone and D.W. Thomas, *J. Chem. Soc., Perkin Trans. 1*, 1985, 1811–1815.
- [5] J.K. Robinson, V. Lee, T.D.W. Claridge, J.E. Baldwin and C.J. Schofield, *Tetrahedron*, 1998, **54**, 981–996.
- [6] J.E. Baldwin, T.D.W. Claridge, F.A. Heupel and R.C. Whitehead, *Tetrahedron Lett.*, 1994, **35**, 7829–7832.
- [7] J.E. Baldwin, L. Bischoff, T.D.W. Claridge, F.A. Heupel, D.R. Spring and R.C. Whitehead, *Tetrahedron*, 1997, **53**, 2271–2290.
- [8] B.T. Farmer, S. Macura and L.R. Brown, *J. Magn. Reson.*, 1988, **80**, 1–22.
- [9] J.K.M. Sanders and J.D. Mersh, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1982, **15**, 353–400.
- [10] J.C. Lindon and A.G. Ferrige, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1980, **14**, 27–66.
- [11] D. Neuhaus, G. Wagner, M. Vasák, J.H.R. Kágy and K. Wüthrich, *Eur. J. Biochem.*, 1985, **151**, 257–273.
- [12] M. Kinns and J.K.M. Sanders, *J. Magn. Reson.*, 1984, **56**, 518–520.
- [13] A.J. Shaka, C. Bauer and R. Freeman, *J. Magn. Reson.*, 1984, **60**, 479–485.
- [14] R.J. Smith, D.H. Williams, J.C.J. Barna, I.R. McDermott, K.D. Haegele, F. Piriou, J. Wagner and W. Higgins, *J. Am. Chem. Soc.*, 1985, **107**, 2849–2857.
- [15] S. Macura, Y. Huang, D. Suter and R.R. Ernst, *J. Magn. Reson.*, 1981, **43**, 259–281.
- [16] N.H. Andersen, K.T. Nguyen, C.J. Hartzell and H.L. Eaton, *J. Magn. Reson.*, 1987, **74**, 195–211.
- [17] E.T. Olejniczak, J.C. Hoch, C.M. Dobson and F.M. Poulsen, *J. Magn. Reson.*, 1985, **64**, 199–206.
- [18] M.P. Williamson and D. Neuhaus, *J. Magn. Reson.*, 1987, **72**, 369–375.
- [19] V. Dötsch, G. Wider and K. Wüthrich, *J. Magn. Reson., Ser. A*, 1994, **109**, 263–264.
- [20] T. Parella, F. Sánchez-Ferrando and A. Virgili, *J. Magn. Reson.*, 1997, **125**, 145–148.
- [21] R. Wagner and S. Berger, *J. Magn. Reson. Ser. A*, 1996, **123**, 119–121.
- [22] L. Mitschang, J. Keeler, A.L. Davis and H. Oschkinat, *J. Biomol. NMR*, 1992, **2**, 545–556.
- [23] G. Otting, *J. Magn. Reson.*, 1990, **86**, 496–508.
- [24] G. Bodenhausen, G. Wagner, M. Rance, O.W. Sorensen, K. Wuthrich and R.R. Ernst, *J. Magn. Reson.*, 1984, **59**, 542–550.
- [25] N.H. Andersen, H.L. Eaton and X. Lai, *Magn. Reson. Chem.*, 1989, **27**, 515–528.
- [26] J.W. Keepers and T.L. James, *J. Magn. Reson.*, 1984, **57**, 404–426.
- [27] A. Kumar, G. Wagner, R.R. Ernst and K. Wüthrich, *J. Am. Chem. Soc.*, 1981, **103**, 3654–3658.
- [28] J.N.S. Evans, *Biomolecular NMR Spectroscopy*, Oxford University Press, Oxford, 1995.
- [29] J. Cavanagh, W.J. Fairbrother, A.G. Palmer, N.J. Skelton and M. Rance, *Protein NMR Spectroscopy: Principles and Practice*, 2nd ed. Academic Press (Elsevier), San Diego, 2006.
- [30] G. Esposito and A. Pastore, *J. Magn. Reson.*, 1988, **76**, 331–336.
- [31] M. Reggelin, H. Hoffman, M. Köck and D.F. Mierke, *J. Am. Chem. Soc.*, 1992, **114**, 3272–3277.
- [32] J. Stonehouse, P. Adell, J. Keeler and A.J. Shaka, *J. Am. Chem. Soc.*, 1994, **116**, 6037–6038.
- [33] K. Stott, J. Keeler, Q.N. Van and A.J. Shaka, *J. Magn. Reson.*, 1997, **125**, 302–324.
- [34] K. Stott, J. Stonehouse, J. Keeler, T.L. Hwang and A.J. Shaka, *J. Am. Chem. Soc.*, 1995, **117**, 4199–4200.
- [35] H. Hu and K. Krishnamurthy, *J. Magn. Reson.*, 2006, **182**, 173–177.
- [36] M.J. Gradwell, H. Kogelberg and T.A. Frenkiel, *J. Magn. Reson.*, 1997, **124**, 267–270.
- [37] H. Hu, S.A. Bradley and K. Krishnamurthy, *J. Magn. Reson.*, 2004, **171**, 201–206.
- [38] N. Fusetani, K. Shinoda and S. Matsunaga, *J. Am. Chem. Soc.*, 1993, **115**, 3977–3981.
- [39] K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, 1986.
- [40] H.J. Dyson and P.E. Wright, *Annu. Rev. Biophys. Biophys. Chem.*, 1991, **20**, 519–538.
- [41] M.P. Williamson and J.P. Waltho, *Chem. Soc. Rev.*, 1992, **21**, 227–236.
- [42] S. Anderson, T.D.W. Claridge and H.L. Anderson, *Angew. Chem. Int. Ed.*, 1997, **36**, 1310–1313.
- [43] L. Fielding, N. Hamilton and R. McGuire, *Magn. Reson. Chem.*, 1997, **35**, 184–190.
- [44] T.D.W. Claridge, J.M. Long, J.M. Brown, D. Hibbs and M.B. Hursthouse, *Tetrahedron*, 1997, **53**, 4035–4050.
- [45] J. Jeener, B.H. Meier, P. Bachmann and R.R. Ernst, *J. Chem. Phys.*, 1979, **71**, 4546–4553.
- [46] I.D. Campbell, C.M. Dobson, R.G. Ratcliffe and R.J.P. Williams, *J. Magn. Reson.*, 1978, **29**, 397–417.

³ This is sometimes evident as a modulation or jumping of the probe-tuning profile and by the appearance of responses in 1D spectra that are symmetric about main resonances at frequencies equal to the spinning speed, yet are indifferent to shimming and of random phase, both of which distinguish them from ‘spinning sidebands’ caused by field inhomogeneity. Owing to their random properties, they tend to be averaged away over many transients but are nonetheless undesirable artefacts.

- [47] J.J. Led and H. Gesmar, *J. Magn. Reson.*, 1982, **49**, 444–463.
- [48] A. Bain, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2003, **43**, 63–103.
- [49] P.J. Hore, *Nuclear Magnetic Resonance*, Vol. 32, Oxford University Press, Oxford, 1995.
- [50] H. Gunther, *NMR Spectroscopy: Basic Principles, Concepts and Applications in Chemistry*, 2nd ed., Wiley, Chichester, 1995.
- [51] A. Bain, *Ann. Rep. NMR. Spectrosc.*, 2008, **63**, 23–48.
- [52] K.G. Orrell and V. Šik, *Annu. Rep. NMR. Spectrosc.*, 1993, **27**, 103–171.
- [53] K.G. Orrell, V. Šik and D. Stephenson, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1990, **22**, 141–208.
- [54] C.L. Perrin and T.J. Dwyer, *Chem. Rev.*, 1990, **90**, 935–967.
- [55] J. Sandström, *Dynamic NMR Spectroscopy*, Academic Press, London, 1982.
- [56] M. Oki, *Applications of Dynamic NMR Spectroscopy to Organic Chemistry*, VCH, Weinheim, 1985.
- [57] D.S. Stephenson and G. Binsch, *J. Magn. Reson.*, 1978, **32**, 145–152.
- [58] E.F. Derose, J. Castillo, D. Saulys and J. Morrison, *J. Magn. Reson.*, 1991, **93**, 347–354.
- [59] R. Ramachandran, C.T.G. Knight, R.J. Kirkpatrick and E. Oldfield, *J. Magn. Reson.*, 1985, **65**, 136–141.
- [60] I. Pianet, E. Fouquet, M. Pereyre, M. Gielen, F. Kayser, M. Biesemans and R. Willem, *Magn. Reson. Chem.*, 1994, **32**, 617–623.
- [61] J.E.H. Buston, T.D.W. Claridge and M.G. Moloney, *J. Chem. Soc., Perkin Trans. 2*, 1995, 639–641.
- [62] C.L. Perrin, *J. Magn. Reson.*, 1989, **82**, 619–621.
- [63] V.S. Dimitrov and N.G. Vassilev, *Magn. Reson. Chem.*, 1995, **33**, 739–744.
- [64] E.W. Abel, T.P.J. Coston, K.G. Orrell, V. Šik and D. Stephenson, *J. Magn. Reson.*, 1986, **70**, 34–53.
- [65] G. Bodenhausen and R.R. Ernst, *J. Am. Chem. Soc.*, 1982, **104**, 1304–1309.
- [66] K.G. Orrell and V. Šik, *Annu. Rep. NMR. Spectrosc.*, 1987, **19**, 79–173.
- [67] K.G. Orrell, *Annu. Rep. NMR. Spectrosc.*, 1999, **37**, 1–74.
- [68] M. Pons and O. Millet, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2001, **38**, 267–324.
- [69] A.A. Bothner-By, R.L. Stephens, J. Lee, C.D. Warren and R.W. Jeanloz, *J. Am. Chem. Soc.*, 1984, **106**, 811–813.
- [70] A. Bax and D.G. Davis, *J. Magn. Reson.*, 1985, **63**, 207–213.
- [71] D. Neuhaus and J. Keeler, *J. Magn. Reson.*, 1986, **68**, 568–574.
- [72] T.M. Chan, D.C. Dalgarno, J.H. Prestegard and C.A. Evans, *J. Magn. Reson.*, 1997, **126**, 183–186.
- [73] H. Kessler, C. Griesinger, R. Kerssebaum, K. Wagner and R.R. Ernst, *J. Am. Chem. Soc.*, 1987, **109**, 607–609.
- [74] A. Bax, *J. Magn. Reson.*, 1988, **77**, 134–147.
- [75] T.L. Hwang, M. Kadkhodaei, A. Mohebbi and A.J. Shaka, *Magn. Reson. Chem.*, 1992, **30**, S24–S34.
- [76] T.L. Hwang and A.J. Shaka, *J. Am. Chem. Soc.*, 1992, **114**, 3157–3159.
- [77] T.L. Hwang and A.J. Shaka, *J. Magn. Reson. Ser B*, 1993, **102**, 155–165.
- [78] C. Griesinger and R.R. Ernst, *J. Magn. Reson.*, 1987, **75**, 261–271.
- [79] P. Adell, T. Parella, F. Sánchez-Ferrando and A. Virgili, *J. Magn. Reson. Ser B*, 1995, **108**, 77–80.
- [80] C. Dalvit and G. Bovermann, *Magn. Reson. Chem.*, 1995, **33**, 156–159.
- [81] W. Bauer, A. Soi and A. Hirsch, *Magn. Reson. Chem.*, 2000, **38**, 500–503.
- [82] M.V. D'Auria, C. Giannini, A. Zampella, L. Minale, C. Debitus and C. Roussakis, *J. Org. Chem.*, 1998, **63**, 7382–7388.
- [83] D. Seebach, M. Overhand, F.N.M. Kühnle, B. Martinoni, L. Oberer, U. Hommel and H. Widmer, *Helv. Chim. Acta*, 1996, **79**, 913–941.
- [84] T.D.W. Claridge, J.M. Goodman, A. Moreno, D. Angus, S.F. Barker, C. Taillefumier, M.P. Watterson and G.W.J. Fleet, *Tetrahedron Lett.*, 2001, **42**, 4251–4255.
- [85] A. Botsi, K. Yannalopoulou, B. Perly and E. Hadjoudis, *J. Org. Chem.*, 1995, **60**, 4017–4023.
- [86] B.-H. Aw, S. Selvaratnam, P.-H. Leung, N.H. Rees and W. McFarlane, *Tetrahedron: Asymmetry*, 1996, **7**, 1753–1762.
- [87] K.E. Kövér and G. Batta, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1987, **19**, 223–266.
- [88] W. Bauer, *Magn. Reson. Chem.*, 1991, **29**, 494–499.
- [89] K. Stott and J. Keeler, *Magn. Reson. Chem.*, 1996, **34**, 554–558.
- [90] P.L. Rinaldi, *J. Am. Chem. Soc.*, 1983, **105**, 5167–5168.
- [91] C. Yu and G.C. Levy, *J. Am. Chem. Soc.*, 1983, **106**, 6533–6537.
- [92] K.E. Kövér and G. Batta, *J. Magn. Reson.*, 1986, **69**, 344–349.
- [93] K.E. Kövér and G. Batta, *J. Magn. Reson.*, 1986, **69**, 519–522.
- [94] W. Bauer, *Magn. Reson. Chem.*, 1996, **34**, 532–537.
- [95] T.M. Alam, D.M. Pedrotty and T.J. Boyle, *Magn. Reson. Chem.*, 2002, **40**, 361–365.
- [96] O. Walker, P. Mutzenhardt and D. Canet, *Magn. Reson. Chem.*, 2003, **41**, 776–781.
- [97] F. Sánchez-Ferrando, *Magn. Reson. Chem.*, 1985, **23**, 185–191.
- [98] F.J. Leeper and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1982, 911–912.
- [99] M.F. Aldersley, F.M. Dean and B.E. Mann, *J. Chem. Soc., Chem. Commun.*, 1983, 107–108.
- [100] C. Cativiela and F. Sánchez-Ferrando, *Magn. Reson. Chem.*, 1985, **23**, 1072–1075.
- [101] A. Macchioni, *Eur. J. Inorg. Chem.*, 2003, 195–205.
- [102] P.S. Pregosin, P.G.A. Kumar and I. Fernandez, *Chem. Rev.*, 2005, **105**, 2977–2998.
- [103] N.G. Stahl, C. Zuccaccia, T.R. Jensen and T.J. Marks, *J. Am. Chem. Soc.*, 2003, **125**, 5256–5257.
- [104] W. Bauer, NMR of Organolithium Compounds: General Aspects and Application of Two-Dimensional Heteronuclear Overhauser Effect Spectroscopy. In: *Lithium Chemistry* (Eds.: A.-M. Sapse and P.V.R. Schleyer), Wiley, New York, 1995, pp. 125–172.
- [105] R.M. Gschwind, P.R. Rajamohanan, M. John and G. Boche, *Organomet.*, 2000, **19**, 2868–2873.
- [106] G. Hilmersson and B. Malmros, *Chemistry*, 2001, **7**, 337–341.
- [107] R.P. Davies, S. Hornauer and P.B. Hitchcock, *Angew. Chem. Int. Ed.*, 2007, **46**, 5191–5194.

Chapter 9

Diffusion NMR spectroscopy

9.1. INTRODUCTION

The study of molecular diffusion in solution potentially offers insights into a range of physical molecular properties including molecular size, shape, aggregation, encapsulation, complexation and hydrogen bonding, and NMR-based measurements have been applied to many areas of chemistry for over four decades [1–3]. However, it is only in more recent years that diffusion measurements have become routinely accessible with conventional high-resolution NMR spectrometers through the introduction of actively shielded pulsed field gradient probe heads. All modern NMR-based diffusion measurements rely on the application of field gradients to encode the physical location of a molecule or complex in solution and so characterize its diffusion along the direction of the applied field gradient, typically the z axis of conventional gradient probe heads. The ability to use standard commercial instrumentation for such measurements on routine samples has obvious advantages over alternative methods for measuring diffusion, such as radioactive tracer studies. NMR techniques allow relatively fast measurements over a range of temperatures and do not require specialised handling of radioactive isotopes.

In this chapter, we shall focus on the use of high-resolution NMR methods for studying diffusion in physically homogeneous (isotropic) solutions. Such self-diffusion arises from the random translational (Brownian) motion of molecules (or their complexes) driven by thermal energy of the system and may be characterised quantitatively by the so-called *self-diffusion coefficient*, D . Whilst in an isotropic system the average displacement of a molecule over time in three dimensions is zero (the sample remains in the tube after all), the mean square displacement is non-zero such that the distance travelled by a molecule in a single direction during a period t is given by:

$$z_{\text{rms}} = (2Dt)^{1/2} \quad (9.1)$$

Here, z_{rms} represents the root mean square distance travelled as a time average for many molecules. The self-diffusion coefficient is therefore a measure of the rate of mean square displacement of a molecule and consequently has units of m^2s^{-1} . It is this physical parameter we aim to measure or utilise when performing diffusion NMR experiments, which from this point on we shall refer to simply as the *diffusion coefficient*.

The characterisation of the rates of diffusion of molecules or their complexes may be related intuitively to, amongst other things, their size and shape and so provides a different, and often complimentary, insight into molecular structures and their solution behaviour over that provided by the familiar parameters of chemical shift and through-bond or through-space spin–spin coupling. Different mobility rates may also be used as the basis for the separation of the spectra of mixtures of compounds in solution, this procedure often being referred to as diffusion ordered spectroscopy or DOSY [4]. As we shall see, this typically produces ‘pseudo’-2D spectra with one axis representing chemical shifts and the second diffusion coefficients, so dispersing the NMR resonances according to the diffusion properties of the parent molecules. The application of high-resolution NMR diffusion measurements to the separation of small-molecule mixtures in this way remains a developing area that has potential use in the analysis of complex matrices such as synthetic and biosynthetic mixtures, biofluids, foods and beverages. However, it is worth noting that the experimental techniques for the direct quantitative measurement of diffusion coefficients and for the production of 2D DOSY spectra are identical, with the different formats for presenting results arising only from different approaches taken in data processing, as will be explained in the sections that follow.

9.1.1. Diffusion and molecular size

It is often the case that diffusion measurements are performed so as to gain information on (relative) molecular sizes, and the diffusion coefficient, D , may itself be related to molecular dimensions through the relationship:

$$D = \frac{k_b T}{f} \quad (9.2)$$

where k_b is the Boltzmann constant, T is the absolute temperature and f represents the hydrodynamic friction coefficient or frictional factor, a term that reflects, amongst other things, the size and shape of the molecular species. For the idealised case of a diffusing sphere, f may be related to the hydrodynamic or Stokes radius of the sphere, r_s , and the solution viscosity, η , through the Stokes equation:

$$f = 6\pi\eta r_s \quad (9.3)$$

Combining these gives the widely quoted *Stokes–Einstein equation*, which for a sphere is:

$$D = \frac{k_b T}{6\pi\eta r_s} \quad (9.4)$$

This demonstrates that the diffusion coefficient is inversely related to the size (radius) of the diffusing species (sphere), as one might expect; that is, larger molecules or complexes will tend to exhibit smaller diffusion coefficients (other conditions being comparable). Whether Eq. (9.4) is valid for your systems will depend on how well their shape can be approximated to that of a sphere, and suggests caution when translating the diffusion coefficient to a hydrodynamic radius (although this has been done in a number of cases; see Section 9.4 below). Furthermore, in the study of smaller molecules, notably where the hydrodynamic radius of the solute tends towards that of the solvent, the scaling factor 6 in Eq. (9.4) becomes invalid and must be corrected; see Section 9.3.4. For larger and more irregular molecular shapes, more complex descriptions of the friction coefficient must be developed, which may become a non-trivial exercise meaning it is more commonly the case that the diffusion coefficients themselves are reported. Note also that these depend not only on the molecular size and shape, as reflected in the friction coefficient, but also on the solution temperature and inversely on its viscosity, which has important bearings on how one should *interpret* the magnitudes of diffusion coefficients. This will be discussed further below, along with other very important practical considerations required when undertaking these measurements. Indeed, whilst it is technically rather straightforward to *execute* diffusion experiments with modern NMR spectrometers, obtaining *meaningful* answers free from interferences can be rather more challenging, as we shall discover. Before discussing the practicalities of such measurements and presenting examples of their applications, we shall consider how one goes about measuring diffusion coefficients.

9.2. MEASURING SELF-DIFFUSION BY NMR

In this section, we shall consider the operation of more widely used diffusion sequences and their variants, and consider methods for processing the acquired data. This essentially falls into two possible approaches, either direct regression analysis to provide values of the diffusion coefficients or treatments that present the pseudo-2D DOSY spectra mentioned above. To understand the process of measuring diffusion coefficients by NMR, we shall first consider the simplest gradient spin-echo experiment.

9.2.1. The PFG spin-echo

The basic scheme for the characterisation of diffusion is the pulsed field gradient spin-echo [5] (Fig. 9.1a). In the absence of gradient pulses, this will act simply as a standard spin-echo and so refocus chemical shift evolution such that the detected signal is attenuated only by transverse relaxation during the 2τ period. When pulsed field gradients are employed, the first gradient pulse will impose a spatially dependent phase on the magnetisation vectors, which can be refocused by a second gradient of equal duration and magnitude; although these pulses are applied in the same sense, their effects will cancel due to the application of the 180° pulse between them. However, *complete* refocusing of the signal will only occur when the local field experienced by a spin is identical during the two

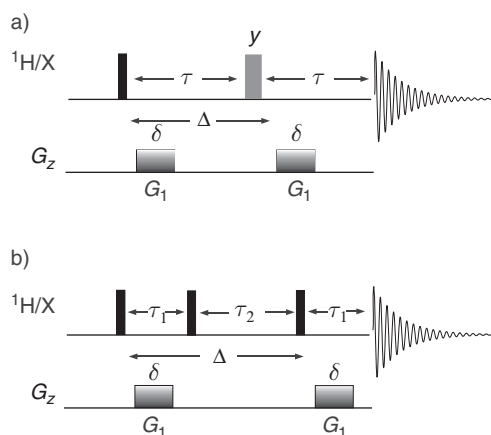


Figure 9.1. Basic sequences for measuring molecular diffusion based on (a) the PFG spin-echo and (b) the PFG stimulated-echo. The diffusion during the period Δ is characterised by a series of measurements with increasing gradient strengths, as represented by the faded gradient pulses.

gradient pulses. Since a field *gradient* is imposed, this refocusing condition is only met if the spin remains in the same physical location when the two PFG pulses are applied. If the molecule were to diffuse away from its initial position during the diffusion delay Δ , then the local field experienced during the second PFG would not exactly match that of the first and only partial refocusing of the signal would occur (Fig. 9.2). The detected signal would therefore be attenuated by an amount dependent on how far the molecule moved during the period Δ , and hence by its diffusion coefficient.

To characterise diffusion rates, it is possible to progressively increase the diffusion period Δ , the length of the gradient pulses δ , or the strength of the gradient pulses G and to monitor the corresponding change in NMR signal intensity. Thus, increasing Δ provides more time for the molecules to move through the bulk sample, whereas increasing δ or G imposes a greater degree of signal dephasing across the sample; in all cases, the amount of signal refocused by the second gradient will have decreased in the presence of self-diffusion and the net NMR signal intensity will have diminished. However, changes made to the overall length of the echo sequence by increasing Δ will introduce additional attenuation arising from increased relaxation losses, so it is now universal practice to increase the diffusion encoding/decoding gradient strengths G whilst keeping all time periods invariant. Whilst T_2 relaxation losses still occur relative to a single-pulse acquisition, they are constant for all echo experiments and thus do not contribute to the progressive signal attenuation that is monitored (Fig. 9.3).

The observed signal intensity at the end of the spin-echo, I_G , for the basic PFG spin-echo experiment is given by the so-called Stejskal–Tanner expression:

$$I_G = I_0 \exp\left(-\frac{2\tau}{T_2}\right) \exp\left(-(\gamma\delta G)^2 D \left(\Delta - \frac{\delta}{3}\right)\right) \quad (9.5)$$

where I_0 is the signal intensity in the absence of the gradient spin-echo (i.e. that after a single 90° pulse), G is the gradient strength, D is the diffusion coefficient, the delays τ , δ and Δ are as in Fig. 9.1, γ is the magnetogyric ratio of the observed nuclide and T_2 is the transverse relaxation rate constant. For the typical case where the total echo time 2τ is held constant and the gradient strength G varied a more useful form is:

$$I_G = I_{G=0} \exp\left(-(\gamma\delta G)^2 D \left(\Delta - \frac{\delta}{3}\right)\right) \quad (9.6)$$

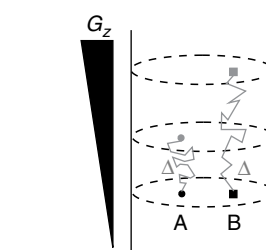
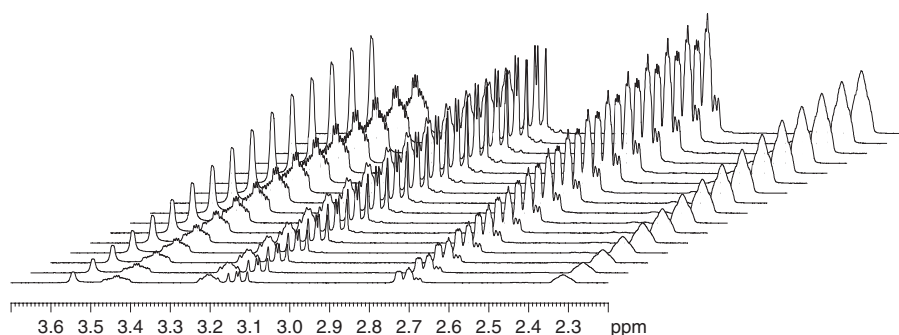


Figure 9.2. A schematic representation of signal attenuation through molecular diffusion. The local field experienced by molecule A during the first gradient pulse (molecules shown in black) does not precisely match that experienced during the second gradient pulse (molecules shown in grey) due to diffusion during the delay Δ . The signal of A does not fully refocus and its response is attenuated. Greater attenuation is observed for the faster moving molecule B due to the greater difference in local fields it experiences during the two gradient pulses.

Figure 9.3. Experimental 1D ^1H diffusion traces showing progressive intensity decay as a function of increasing gradient strength (from back to front).

where $I_{G=0}$ represents the signal intensity of the spin-echo at zero gradient strength. As the constants γ , δ and Δ are known, the diffusion coefficient may be calculated directly from appropriate linear or non-linear regression, and optionally the data presented in the DOSY format; see Section 9.2.5 below.

9.2.2. The PFG stimulated-echo

The PFG spin-echo sequence described above is limited in practice by the aforementioned relaxation losses and is now less used for diffusion measurements. Because magnetisation is transverse during the diffusion period, these losses are dictated by the transverse (T_2) relaxation rates, which themselves increase with molecular size. Since larger molecules tend to require longer diffusion periods to translate significant distances, the use of long Δ delays (which are typically in the region of 10 ms to 1 s) can lead to unacceptable signal-to-noise degradation. The spin-echo sequence may also be subject to J -modulation (coupling evolution) when magnetisation is transverse, which can lead to undesirable signal distortion, especially for strongly coupled spin systems. In the alternative stimulated-echo (STE) sequence [6] (Fig. 9.1b), magnetisation is transverse only during the relatively short gradient encoding periods τ_1 (1–10 ms) but longitudinal during the remainder of the diffusion period τ_2 by virtue of the second 90° pulse, meaning signal loss is dictated more by the potentially slower longitudinal (T_1) relaxation rates instead. Following the diffusion period, the magnetisation is returned to the transverse plane by the third 90° pulse for refocusing and detection. The observed signal intensity for the stimulated-echo, I_G , is now given by:

$$I_G = \frac{I_0}{2} \exp\left(-\frac{2\tau_1}{T_2} - \frac{\tau_2}{T_1}\right) \exp\left(-(\gamma\delta G)^2 D \left(\Delta - \frac{\delta}{3}\right)\right) \quad (9.7)$$

or again when varying gradient strength alone:

$$I_G = I_{G=0} \exp\left(-(\gamma\delta G)^2 D \left(\Delta - \frac{\delta}{3}\right)\right) \quad (9.8)$$

In addition to the dependence on T_1 it may be seen in Eq. (9.7) that the signal intensity is now reduced by a factor of 2 relative to that of the spin-echo sequence. This is a consequence of the second 90° pulse placing only one of the two orthogonal components of the transverse x – y magnetisation back along the longitudinal z axis during the diffusion period, the remaining component being removed by phase cycling or additional gradient purging. This signal loss is a necessary evil of this stimulated-echo sequence but is often tolerated because of its improved behaviour; the more recently introduced diffusion sequences described below tend to be derived from this basic stimulated-echo sequence. One further feature of the stimulated-echo that has consequences for data analysis is that the intensity of the echo is also dependent on the chemical shift offset of each resonance when the applied gradient strength is zero, that is, with no gradient pulses applied (see the reference by Johnson [2] for details). This dependence is removed for non-zero gradients, so in practical implementations, it is usual either to ignore the spectra with $G=0$ for data analysis or more typically to begin the experiment with a small but non-zero value for G .

9.2.3. Enhancements to the stimulated-echo

A number of enhancements to the basic stimulated-echo sequence have been suggested over the years aimed at minimising lineshape distortions and reducing experiment time by minimising phase-cycling requirements. Resonance perturbations arise from the application of strong field gradient pulses, causing eddy currents in metal work surrounding the sample and disturbance of the field-frequency lock. The use of actively shielded field gradient probe heads combined with shaped gradient pulses significantly reduces eddy current generation but diffusion experiments can remain sensitive to small residual effects. Some of the more commonly employed sequences are presented below that address these issues, and further variants are discussed in Section 9.3.1 when the thorny issue of convection, the bane of diffusion measurements, is addressed.

The BPP-STE sequence

The key feature of the bipolar pulse pair STE (BPP-STE) sequence (Fig. 9.4a) is the use of bipolar gradient pulse pairs. In this a single gradient pulse in the conventional STE sequence is replaced by two pulses of half the duration and opposing signs that are separated by a non-selective 180° pulse [7] (Fig. 9.5). The two equal but opposite gradient pulses impose a net additive dephasing effect for all spins experiencing the central 180° pulse and so mirror the effect of a single pulse. However, they generate less eddy current

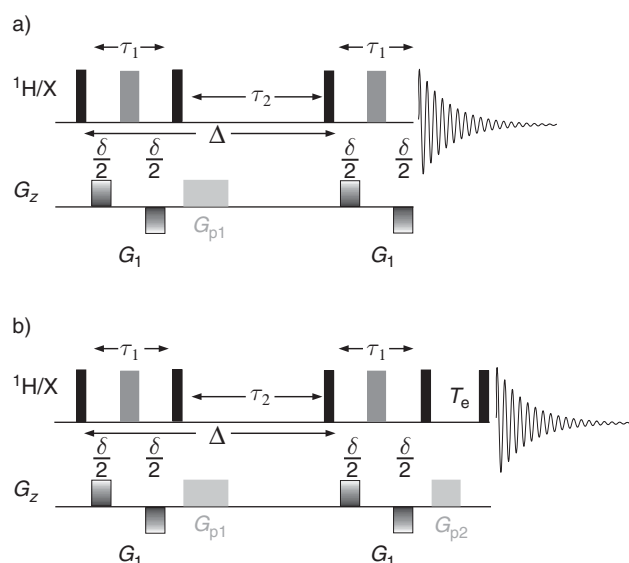


Figure 9.4. The (a) BPP-STE and (b) BPP-LED diffusion sequences. The encoding gradients of the stimulated-echo are applied as symmetrical bipolar pulse pairs of total duration δ and the LED sequence is extended with an eddy current delay period T_e . Optional purging pulses G_{p1} and G_{p2} may also be employed.

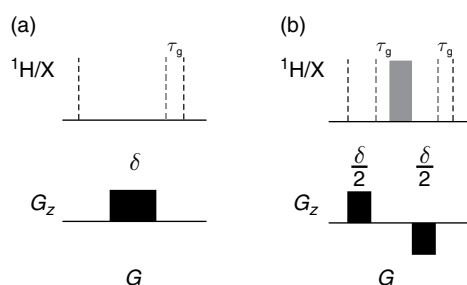


Figure 9.5. (a) Monopolar gradient pulse and (b) the equivalent bipolar pulse pair (BPP). Dashed vertical lines indicate time points and the period τ_g is the gradient recovery delay.

distortions since the two pulses are applied in opposite senses. Furthermore, since the deuterium nuclei do not experience the 180° rf pulse, the bipolar pair has a net zero effect on the lock system unlike a single gradient pulse that would also dephase the lock resonance. Thus, perturbations of the lock circuitry are also reduced with the bipolar pair (Fig. 9.6), all of which can lead to reduced lineshape disturbances in spectra (Fig. 9.7).

To allow for gradient pulse recovery delays τ_g in the bipolar pairs, Eq. (9.8) for defining the signal amplitude of the stimulated-echo is modified slightly:

$$I_G = I_{G=0} \exp\left(-(\gamma\delta G)^2 D \left(\Delta - \frac{\delta}{3} - \frac{\tau_g}{2}\right)\right) \quad (9.9)$$

One consequence of employing the bipolar pulse pairs is the need for suitable phase cycling of pulses to select only the magnetisation that experiences the 180° pulses and thus exhibits the correct diffusion weighting [8]. This requirement for extended phase cycling may be reduced somewhat by the use of additional gradient purge pulses applied when the desired magnetisation is longitudinal (Fig. 9.4a), which leads to a minimum 8-step cycle for the BPP-STE sequence (Table 9.1). In this, the first four steps select transverse magnetisation produced by the 90° pulse of the last spin-echo whilst the next four compensate for errors in the final 180° pulse.

The BPP-LED sequence

The bipolar pulse pair longitudinal eddy current delay (BPP-LED¹) sequence [9, 10] (Fig. 9.4b) has been one of the most commonly used sequences and employs the BPP-STE with an additional eddy current delay period T_e in which the magnetisation is stored longitudinally prior to detection whilst eddy currents are allowed to decay. Following a suitable recovery period, the longitudinal magnetisation is placed transverse by the final 90° pulse for detection. The duration T_e will depend on the hardware in use, and the LED extension may indeed be unnecessary on modern, well-shielded probe heads operating with relatively low gradient strengths. Otherwise, T_e may typically be some milliseconds or tens

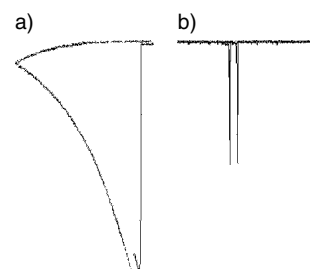


Figure 9.6. Deuterium lock signal behaviour during the STE sequence recorded with (a) monopolar and (b) bipolar gradient pulses. Lock signal refocusing is apparent in (b) leading to a minimal and only momentary perturbation from the two gradient pulse pairs.

¹ In the original paper describing this technique [9], the acronym *LED* appears to have been derived from the term longitudinal encode-decode, a reference to the pulse-gradient combinations employed, whereas this term is now taken to mean longitudinal eddy-current delay [10].

Figure 9.7. ^1H lineshapes observed for the STE sequence recorded with (a) monopolar and (b) bipolar gradient pulses.

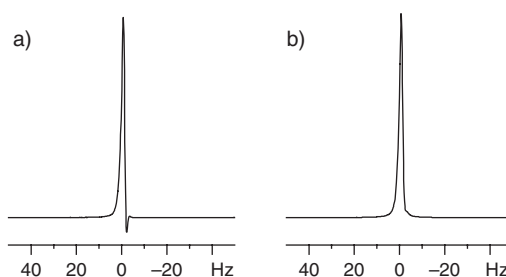


Table 9.1. A suitable minimum phase cycle for the gradient-purged BPP-STE experiment where pulses P_1 to P_5 represent the five rf pulses of the sequence in the order they are applied and ϕ_R the receiver phase

Transient	P_1	P_2	P_3	P_4	P_5	ϕ_R
1	x	x	x	x	x	x
2	x	x	x	y	x	-y
3	x	x	x	-x	x	x
4	x	x	x	-y	x	y
5	x	-x	x	x	-x	x
6	x	-x	x	y	-x	-y
7	x	-x	x	-x	-x	x
8	x	-x	x	-y	-x	y

Table 9.2. The minimum phase cycle for BPP-LED experiment where pulses P_1 to P_5 represent the five 90° pulses of the sequence in the order they are applied and ϕ_R the receiver phase

Transient	P_1	P_2	P_3	P_4	P_5	ϕ_R
1	x	x	x	x	x	x
2	x	x	x	-x	x	-x
3	x	-x	x	x	x	-x
4	x	-x	x	-x	x	x
5	x	x	-x	-x	-x	-x
6	x	x	-x	x	-x	x
7	x	-x	-x	-x	-x	x
8	x	-x	-x	x	-x	-x

of milliseconds but in any case must be short relative to the longitudinal relaxation times of the nuclei being observed so as to retain signal intensity.

The bipolar pulse pairs here lead to a minimum 8-step (preferably 16-step) cycle [10] for the BPP-LED (Table 9.2). As for the BPP-STE experiment, an additional element in common usage is the incorporation of purging gradient pulses to destroy any residual transverse magnetisation during the diffusion period and potentially also during the eddy current recovery period where the desired magnetisation is longitudinal. This will however invalidate the refocusing effects on the lock resonance since these purge pulses are not applied as bipolar pairs, so their use may in fact be detrimental on some instruments. In this case, one must also be aware of the possibility of accidental refocusing by these purge pulses of unwanted coherences that may interfere with peak intensities and, if used, some trial and error in the selection of the purge gradient strengths and durations may become necessary. An improved implementation of purging gradients is as balanced pulses, as in the one-shot sequence below.

The 'one-shot' sequence

To avoid the need for extensive phase cycling yet retain the benefits of bipolar pulse pairs, Morris *et al.* have suggested the 'one-shot' sequence [11], which requires only a single transient per gradient value, signal-to-noise permitting, so allowing the very rapid collection of diffusion data. A key feature of the sequence is the use of balanced asymmetrical bipolar gradient pairs to minimise the potential for resonance distortion arising from eddy currents and lock disturbance (Fig. 9.8).

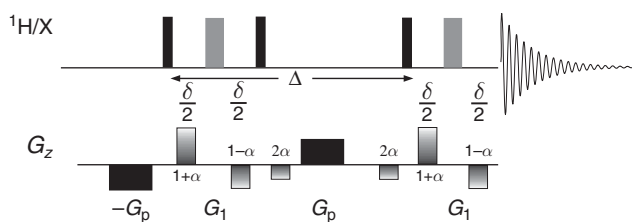


Figure 9.8. The ‘one-shot’ diffusion sequence. The bipolar pulses G_1 are applied as unbalanced pairs in a ratio $1 + \alpha : 1 - \alpha$ with additional balancing pulses of relative intensity 2α . An intense purge gradient G_p is applied during the diffusion period D and is balanced by the gradient pulse $-G_p$ during the relaxation delay.

Table 9.3. An 8-step phase cycle for the ‘one-shot’ experiment where P_1 to P_3 represent the three 90° pulses of the sequence in the order they are applied and ϕ_R the receiver phase

Transient	P_1	P_2	P_3	ϕ_R
1	x	x	x	x
2	-x	x	x	-x
3	x	x	-x	-x
4	-x	x	-x	x
5	y	x	x	y
6	-y	x	x	-y
7	y	x	-x	-y
8	-y	x	-x	y

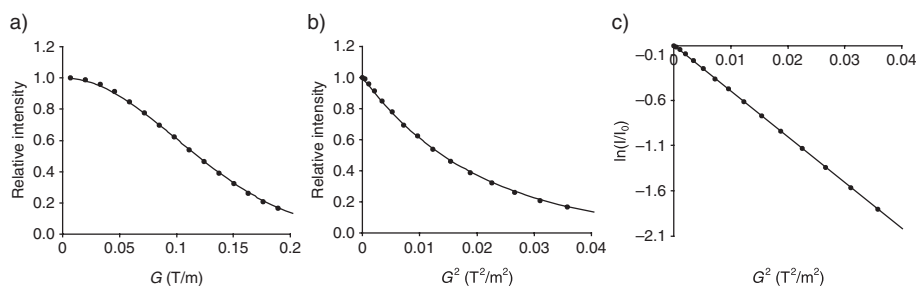
The bipolar pulse pairs themselves are unbalanced with an intensity ratio of $1 + \alpha : 1 - \alpha$ to dephase any magnetisation that is not refocused by the 180° pulse (which would otherwise require suppression through EXORCYCLE phase cycling of the 180° pulses as for a standard spin-echo; Section 7.2.2). To counteract the resultant imbalance in these opposing gradient pulses, and thus counter the destruction of the lock, additional balancing pulses each of intensity 2α are applied at the start and end of the diffusion period in which magnetisation is longitudinal. Finally, an additional intense purge pulse G_p is applied also during this diffusion period to select pure longitudinal magnetisation, and this is similarly balanced with an equal but opposite gradient during the relaxation delay. Thus, the resultant is retention of the desired magnetisation through the sequence by gradient selection in a single transient but with no net destruction of the lock signal owing to the balancing throughout of positive with negative gradient pulses. If signal-to-noise considerations demand more than a single transient per experiment, phase cycling may also be employed advantageously for which a basic 8-step cycle is presented in Table 9.3; this may be further extended as in reference [11]. The use of the imbalance factor α (typically in the region 0.1–0.2) demands a slight modification of the expression for signal attenuation, which is also described in reference [11].

9.2.4. Data analysis: regression fitting

Having collected the diffusion data set consisting of a series of 1D traces recorded with increasing gradient amplitudes, it remains to extract from this the diffusion coefficients for the species of interest. These data will take the form of typically 8–32 1D spectra, and for all sequences described above, the data fitting procedures rely on the measurement of the decay in signal intensity (recorded either as peak height or integrated area) as a function of the applied gradient strength. This process relies on the resonances being monitored having arisen from a single species only and thus being free from overlap with those of other molecules such that each decay profile reflects the behaviour of only one molecular component. As such, maximum resonance dispersion is essential for reliable measurements of individual diffusion coefficients. It is also vitally important to ensure baselines are flat and have no offset prior to measuring peak amplitudes meaning baseline correction routines are likely to prove beneficial.

Diffusion coefficients may be extracted directly from regression fits of the peak intensities versus gradient strengths. This typically takes the form of plotting I_G against G for which a Gaussian decay profile is obtained (Fig. 9.9a) or I_G against G^2 for which an exponential profile is obtained (Fig. 9.9b) and D extracted by an appropriate non-linear fit since all other constants γ , δ , and Δ (in Eq. (9.8) for example) are known. In this approach, $I_{G=0}$ may also be a variable in the fit since this is not usually determined for the stimulated-echo (Section 9.2.2). An alternative approach favoured by some is to plot $\ln(I_G/I_{G=0})$ against G^2 for which a linear least-squares fit is appropriate (Fig. 9.9c) with the slope proportional to $-D$.

Figure 9.9. Regression analysis to obtain the diffusion coefficient by fitting of (a) Gaussian decay profile (I_G vs G) (b) Exponential decay profile (I_G vs G^2) or (c) Linear decay profile ($\ln(I_G/I_{G=0})$ vs G^2); see text.



In situations where two peaks cannot be resolved but arise from separate molecules with differing diffusion behaviour, an apparent diffusion coefficient will result from fitting for a single value of D that has a magnitude intermediate between those of the overlapping components. In such cases, it is theoretically possible to perform biexponential fits to extract the two diffusion coefficients although resolving superimposed exponential decays is notoriously difficult and achieving reliable results can be problematic; see discussions of Nilsson *et al.* [12]. Coincidental overlap of even more components would demand multi-exponential fits making extraction of meaningful diffusion coefficients all but impossible for routine applications; an experimental alternative in such cases is the use of hybrid diffusion sequences to improve resonance dispersion as described in Section 9.5 below.

9.2.5. Data analysis: pseudo-2D DOSY presentation

An aesthetically different approach to presenting the results of diffusion experiments is as a pseudo-2D contour presentation, which has been termed DOSY (diffusion-ordered spectroscopy). In this, one dimension represents chemical shifts and the other the diffusion coefficients of each component as exemplified in Fig. 9.10. The most general and widely used data processing scheme is direct exponential fitting of decay intensities, as described above, from which the 2D display is synthesised such that the centre of a crosspeak in the diffusion dimension represents the magnitude of the coefficient itself and the peak width in the same dimension reflects the magnitude of the fitting error, presented typically as a Gaussian profile. More generally, procedures suitable for extracting diffusion coefficients are equally applicable to the generation of DOSY plots, and there exist a range of more complex mathematical procedures for transforming diffusion decays into 2D DOSY spectra when peak overlap is present including CONTIN, SPLMOD, DECRA, CORE and GIFA, and the interested reader is referred to selected review papers [2, 13, 14]. In all cases, high-quality data are a prerequisite for successful analysis. In practice, one is most likely to make use of the tools available in standard NMR processing packages for routine work, and it is likely that developments in this will continue to appear. The DOSY presentation clearly lends itself to experiments on multi-component systems for which it shares some parallels in chromatographic separation of mixtures, although the underlying physical processes giving rise to the separation are, of course, very different.

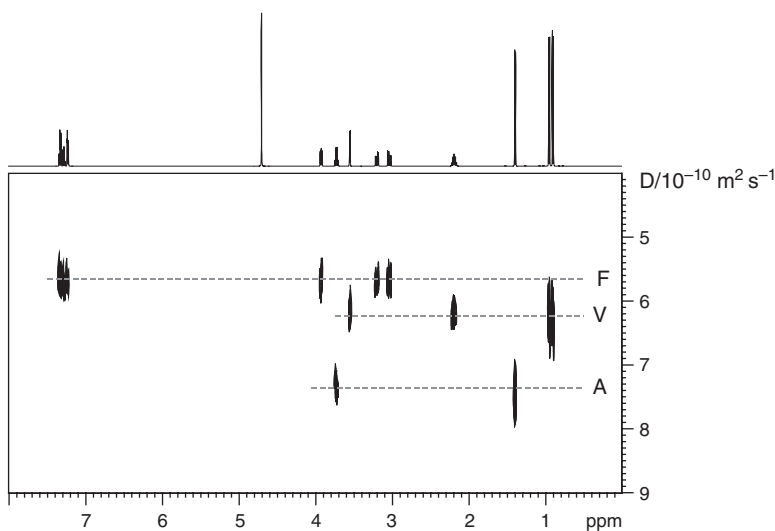


Figure 9.10. 2D DOSY presentation. The spectrum illustrates the separation of resonances from an equimolar mixture of three L-amino acids alanine (A), valine (V) and phenylalanine (F) in aqueous solution at 298 K according to their diffusion coefficients, plotted along the vertical axis. Diffusion coefficients obtained from regression analysis of the data indicated values of 5.67 (F), 6.25 (V) and 7.37 (A) $\times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

9.3. PRACTICAL ASPECTS OF DIFFUSION NMR SPECTROSCOPY

In this section, we shall explore some of the procedures needed for the recording of reliable diffusion data including instrument calibration, sample preparation and equilibration requirements. We shall also address some of the most important factors that can lead to the collection of inaccurate data, something to which diffusion measurements can be especially prone. Indeed, the set-up of a diffusion sequence is no more demanding than most other 2D experiments but the potential for erroneous results is considerably greater in most cases and, worst still, is not always readily apparent if one is not aware of the potential pitfalls and how to avoid them.

9.3.1. The problem of convection

There can be little doubt that the greatest barrier to the reliable determination of diffusion coefficients on a modern high-resolution NMR spectrometer stems from thermal (Rayleigh–Bénard) convection arising in the NMR sample. This typically takes the form of convective flow along the length of the NMR tube, which is driven by temperature gradients in the sample that cause the upward flow of the warmer, less dense fluid. Such temperature gradients are generated by the temperature regulation methods used by NMR spectrometers. The most widely adopted method on commercial instrumentation involves the use of a gaseous flow, typically air or nitrogen, entering through the base of the NMR probe and passing over the NMR tube. To maintain sample temperature, the gas is usually heated prior to the sample with the heater current controlled via a feedback mechanism to regulate the resulting sample temperature, as monitored by a thermocouple placed close to the base of the NMR tube. Even though this may maintain a very stable mean sample temperature, there remains the likelihood that temperature gradients exist within the sample that may initiate convective flow. Since this provides an alternative mechanism to diffusion for the translational motion of molecules, its presence will lead to enhanced attenuation of the signal decay in diffusion experiments and hence to larger but erroneous (apparent) diffusion coefficients.

Problems arising from convection are expected to be greatest for low viscosity solvents and at sample temperatures furthest from the ambient probe temperature. As discussed in the sections that follow, convection can be significant at temperatures only slightly higher than ambient for commonly used low viscosity solvents. Indeed, for a solvent such as chloroform, a temperature gradient as small as 0.2 K along a typical NMR sample can be sufficient to induce convection [15]. Conversely, there is anecdotal evidence that convection can be less severe although still present when operating at very low temperatures; this is most likely because of increases in solution viscosity and the fact that cold rather than hot gas is flowing over the base of the tube. In any case, it is vitally important that one can recognise the influence of convection in an NMR sample before diffusion measurements are undertaken and, if present, take measures to either avoid this or to compensate for its influence in the techniques used.

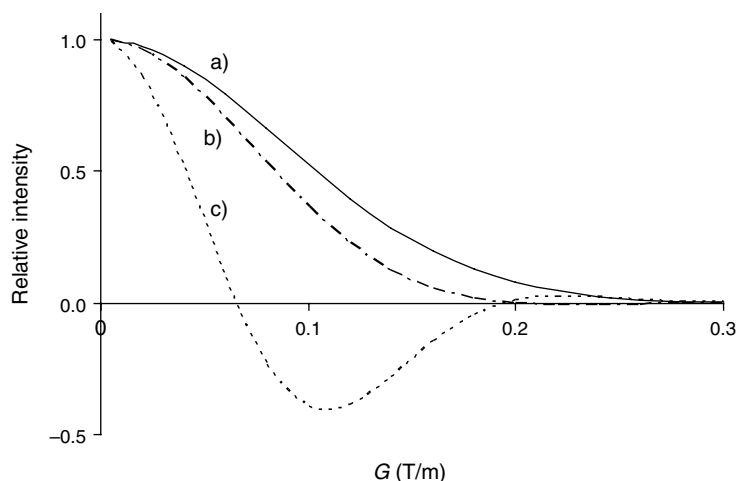
Diagnosing convection

In the case of severe thermal gradients (and assuming no other potentially complicating factors are present such as resonance overlap of different components or chemical exchange processes), the influence of convection may be seen as distortions to the usual signal amplitude Gaussian decay profiles. In cases where convection dominates over diffusion, it may also be that signal amplitudes become negative before again displaying positive intensities since convection introduces what approximates to a cosine modulation into the diffusion signal attenuation [16] thus:

$$I_G \approx \underbrace{I_{G=0}}_{\text{convection}} \cos(\gamma \delta G \Delta v) \underbrace{\exp\left(-(\gamma \delta G)^2 D \left(\Delta - \frac{\delta}{3}\right)\right)}_{\text{diffusion}} \quad (9.10)$$

where v represents the convective flow velocity. This effect may be seen in Fig. 9.11 where signal attenuations are presented without and with such contributions from convection. Although in the case of significant and dominant flow (Fig. 9.11c), the distortion is obvious and hence indicative of convection, the situation with a more moderate contribution from flow is far less clear (Fig. 9.11b) owing to the similarities between the Gaussian and cosine

Figure 9.11. Simulated diffusion attenuation profiles illustrating the influence of convection. Trace (a) illustrates signal decay for a diffusion coefficient of $10 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ in the absence of convection whilst in trace (b) shows how a moderate flow velocity (0.1 mm s^{-1}) accelerates signal attenuation. In trace (c) a relatively high flow velocity (0.3 mm s^{-1}) induces pronounced negative going transitions.

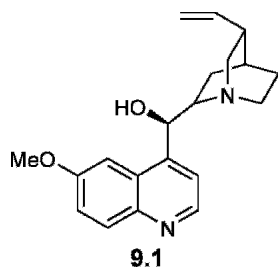


function profiles. Hence, convection-moderated data can appear to fit the usual exponential function characterising diffusion rather well but the apparent diffusion coefficients D_{app} so determined will be greater than the true value D and may be approximated as [16]

$$D_{\text{app}} = D + \frac{\Delta v^2}{2} \quad (9.11)$$

Note that flow velocities as little as 0.05 mm s^{-1} can be sufficient to lead to an erroneous apparent diffusion coefficient for a species whose actual value of D is $1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

A more sensitive and reliable test for the presence of convection is to record two or more diffusion experiments with differing values of the diffusion period Δ under otherwise identical conditions. In the absence of convection (or where its influence has been suppressed), the value of D obtained should not differ between data sets. In contrast, the *apparent* diffusion coefficients measured in the presence of convection will vary with Δ , as indicated by Eq. (9.11), and produce progressively larger values of D_{app} with longer diffusion periods. This influence is readily apparent for quinine **9.1** in CDCl_3 recorded at the slightly elevated temperature of 313 K but may also be observed at a much reduced level at 298 K where probe temperature regulation is employed (Fig. 9.12).



A further approach to establishing the presence of convection is to record a series of 1D diffusion traces with an *asymmetrical* version of the double stimulated-echo experiment introduced to compensate for convection (a description of which is given below that you may wish to consider before proceeding). In the compensated sequence, the total diffusion period Δ is split into two *equal* periods (here denoted Δ_1 and Δ_2 with $\Delta_1 + \Delta_2 = \Delta$) such that contributions from convection arising during the first echo are exactly cancelled by those in the second. It then follows that an asymmetrical double echo in which $\Delta_1 \neq \Delta_2$ will lead to only partial cancellation of these contributions according to the imbalance in the ratio $\Delta_1:\Delta_2$. Thus, a sequence of 1D experiments in which Δ is held constant but Δ_1 and Δ_2 are systematically varied will present spectra with increasing signal attenuation in the presence of convection as the imbalance in the two diffusion periods increases due to the progressively larger contributions to signal attenuation from uncompensated convection. Conversely, uniform intensity will be observed for all spectra in the absence of convection

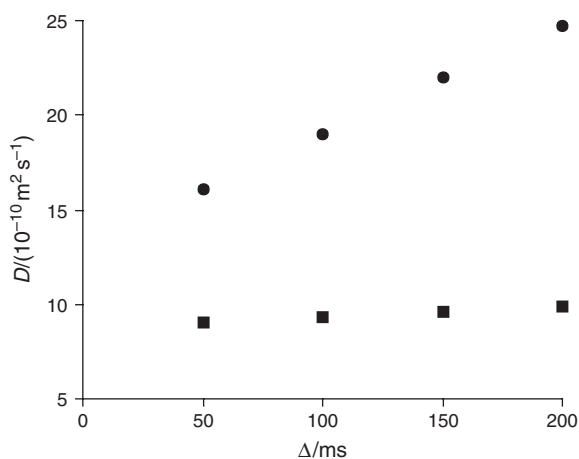


Figure 9.12. Diffusion coefficients for quinine **9.1** in CDCl_3 as a function of diffusion time Δ recorded at 298 K (squares) and 313 K (circles) demonstrating the influence of convection.

since the total diffusion period Δ remains invariant (Fig. 9.13). This method may also be employed to determine flow velocities within NMR tubes as detailed in reference [15].

Dealing with convection

As indicated above, when convection can be shown to be present within a sample, there are essentially two approaches for negating this; one may seek to suppress convection itself or one can employ methods that are designed to compensate for it. The optimum approach will depend on a number of factors relating to the nature of the sample and the behaviour of the instrumentation, so both these approaches will be considered before some general recommendations are presented.

Before proceeding, it is instructive to consider the conditions under which convection arises. The onset of convection, as driven by temperature gradients along the length of the NMR sample, is characterised by the Rayleigh number, R , which for a long cylindrical sample may be defined as

$$R = \frac{g\alpha}{\kappa\nu} r^4 T^* \quad (9.12)$$

where g is the acceleration of gravity, α is the thermal expansivity, κ is the thermal diffusivity, ν is the kinematic viscosity², r is the (inner) radius of the NMR tube and T^* is the temperature gradient over the length of the sample (equivalent to $\Delta T/l$ where ΔT is the temperature difference across a sample height l). According to theory, convection will arise when the Rayleigh number for the solution exceeds a critical threshold, which itself depends on the thermal conductivity of the sample container walls (i.e. the NMR tube walls). Although in practice the onset of convection may deviate from this idealised expression, it does serve to suggest how one may at least deter this onset, as discussed below.

Temperature gradients and decoupling

It is apparent from above that one option to delay the onset of convection is to reduce the temperature gradient along the sample column. A crude approach to this is to not use active temperature regulation of the sample at all; that is, have no gas flow over the sample and to ensure it is left to fully equilibrate, although this is clearly somewhat limiting and possibly impractical. Certainly, it is critical to allow samples to equilibrate under whatever conditions are used prior to diffusion measurements, and the timescales for this are considerably greater than would typically be considered sufficient for most high-resolution NMR experiments; equilibration periods of 30 min or more are often appropriate.

When active temperature regulation is employed, any temperature gradients generated are, at least in part, dictated by probe design, for which most of us have no immediate control. However, it has been demonstrated that temperature gradients along NMR samples may be substantially reduced by using high gas flow rates over the sample [15, 17, 18]. Whilst higher flow rates may be better, these may induce undesirable sample vibrations or even lift the sample out of the probe head if too large and a good quality, heavy sample turbine may be beneficial in this respect. An additional insidious cause of temperature gradients arises from non-uniform rf heating of a sample through the use of broadband decoupling, as would be required, for example, in the case of diffusion measurements of heteronuclear species benefiting from proton decoupling. Indeed, even conventional proton decoupling using composite decoupling schemes such as WALTZ-16 (see Section 10.3) can induce measurable changes in diffusion coefficients, even at ambient temperatures for low-viscosity solvents. In such cases, reduced decoupler powers may be required, with correspondingly longer decoupler pulse widths. The use of extended relaxation delays during which the decoupler is gated off also proves beneficial.

Additional methods to eliminate temperature gradients include the use of coaxial inserts to hold the solution within a conventional NMR tube so as to jacket the sample, with the intermediate space filled with a perfluorinated hydrocarbon oil [19], an NMR silent solvent [20] or simply just empty space [21], the last of these having been demonstrated specifically for low-temperature (~ 230 K) studies.

Tube diameters

As indicated by Eq. (9.12), an alternative approach to delaying the onset of convection is to reduce the diameter of the NMR tube [18] since the Rayleigh number scales with r^4 . Thus, reducing the tube diameter from 5 to 3 mm will require temperature gradients to be in excess of an order of magnitude greater for convection to arise in the narrower tube as

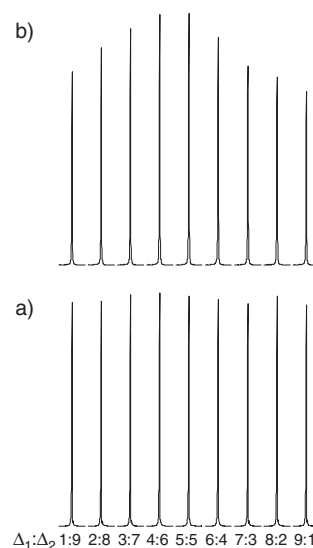


Figure 9.13. Signal profiles recorded with the asymmetrical double stimulated-echo (aDSTE) sequence as a test for convection as the ratio $\Delta_1:\Delta_2$ is varied. (a) A uniform profile indicates the absence of convection whereas in (b) the asymmetrical profile demonstrates convection to be present.

² Thermal expansivity is a measure of the expansion of a sample as a result of heating, thermal diffusivity is a measure of the rate at which a temperature disturbance applied to one part of a sample travels to another and kinematic viscosity reflects the viscosity–density ratio.

compared to those in the wider tube. The disadvantage of such an approach is the reduction in signal intensity with the smaller sample volume (for a fixed sample concentration), so may only be appropriate when sample strength is not a limiting factor. A related approach is to employ a reduced sample column height to reduce the total temperature gradient across the sample and purpose designed, susceptibility matched tubes have been developed for this [22]. Whilst these may be impractical for routine work, ensuring solution depths are not excessive appears a prudent experimental procedure.

Solvent viscosity

Eq. (9.12) further indicates that a solvent with greater viscosity will be less likely to suffer from convection, as one may anticipate. Thus, lower viscosity solvents (Table 9.4) such as acetone and acetonitrile may suffer from convection even at temperatures within a few degrees of ambient (unregulated) probe temperatures whereas more viscous solvents such as dimethylsulfoxide and water are more robust in this respect. However, solvent selection is more usually dictated by the solubility and stability of the compounds of interest and may offer limited scope for dealing with convection.

Sample spinning

The rotation of an NMR sample tube has also been demonstrated to reduce convection owing to the induced stabilising forces acting to retard the onset of laminar flow [23, 24]. This effect is illustrated in Fig. 9.14, which shows the measured diffusion coefficients of quinine **9.1** in CDCl_3 recorded at 313 K for a static sample and sample spinning at 20 Hz, as a function of Δ . Spinning the sample produces a reliable measure of the diffusion coefficient that is independent of Δ . However, an absolute requirement for this approach to be effective is that the spinning rate and diffusion periods must be synchronised such that the sample tube prescribes an integer number of rotations during the diffusion period. Thus, for a sample spinning at 20 Hz, appropriate diffusion periods would be 50 ms or multiples thereof. This is to prevent any net displacement of the solute molecules along the direction of the applied gradient due to the physical relocation of the particle through rotation. If such a displacement were to occur in the period between the defocusing and refocusing gradient pulses, this would contribute to signal attenuation in addition to that arising from diffusion itself and so result in an erroneously high apparent diffusion coefficient. This process is illustrated in exaggerated form in Fig. 9.15; a lack of perfect concentricity between the sample tube and the gradient coils means the solute

Table 9.4. Viscosities of some common solvents at 298 K. The SI units of mPa.s are equivalent to the widely quoted centipoise (cP)

Solvent	Viscosity (mPa.s)
Acetone	0.31
Acetonitrile	0.35
Benzene	0.60
Chloroform	0.54
Dichloromethane	0.41
Dimethylsulfoxide	1.99
Methanol	0.54
Tetrahydrofuran	0.46
Toluene	0.56
Water	0.89

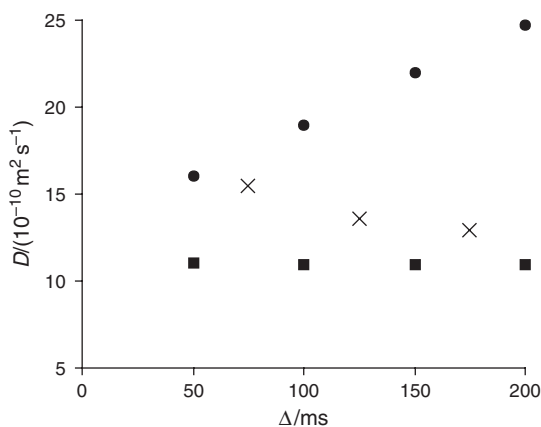


Figure 9.14. Observed diffusion coefficients of quinine **9.1** in CDCl_3 at 313 K recorded as a function of Δ for a static sample (circles) and for the sample spinning at 20 Hz (squares). When the spin rate is not synchronised with diffusion time the apparent diffusion coefficients may be erroneous and dependent on Δ (crosses).

particle effectively travels along the gradient direction as the sample rotates, whereas no net displacement arises following a complete revolution. Since NMR diffusion measurements are sensitive to translational motions on the micrometer scale, only a miniscule displacement of a particle by rotation is sufficient to interfere with diffusion measurements. The effect of mismatched sample spinning is also illustrated in Fig. 9.14 wherein erroneously high apparent diffusion coefficients are measured when the diffusion period Δ and the spin rate are no longer synchronised; in this case where Δ is not a multiple of 50 ms. Thus, whilst sample spinning can be an effective means of suppressing convection, it demands cautious implementation, stable sample rotation and benefits from high-quality tubes. Furthermore, at temperature extremes in particular, spinning may not lead to complete convection suppression and a check for the presence of this, as described above, is still desirable.

Convection compensating sequences

The most effective approach to removing the deleterious effects of convection is to employ sequences that directly compensate for this effect by cancelling the influence of flow in the sample – the so-called double-echo experiment. It has already been stated above that convection imposes an attenuation factor on the NMR response in addition to that arising from diffusion itself, as was expressed in Eq. (9.10) above. More precisely, the presence of linear flow during a gradient echo sequence imposes a *velocity*-dependent phase on the NMR signal that arises because the spins move during the gradient pulse and so experience a varying magnetic field. This velocity-dependent term acts in addition to the spatially dependent phase imposed by the field gradient pulse. In any convecting NMR sample, molecules will be moving in both upward and downward directions and will exhibit a spread of flow velocities such that the net effect of the various imposed phases is the aforementioned attenuation of the detected NMR response.

In a basic gradient echo refocusing experiment such as that in Fig. 9.16a, the *spatially* dependent phase imposed by the first gradient may be refocused simply by applying a second equal but opposite gradient to produce the echo. However, in the presence of convective flow there remains a velocity-induced phase after the second gradient pulse, which depends on the duration of the gradient pulses, the time between these and the velocity itself. In the typical situation where the gradient durations are small relative to the time between them, the remaining phase is primarily due to the linear flow that occurs between the gradient pulses meaning different local fields are experienced by spins during these pulses. This unwanted residual phase may however be cancelled by repeating the echo sequence but with inversion of the sign of the two gradient pulses (Fig. 9.16b). The spatially dependent phase is again refocused within this second gradient pair but, critically, the residual flow-induced phase is now equal but opposite to that produced by the first gradient pair. Thus, the velocity-dependent phase arising from the first pair of gradients is exactly cancelled by that arising from the second pair, and the desired convection compensation is achieved using a ‘double-echo’.

In this, it has been assumed that the coherence order, p , of magnetisation (Section 5.4) has remained constant (at either +1 or -1) throughout the echo sequence. In fact, the velocity-induced phase is also sensitive to the sign of the coherence suggesting an equivalent approach to convection compensation is to repeat the echo with gradients of the same sign but with an associated change of coherence order (brought about by appropriate rf pulses) between the two echoes (e.g. $p = +1$ to $p = -1$) as in Fig. 9.1c. This approach has been incorporated in the mostly widely used convection compensated sequences. In the double stimulated-echo (D-STE) sequence (Fig. 9.17a), the total diffusion period Δ is now divided in two such that each half comprises the basic stimulated-echo sequence. The

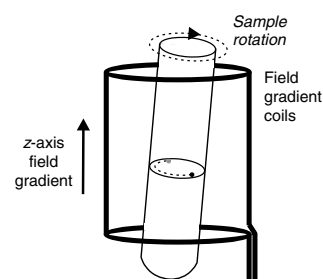


Figure 9.15. Schematic (and exaggerated) illustration of molecular translation brought about by sample spinning in cases where the axes of the sample tube and gradient coils are not perfectly aligned.

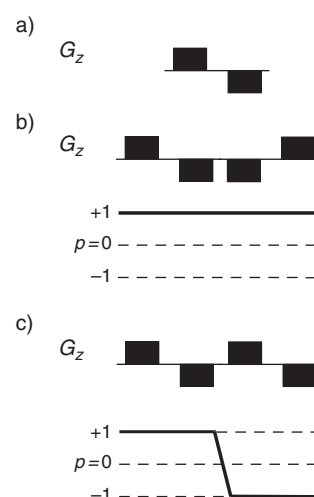


Figure 9.16. Generic gradient schemes for convection compensation. (a) A basic refocusing gradient echo scheme, (b) a double-echo scheme to refocus velocity-dependent phase (with retention of coherence order at +1) and (c) a double-echo scheme to refocus velocity-dependent phase (with inversion of coherence order +1 to -1). The change in coherence order is only possible by the application of suitable radiofrequency (rf) pulses (not shown).

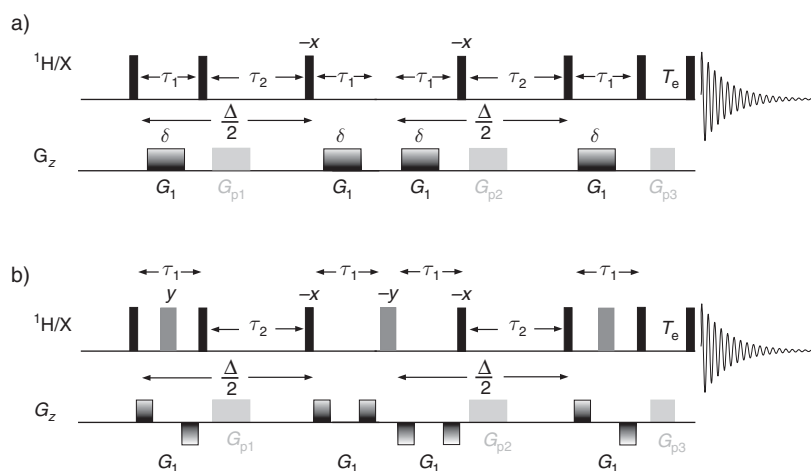
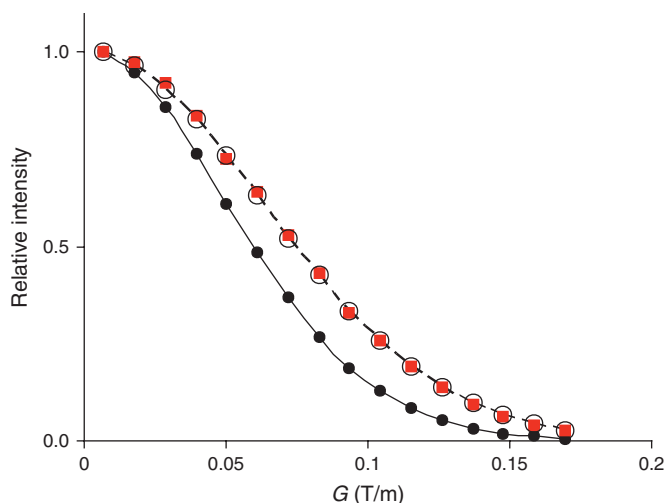


Figure 9.17. Convection compensated diffusion sequences. (a) The double stimulated-echo DSTE, (b) the double stimulated-echo with bipolar pulses, BPP-DSTE. Delays are as for the stimulated-echo scheme but the total diffusion period Δ is split and applied as two echo sequences. Optional gradient pulses (denoted G_p) and the LED scheme are also shown; see [25] for details of the necessary phase cycling.

Figure 9.18. Diffusion decay profiles for quinine **9.1** in CDCl_3 at 313 K recorded with the BPP-STE sequence (closed circles), the double-BPP-STE sequence (red squares) and the BPP-STE sequence with the sample spinning at 20 Hz (open circles). The total diffusion time Δ in each case was 100 ms with gradient pulses δ totalling 4 ms each.



gradient pulses are applied with the same sign throughout demanding the coherence orders selected by phase cycling are [25] $p = -1, +1, +1, -1$ when these are applied to give the desired convection compensation. As for the non-compensated sequences described above, the use of an eddy current delay period may also be advantageous, and additional purging gradients may be employed during the diffusion or LED periods when magnetisation is longitudinal. A further refinement is the incorporation of bipolar pulses (Fig. 9.17b); in this scheme, the two adjacent sets of BPPs at the centre of the sequence have been combined such that only a single 180° refocusing pulse is necessary. Fitting to the Stejskal–Tanner equation is as for the stimulated-echo.

Figure 9.18 compares the diffusion decay profiles of the uncompensated BPP-STE and the compensated BPP-DSTE sequences for a sample of quinine **9.1** in CDCl_3 at 313 K. The apparent diffusion coefficient from the STE data of $17.1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ is erroneously high due to the contribution from convection whereas a lower value of $11.1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ is provided both by the double stimulated-echo method and for the STE with sample rotation. Whilst providing effective compensation for convection, the double stimulated-echo sequences suffer from the disadvantage of reduced sensitivity as each echo sacrifices half of the available signal, which may make the sequences less attractive in some instances. In contrast, double-echo sequences based on spin-echoes rather than stimulated-echoes do not suffer such sensitivity losses and have been employed in some combined 3D-DOSY sequences [26].

Summary

When executing diffusion experiments, it is critically important that convection does not contribute to the observed signal decay intensities, and its presence should always be considered a possibility. This is most likely to arise for lower viscosity solvents or when operating at elevated or reduced sample temperatures. A simple approach to test for this is to perform the experiment with two or more diffusion periods Δ and to compare the measured diffusion coefficients. In the absence of convection, these will be the same. When convection is present it may be suppressed by appropriate physical measures such as synchronised sample spinning or the use of narrower NMR tubes. Alternatively, its influence on recorded data may be compensated by the use of suitable pulse sequences.

9.3.2. Calibrating gradient amplitudes

A fundamental requirement for the accurate determination of diffusion coefficients is a measure of the gradient strengths employed, and there are essentially two procedures that find widespread use for calibrating the gradient amplitudes produced by an instrument. This amplitude will represent the combined capabilities of the gradient amplifier and the gradient coils themselves and thus may vary between consoles and between probes. In principle, the actual field gradients delivered by a probe can be calculated from the coil geometry, and knowledge of the gradient amplifier output and vendors often quote the maximum field gradient a probe can produce based on this. Nevertheless, a basic calibration is recommended if only to confirm this to be the case for your system.

The first method is based on taking a 1D image of a ‘phantom’ in an NMR tube and calculating the applied gradient strength from this; this procedure is described in Section 3.5.2. The second approach is to determine the strength from a diffusion experiment performed at a defined temperature by using a suitable calibration sample of known diffusion coefficient. In this, single-diffusion traces are acquired with a nominal fixed gradient strength applied (often defined

as some fraction of the maximum gradient amplifier output) and the resonance intensity of the calibrant compared with that in the absence of gradient pulses. The simple gradient spin-echo scheme of Section 9.2.1 is well suited for this. The applied gradient amplitude G may then be back-calculated from the Stejskal–Tanner equation (Eq. (9.6)) since the diffusion coefficient of the calibrant is known. A commonly used sample for this is H_2O in D_2O for which the diffusion coefficient at 298 K is given by [20, 27]:

$$D_{\text{HDO}(298\text{K})} = 2.30 - 0.4652x + 0.0672x^2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1} \quad (9.13)$$

where x represents the mole fraction of D_2O in the sample. Thus, the residual HDO in ‘pure’ D_2O (99.9% D) has a diffusion coefficient of $1.902 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ and provides a suitable calibrant for the back calculation of G . Whilst this method may appear more straightforward than the use of a phantom, it does require that the sample temperature has also been accurately calibrated (see Section 3.5.3) and regulated, and that convection does not hamper the amplitude intensities. Its notable advantage is that, provided the calibration uses the same sequence as subsequent measurements, this procedure allows for any non-ideal behaviour in the field gradients produced. Reference data for a selection of calibration samples for nuclei other than ^1H have also been reported should these ever be required and include ^7Li , ^{13}C , ^{19}F and ^{31}P [20].

9.3.3. Optimising diffusion parameters

Diffusion coefficients depend on many parameters including the size and shape of the solute under investigation, properties of the solvent such as its viscosity, sample temperature and concentration and so on, meaning it is most often the case that optimum parameters for diffusion experiments have to be determined on a sample-to-sample basis. When the physical conditions for the experiment have been decided (solvent, temperature, etc) the principal parameters that remain to be defined are the diffusion period Δ , the gradient pulse duration δ (and possibly its shape) and the range of gradient strengths to be employed, $G_{\min}$ to $G_{\max}$. The aim is to obtain a well-characterised decay profile to allow reliable data fitting (Fig. 9.19). The optimisation may be done following an iterative process along the following lines for a standard high-resolution probe head:

- Select a value for Δ based on prior knowledge or literature precedence; 50–100 ms being a typical value for ‘small’ molecules.
- Select a value for δ using similar guidance; 2–4 ms is often appropriate.
- Set the gradient output amplitude to a small value, for example $\sim 2\%$ of maximum output.
- Collect a single 1D diffusion trace as a reference point (trace A) using sufficient transients to clearly see all peaks of interest. This would equate to the first data point of the full diffusion experiment.
- In a second data set, repeat the above acquisition with the gradient amplitude set close to the maximum possible value ($\sim 90\%$ of maximum output) but with otherwise identical acquisition parameters to provide a test spectrum (trace B).
- Compare trace B with the reference trace A; one should expect to observe a significant reduction in signal intensity.

The aim is for the signal intensity in trace B to be $\sim 10\%$ of reference trace A for the solutes of interest as this will represent the final data point in the full diffusion experiment (Fig. 9.20).

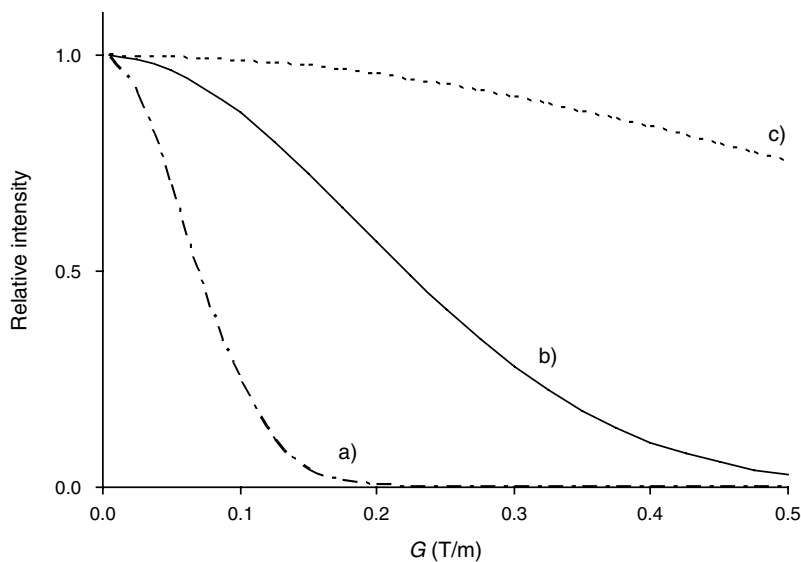
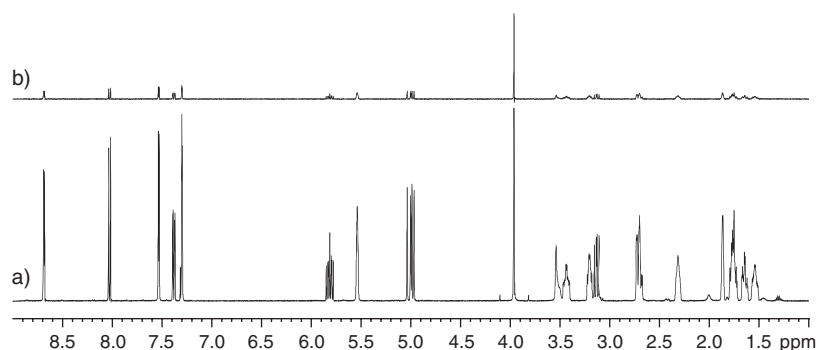


Figure 9.19. Diffusion decay profiles. When the signal attenuation is too fast (trace (a)), the later data points do not contribute to the profile, when it is too slow (trace (c)) there is insufficient attenuation to provide an accurate determination of D . Trace (b) shows an optimum profile where all data points would contribute to characterisation of the decay.

Figure 9.20. Experimental (1D) ^1H diffusion traces for quinine **9.1** showing gradient optimisation with (a) an initial minimum gradient value $G_{\min}$ of 2% (of maximum output) and (b) an maximum gradient value $G_{\max}$ of 70%. The residual signal in (b) is 7% of that in (a).



This process is iterated with suitable parameter modification to achieve the desired attenuation. Thus if the signal has been completely attenuated in trace B, then the upper gradient amplitude and/or the diffusion time may be reduced. Shortening Δ may be advantageous as this reduces relaxation losses, although its selection may be constrained if sample rotation is employed; see Section 9.3.1 above. If the signal attenuation in trace B is insufficient, then a longer diffusion period may be employed although this must remain shorter than, or comparable to, the T_1 relaxation time constant (or T_2 for the spin-echo-based sequences) so that signal intensity is not overly sacrificed prior to further diffusion-based attenuation. An alternative approach in such cases is to increase the net gradient power applied by increasing δ , noting here that signal attenuation scales with δ^2 but is linear in Δ . The profiles of Fig. 9.21 are simulated for $D = 10 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and illustrate the influence of the Δ and δ parameters on signal attenuation and may offer some guidance on parameter selection.

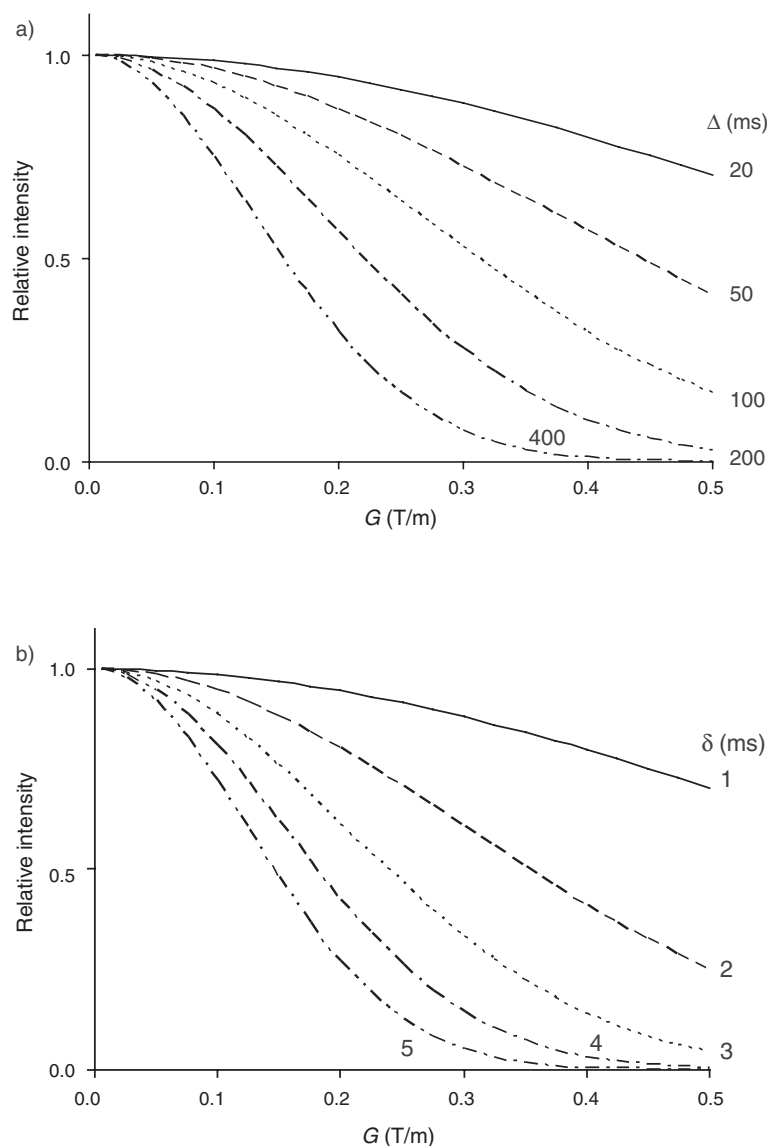


Figure 9.21. Diffusion decay profiles illustrating the influence of the key experimental parameters Δ and δ ; (a) variation as a function of Δ ($D = 10 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and $\delta = 1 \text{ ms}$), (b) variation as a function of δ ($D = 10 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and $\Delta = 20 \text{ ms}$).

Ultimately, one may need to alter Δ , δ and $G_{\max}$ to achieve optimum signal decay profiles. The simulation of diffusion decay profiles can be usefully employed when setting-up diffusion experiments on new systems by suggesting appropriate initial parameters prior to optimisation. Typical values for Δ are milliseconds to hundreds of milliseconds and those for δ are in the range 1–10 ms.

During the set-up procedure, and depending on the nature of the systems under study, one should remain alert to the possibility of damaging hardware if the gradient amplifier is driven too high for too long, and it is wise to be guided by vendor specifications appropriate to your system for selecting δ and $G_{\max}$. Indeed, for the study of very slowly moving particles such as large macromolecular species for example, purpose built gradient amplifiers and water-cooled probe heads are commercially available that are able to deliver far larger gradient strengths than conventional high-resolution probe heads. You may also choose to use non-rectangular gradient profiles to reduce eddy current generation in which case the resultant gradient amplitudes will be less than those for rectangular pulses of equivalent duration. An appropriate gradient shape factor must then be employed in the fitting the Stejskal–Tanner equation to account for this. Equivalently, one can simply apply a correction factor to the gradient amplitudes used in the fit to allow for the difference in integrated intensities of the shape profiles; the shape factor for the commonly employed half-sine bell shape is $2/\pi$ or 0.6366. Of course, the chosen gradient shape must also be employed during optimisation of the parameters for the diffusion experiment. Finally, if using the LED extension it remains to determine what duration is needed to remove residual lineshape distortions. This is best done through ‘trial and error’ by examining 1D diffusion traces recorded with a high gradient strength (i.e. equating to the parameters for the final spectrum of the diffusion experiment) and increasing T_e from, say, 1 ms to find a suitable value. Periods of 5–10 ms are most commonly reported in the literature although the use of modern shielded probe heads and shaped gradients may make this stage superfluous.

When selecting parameters one may also need to be aware of the possibility of solutes exhibiting a range of diffusion coefficients if dealing with sample mixtures, and it may not be possible to characterise reliably the diffusion of all components with a single diffusion parameter set. Thus, for example, if one were looking to characterise both macromolecules and small molecules in the same sample, it may be that the attenuation of the small molecules is far too rapid to characterise adequately when diffusion parameters are optimised for the macromolecule. In practice, it is easily possible to measure diffusion coefficients varying over an order of magnitude from a single data set, as illustrated in Fig. 9.22, and possibly over two orders for good quality data, whereas larger differences are likely to demand multiple experiments with appropriately optimised parameters. The diffusion coefficients of many small molecules and their complexes often fall in the region of $1\text{--}20 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and may therefore be readily characterised by ^1H diffusion experiments using standard gradient hardware.

Besides ^1H measurements, there are potential benefits in the observation of other nuclides for diffusion measurements. There may be fewer problems with resonance overlap of different species or less interference from solvent or impurity resonances, for example, or the compound of interest may simply lack hydrogen as is often the case with counter-ions. In this case, one should be aware that optimum parameters for ^1H measurement may not be suitable for nuclides of lower magnetogyric ratio, γ . This is because the total *effective* gradient strengths employed also depend on this parameter and, as can be seen from the Stejskal–Tanner equation (Eq. (9.6)), the degree of attenuation of resonance intensity will be reduced as γ becomes smaller. This is illustrated in Fig. 9.23 where the decay profiles for four different nuclides are shown, having been calculated with identical diffusion parameters but with the appropriate γ

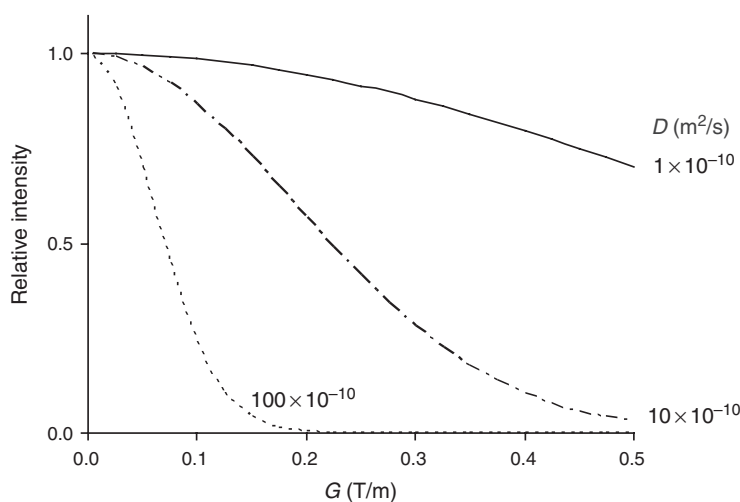
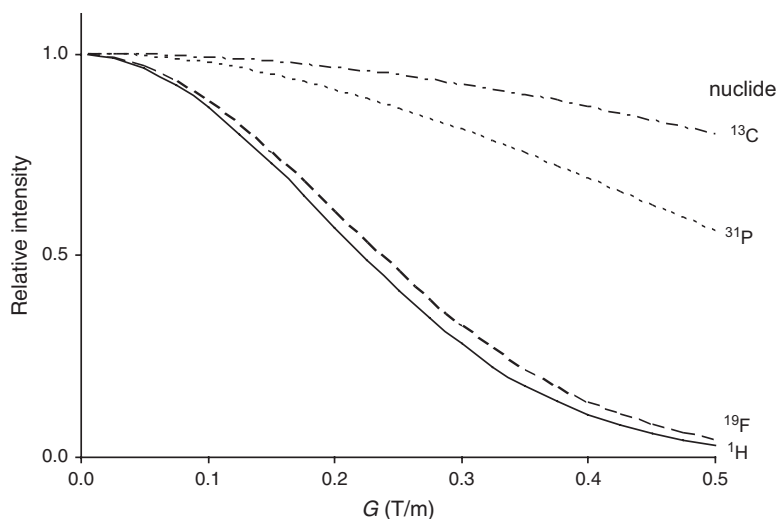


Figure 9.22. Diffusion decay profiles for three diffusion coefficients spanning two orders of magnitude (1 , 10 and $100 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$). Diffusion parameters were $\Delta = 50 \text{ ms}$ and $\delta = 2 \text{ ms}$.

Figure 9.23. Diffusion decay profiles calculated for when differing nuclides are being observed. The parameters are optimum for ^1H observations ($D = 10 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$, $\Delta = 50 \text{ ms}$ and $\delta = 2 \text{ ms}$) but the profiles change according to the magnetogyric ratio (γ) of the observed nuclide, becoming sub-optimal for smaller values of γ .



value employed. Clearly, whilst the profiles for ^1H and ^{19}F are perfectly adequate, those for ^{31}P and ^{13}C are less so. This means that larger diffusion times and/or gradient pulse durations will be required relative to those suitable for ^1H observations when lower γ nuclides are studied and may thus demand a separate optimisation procedure for each nuclide to be observed. A problem that is likely to be relevant only to proton observation, although is likely to be rarely encountered, is that of radiation damping caused by very concentrated samples. This has been shown to interfere severely with diffusion measurements, although the simple expedient of using a reduced excitation flip angle can greatly reduce the problem [28].

9.3.4. Hydrodynamic radii and molecular weights

Having made careful measurements of diffusion coefficients, it may be inviting in some instances to use these to calculate hydrodynamic radii and molecular volumes, and there are many reports of this in the literature, as highlighted in the following section. In doing so, there are a number of issues to be addressed, some of which may influence the experimental protocol one employs. The hydrodynamic radius of a molecule or complex may be derived directly from the diffusion coefficient using the Stokes–Einstein equation (Eq. (9.4)), which itself includes terms for the sample temperature T and the solution viscosity η . Whilst the temperature may be regulated following appropriate calibration, knowledge of the solution viscosity may be less readily available and there are two general approaches to obviating the need for direct viscosity measurements. The first is simply to assume the solution viscosity is that of the pure solvent under the conditions employed, which may only be applicable for dilute solutions, perhaps low mM. The second is to utilise a calibrant molecule of known hydrodynamic radius placed in the solution and use the measured diffusion coefficient of this to calculate η or alternatively to negate the need for this parameter. This requires that the internal reference material is of similar size to the species being investigated such that the same Stokes–Einstein scaling factor applies (see below). In this case the hydrodynamic radius of the solute r_s may be determined from that of the reference r_{ref} by comparison of the respective diffusion coefficients D_s and D_{ref} :

$$\frac{D_s}{D_{\text{ref}}} = \frac{r_{\text{ref}}}{r_s} \quad (9.14)$$

Both tetramethylsilane (TMS) and terakis(trimethylsilyl)silane (TMSS) have been suggested as internal calibrants no doubt owing to their being chemically inert and in approximating well to the desired spherical shape. The use of an internal reference such as this can also be useful in indicating viscosity changes on a sample to sample basis even if r_s itself is not required and may also act as an indicator of convective flow if their diffusion coefficients differ greatly.

If solution viscosity is known then r_s may be calculated directly from the Stokes–Einstein equation, thus:

$$r_s = \frac{k_b T}{c \pi \eta D_s} \quad (9.15)$$

where here the scaling factor in the denominator, c , typically has a value of 6. In fact this is only applicable for molecules or complexes that are significantly larger than the solvent molecules surrounding the particle, falling to around 4 when these become comparable. It has been

suggested that $c=6$ is applicable if $r_s > 0.5$ nm but may require correction for smaller molecules. The following equation has been proposed [29] based on a semi-empirical modification of that arising from friction theory, which includes explicitly the solvent hydrodynamic radius r_{solv} :

$$c = \frac{6}{\left[1 + 0.695 \left(\frac{r_{\text{solv}}}{r_s}\right)^{2.234}\right]} \quad (9.16)$$

It is further possible to relate the hydrodynamic radius of a particle to its partial specific volume, $\bar{v}$, which in turn allows one to correlate the diffusion coefficient of the particle with its molecular weight, M . Thus, for the case of a spherical particle the following relationship holds [30]:

$$r_s = \sqrt[3]{\frac{3M\bar{v}}{4\pi N_A}} \quad (9.17)$$

in which N_A is Avogadro's number. This indicates the diffusion coefficient itself has a reciprocal cube-root dependence on molecular weight. This may be used in a relative sense to relate the diffusion coefficients of two molecules D_s and D_{ref} to their molecular weights M_s and M_{ref} :

$$\frac{D_s}{D_{\text{ref}}} = \sqrt[3]{\frac{M_{\text{ref}}}{M_s}} \quad (9.18)$$

Again, this applies to idealised spherical molecules but may nonetheless provide a useful approximation for comparison between solutes, whereas deviations from such idealised geometry may demand the use of corrective shape factors; see reference [30] and references therein.

9.4. APPLICATIONS OF DIFFUSION NMR SPECTROSCOPY

In the sections above, we have reviewed the most widely used diffusion sequences and have explored the important experimental parameters and procedures for recording meaningful diffusion data. This section will illustrate some of the applications of diffusion NMR spectroscopy that have appeared in the literature in recent years and whilst not exhaustive is meant to give sufficient insight to allow one to realise opportunities where these methods may be applicable to your own work. A number of extensive reviews are available that deal with such applications [3, 31–33], and the interested reader is encouraged to refer to these. It is noteworthy that many of these reviews deal simultaneously with diffusion and the NOE and stands testament to the manner in which these methods may be combined in the elucidation of intermolecular interactions in particular, a recurrent theme in the examples below.

9.4.1. Signal suppression

A rather simple but sometimes highly effective application of diffusion NMR methods is in the editing of the NMR spectrum to suppress unwanted resonances, whereby use is made of the differing diffusion properties of species in the solution even though a measurement of diffusion coefficients themselves is not necessary. A typical application might be the selective removal of the resonance(s) of a solvent. In this, a set of 1D diffusion parameters are established in which the faster moving solvent has been completely attenuated whereas the slower diffusing solutes of interest are themselves also attenuated but to a lesser degree and thus remain in the resulting 1D spectrum. This approach is illustrated in Fig. 9.24 for an

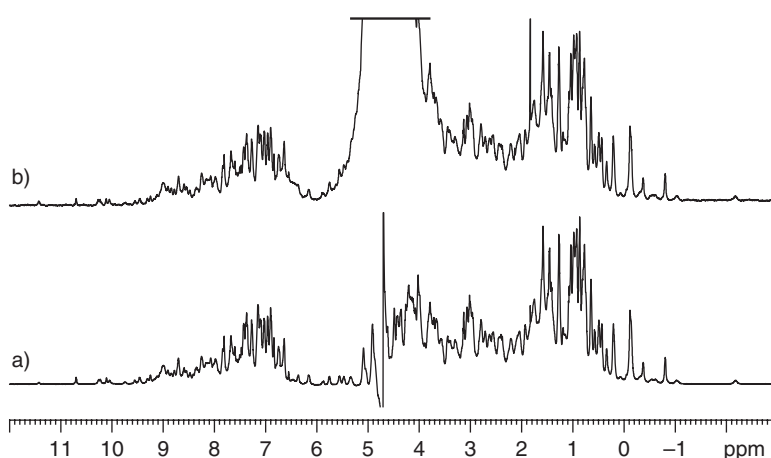
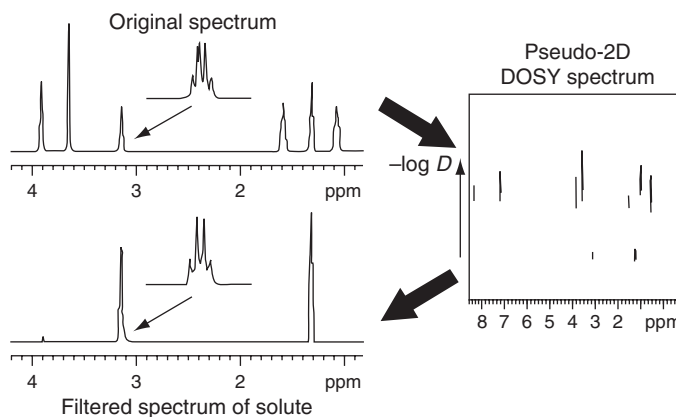


Figure 9.24. Solvent signal suppression by a diffusion filter. The sample is 1 mM lysozyme in 50:50 $\text{H}_2\text{O}:\text{D}_2\text{O}$ and suppression in (a) was achieved using a ^1H stimulated-echo-based sequence employing a diffusion delay Δ of 50 ms, bipolar gradient pairs of 4 ms total duration (δ) and gradient strengths of 0.43 Tm^{-1} . The standard ^1H spectrum is shown in (b).

Figure 9.25. The process of signal editing with 2D DOSY. The ^1H spectrum of the solute ethyl bromide can be extracted free from contributions from the neat ionic liquid solvent (reproduced with permission from [34], Copyright Wiley-VCH Verlag GmbH & Co. KGaA.).



aqueous protein solution (lysozyme, M_r 14.4 kDa, in 50:50 $\text{H}_2\text{O}:\text{D}_2\text{O}$) in which the water is essentially fully suppressed but the protein resonances remain visible, even those that are completely masked by the solvent in the absence of suppression (Fig. 9.24b; see Section 10.5 for discussions of alternative solvent suppression schemes).

As a solvent suppression method this requires there to be a sufficiently large difference in the diffusion coefficients of solvent and solute and may therefore be limited to investigations of larger systems or complexes. It does, however, have the notable advantage relative to more conventional signal suppression schemes of being able to suppress multiple signals irrespective of chemical shift and signal overlap. A slightly unusual example in this context is the use of DOSY editing for NMR spectroscopy in neat ionic liquids [34], systems of increasing commercial interest as 'green' solvents. In these systems, the use of deuterated solvents is often impractical or too expensive meaning the solvents often present multiple resonances and are thus poorly suited to conventional selective suppression schemes. However, application of the DOSY approach allows the extraction of a trace from the pseudo-2D plot through the solute alone (which may itself diffuse *faster* than the ionic solvent) to reveal a 1D spectrum devoid of the ionic solvent peak. This concept is illustrated in Fig. 9.25 for ethyl bromide in an imidazolium ionic solvent system. This has the added advantage over coefficient measurements that precise diffusion coefficients are not required so that accurate gradient calibrations are not needed, making implementation straightforward.

9.4.2. Hydrogen bonding

The presence of intermolecular hydrogen bonding has also been investigated successfully with diffusion methods and the relative strength of such bonds probed in a qualitative manner. Thus, in a mixture of phenol and cyclohexanol in CDCl_3 the cyclohexanol exhibits a lower diffusion coefficient than phenol (Fig. 9.26a) owing to the larger bulk of this system and potentially to differences in intermolecular hydrogen bonding. On addition of the hydrogen-bond acceptor DMSO, the phenol is observed to be retarded by a greater extent than the alcohol (Fig. 9.26b), suggesting a greater propensity for the phenol to exist in a H-bonding complex with the DMSO, attributable to its greater acidity relative to the alcohol [35]. In the same report, ^{31}P DOSY was used in a similar manner to study hydrogen bonding of various phosphorus-containing compounds.

In a similar approach, and as part of a larger study [36], hydrogen bonding between *p*-cresol and piperazine has been shown to occur in CDCl_3 with the changes in diffusion coefficients relative to the pure, dilute materials in the same solvent (Table 9.5) being consistent with the 2:1 binding stoichiometry observed in the crystal structure (Fig. 9.27).

Figure 9.26. ^1H DOSY spectra of phenol (1) and cyclohexanol (2) in (a) CDCl_3 and (b) CDCl_3 containing 1 molar equivalent DMSO- d_6 . In CDCl_3 , phenol diffuses more rapidly than cyclohexanol whereas stronger hydrogen bonding with DMSO causes this to be retarded more than the alcohol (reproduced with permission from [35], Copyright 2000, Elsevier).

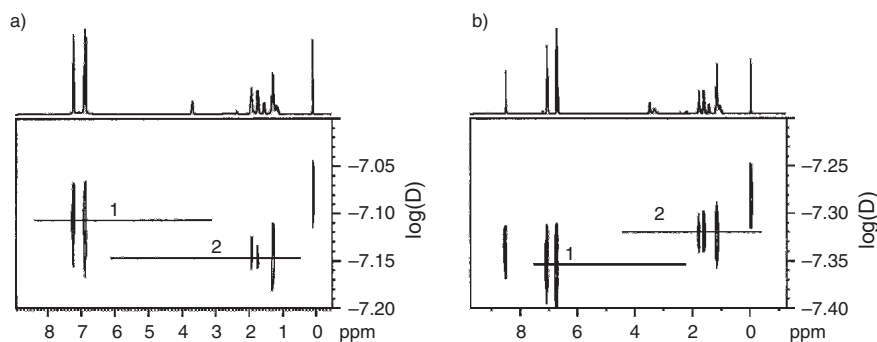
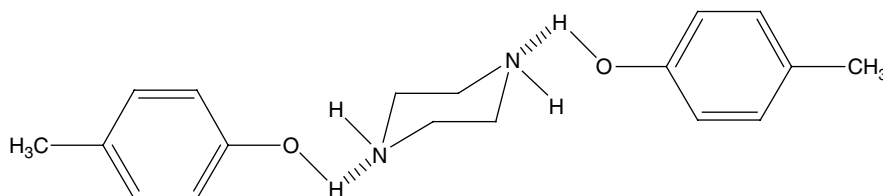


Table 9.5. Diffusion coefficients for pure *p*-cresol and piperazine in CDCl₃ and in the hydrogen-bonded complex

	D (10 ⁻¹⁰ m ² s ⁻¹)	
	Pure	Complex
<i>p</i> -cresol	16.7	9.70
Piperazine	25.3	9.91

Adapted with permission from [36].

**Figure 9.27.** The structure of the *p*-cresol:piperazine hydrogen-bonded complex derived from X-ray data (adapted with permission from [36], Copyright 2003, Elsevier).

The similarity of diffusion coefficients for both molecules in the complex suggests they exist predominantly in the hydrogen-bonded state. In such studies of intermolecular interactions, it has been suggested [37] that TMS offers a suitable internal reference material to allow corrections for viscosity changes to be made as different components are mixed and so isolate those changes in diffusion coefficients arising from hydrogen-bonding interactions.

9.4.3. Host–guest complexes

Host–guest complexes, in which a smaller guest molecule becomes encapsulated within a larger host, present another form of intermolecular association that is well suited to study by diffusion NMR methods. One may observe a measurable change in the diffusion coefficient of the guest molecule as it is taken up by the host providing not only direct evidence for the binding event but also, in favourable situations, allowing the determination of association constants from the diffusion data. In a fast exchange process between the free and bound states of the guest, its observed diffusion coefficient represents the population-weighted average of the diffusion properties of these two states. Monitoring the change in diffusion coefficient *D* as a function of the host–guest ratio allows the determination of association constants, *K_a*; the use of *D* replaces the more usual chemical shift as the NMR observable in such titrations [38].

In the world of host–guest complexes, the cyclodextrins represent one of the most studied host systems by virtue of their open hydrophobic cavities and not surprisingly have been studied by diffusion NMR. One example of the determination of association constants by diffusion measurements is the binding of cyclohexylacetic acid **9.2** and the bile acid cholic acid **9.3** to β -cyclodextrin **9.4** [39]. Fig. 9.28 shows the variation in diffusion coefficients as the hosts are titrated against differing guest concentrations. For the smaller guest **9.2** (Fig. 9.28a), there is an appreciable change in *D* as the guest concentration varies, consistent with the binding process, whereas the diffusion behaviour

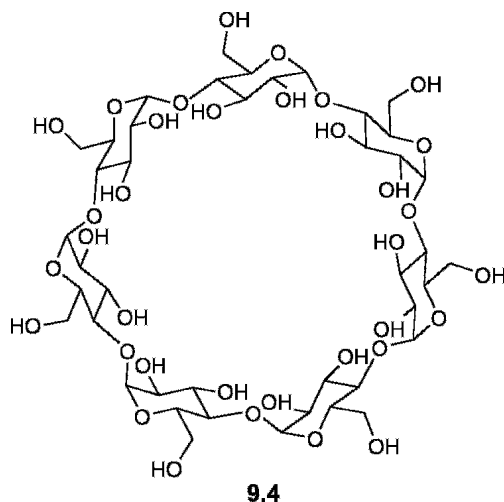
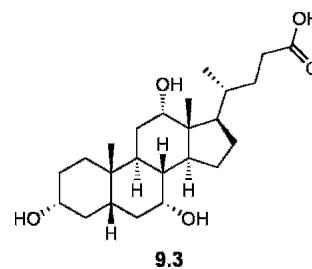
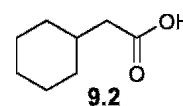


Figure 9.28. The diffusion titration curves for the binding of the host β -CD **9.4** with the guests (a) cyclohexylacetic acid **9.2** ($K_a = 1800 \text{ M}^{-1}$) and (b) cholic acid **9.3** ($K_a = 5900 \text{ M}^{-1}$) (adapted with permission from [39], Copyright 2001, American Chemical Society).

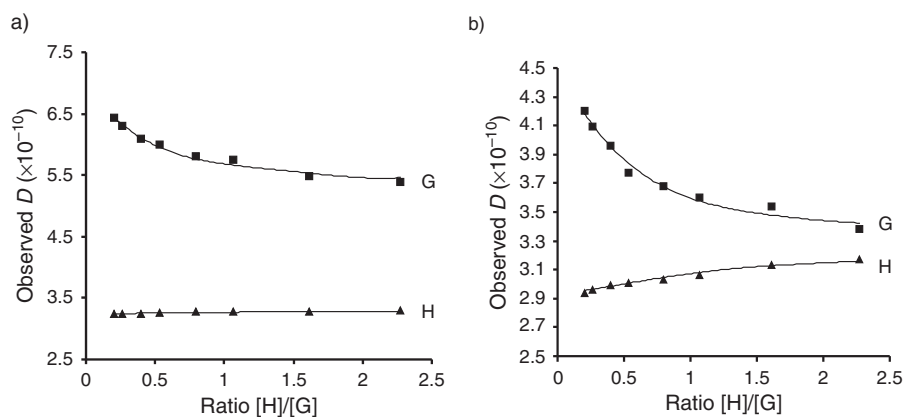


Table 9.6. Diffusion coefficients for the free diaminoalkanes **9.5–9.9** and their α CD complexes in D_2O at 298 K

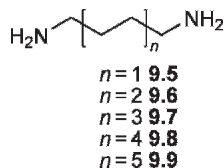
System	Free		Complex		X	$K_a \text{ (M}^{-1}\text{)}$
	D_{amine} ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)		D_{amine} ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)	$D_{\alpha\text{CD}}$ ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)		
α CD	—		—	3.0		
9.5 $n=1$	7.6		7.1	2.9	0.11	15
9.6 $n=2$	6.5		4.9	3.0	0.46	65
9.7 $n=3$	6.0		4.7	2.9	0.42	680
9.8 $n=4$	5.5		3.8	2.9	0.65	13,500
9.9 $n=5$	5.0		3.2	2.6	0.75	7,080

Bound molar fractions X are determined from the measured diffusion coefficients as weighted averages of the values for the bound and free states and association constants K_a are derived from changes in diffusion coefficients as a function of the host–guest ratio.

Adapted with permission from [41], Copyright 2002, American Chemical Society.

of the cyclodextrin host itself is largely invariant to the binding as it is able to consume the small guest. In contrast, addition of the larger steroid guest **9.3** produces a significant change in both the guest and the host diffusion profiles (Fig. 9.28b). The host variation must also be accounted for when determining K_a since the simpler assumption of the host behaviour being unaffected by the presence of the guest is clearly no longer valid. In both cases, the solid curves represent the fitted values for the association constants determined from the simultaneous fitting of both the host and the guest diffusion coefficients.

In a study of camphor binding with cyclodextrins, both chemical shift and diffusion titrations were shown to be equally suitable for the determination of binding constants [40]. However, chemical shift changes are not always resolvable or of sufficient magnitude to be reliable for K_a determination. Thus, the diffusion-based approach was taken in the investigation of the binding of α,ω -diaminoalkanes of various lengths **9.5–9.9** in α CD cavities [41] with a view to determining whether protonation of the nitrogen prevents the diamines from escaping from the cavity and so forming rotaxanes from the initially formed pseudorotaxanes (in which the diamines exchange between the free and complexed states). Owing to the small chemical shift changes on binding, diffusion coefficients were considered a favoured parameter with which to monitor binding events. Table 9.6 shows the variation in diffusion coefficients and association constants as a function of chain length with a general trend of increased binding for longer chains. The **9.5** $n=1$ diamine demonstrates very weak binding with the α CD whereas the exceptional binding for the **9.8** $n=4$ diamine is attributed to additional favourable interactions between the terminal amine and hydroxyls of the cyclodextrin. Further studies on these systems demonstrated that protonation of the amines resulted in weakened binding with the α CD.



9.4.4. Ion pairing

In the field of organometallic chemistry in particular, diffusion measurements have been used extensively by a number of pioneering groups in various studies of complex

Table 9.7. Diffusion coefficients for tetrabutylammonium tetrahydroborate in CDCl₃ as a function of concentration with tetrabutylsilane as internal reference

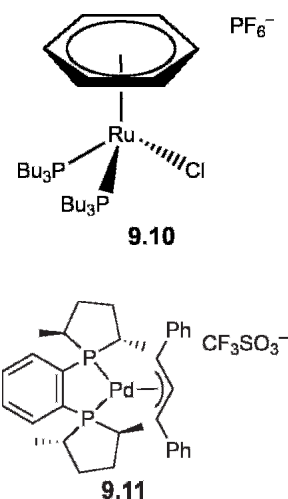
Conc. (mM)	D (10 ⁻¹⁰ m ² s ⁻¹)			Aggregation number
	TBA ⁺	BH ₄ ⁻	Si(Bu) ₄	
200	4.99	5.34	8.64	5.2
40	6.83	7.35	10.3	3.4
8	8.10	8.42	10.5	3.3
2	8.48	8.73	10.6	2.0
0.5	8.34	8.64	10.5	2.0

Adapted with permission from [42], Copyright 1997, American Chemical Society.

coordination and ion pairing [31, 32]. The notion is that when held in an ion pair, the observed diffusion of the ions is retarded relative to that of the dissociated states and in the usual case of fast exchange between the coordinated and dissociated ions a population-weighted average of the individual diffusion coefficients will be observed. In the first application of diffusion NMR to the simultaneous observation of cations and anions as ion pairs [42], it was demonstrated that the tetrabutylammonium (TBA⁺) and tetrahydroborate (BH₄⁻) ions exist as a tight pair in chloroform solution as evidenced by their similar diffusion coefficients and the fact that these were significantly smaller than that of the non-aggregating internal reference tetrabutylsilane. The concentration dependence of the diffusion coefficients indicated the ion pairs formed aggregates, and from comparisons with the diffusion data for Si(Bu)₄, it was possible to estimate the aggregate size derived from estimated molecular volumes (Table 9.7).

In a number of examples, it has also been shown that ion pairing is more strongly favoured in chloroform solutions for many organometallic salts as illustrated by the diffusion data collected for the two complexes **9.10** and **9.11** (Table 9.8) [43]. Thus, in chloroform the anions and cations of both complexes exhibit very similar diffusion coefficients, consistent with them existing as tight ion pairs. However, the different coefficients recorded in dichloromethane suggest the pairing to be weakened and incomplete with the anions now moving significantly faster on average than the larger cations. Nevertheless, comparisons with the hydrodynamic radii of ca. 2.6 Å (PF₆⁻) and 3.2 Å (OTf⁻) for the two anions determined in methanol (a strongly coordinating solvent likely to disrupt the ion pairing) suggest a significant amount of pairing still exists in dichloromethane. These data also serve to illustrate the combined application of ¹H and ¹⁹F diffusion studies, as required for the cations and anions respectively. In a similar multinuclear approach to chiral recognition, it has even been suggested that diffusion measurements are sensitive enough to distinguish diastereomeric complexes through their differing degrees of ion pairing [44].

In a separate study on a series of zirconium-based metallocenes complexed with [MeB(C₆F₅)₃]⁻, there has been shown a good correlation between their hydrodynamic volumes estimated from diffusion measurements (using Si(*p*-tolyl)₄ as internal reference in benzene) and the van der Waals volumes determined for the 1:1 ion pairs from their crystal structures [45] (Fig. 9.29), suggesting that appropriate application of the diffusion NMR methodology can be an effective means of determining the sizes of molecular complexes in solution.

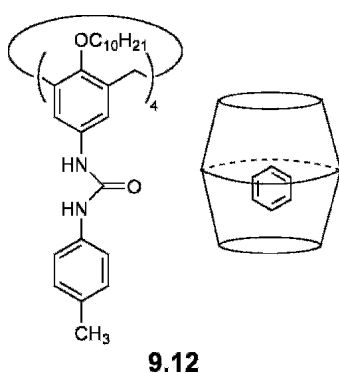
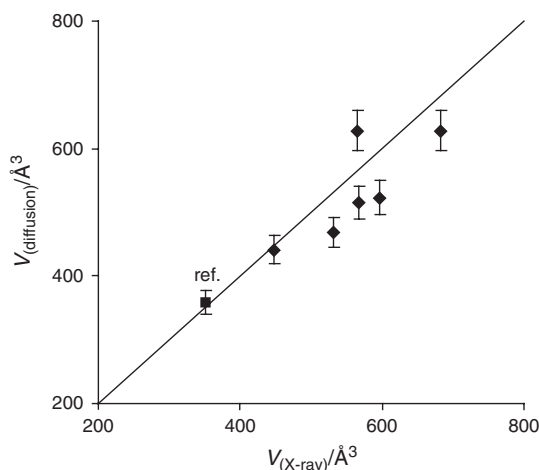
**Table 9.8.** Diffusion coefficients for the ionic complexes **9.10** and **9.11** as a function of solvent

		CDCl ₃		CD ₂ Cl ₂	
		D (10 ⁻¹⁰ m ² s ⁻¹)	r _s (Å)	D (10 ⁻¹⁰ m ² s ⁻¹)	r _s (Å)
9.10	Cation	6.25	6.3	8.74	6.2
	PF ₆ ⁻	6.27	6.3	10.17	5.3
9.11	Cation	6.64	6.0	9.14	5.9
	OTf ⁻	6.45	6.1	11.69	4.7

Coefficients for the metal complexes and their fluorinated counterions were determined from ¹H and ¹⁹F diffusion experiments respectively and the hydrodynamic radii r_s calculated from these values assuming solution viscosities matched those of the pure solvents.

Adapted with permission from [43], Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

Figure 9.29. The plot of hydrodynamic volumes determined from diffusion NMR measurements and van der Waals volumes computed from 1:1 ion pairs in the corresponding crystal structures for a series of metallocenes. The point labelled **ref.** is for the internal diffusion reference $\text{Si}(p\text{-tolyl})_4$ (adapted with permission from [45], Copyright 2003, American Chemical Society).



9.4.5. Supramolecular assemblies

As a simple yet powerful illustration of the capabilities of diffusion methodology, the encapsulation of benzene within the cage of the tetraurea calix[4]arene **9.12** was proven when the resonance at 4.4 ppm believed to arise from the encapsulated solvent was shown to possess an identical diffusion coefficient to the dimeric assembly itself ($3.4 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$) and that this was considerably less than that of free benzene in the solution ($22.1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$) [46]. The characterisation of hydrogen-bonded supramolecular assemblies known as rosettes has also been undertaken and single, double and tetra rosette complexes have been classified according to their diffusion behaviour (Fig. 9.30) [47]. Moreover, these experiments were able to demonstrate that the single rosettes are dynamic assemblies in which the components exchange rapidly on the diffusion timescale whereas some of the double rosettes were more robust and undergo a slow exchange in the presence of an excess of one of the constituent components.

In a similar approach, double-stranded helicates of various lengths that were derived from copper and silver-based metallocsupramolecular architectures have also been classified by their diffusion properties and estimates of the molecular sizes made [48]. Owing to the ellipsoidal structures, it was necessary to introduce appropriate shape factors to translate the hydrodynamic radii determined directly from the unmodified Stoke–Einstein equation into dimensions that were meaningful for these assemblies. Thus, knowledge of the width of the helicates (determined from the X-ray structure of a single complex in this case) allowed the determination of their lengths from the hydrodynamic radii. The results for a series of these helicates is summarised in Table 9.9. It was further shown that 2D DOSY spectra could be employed to differentiate the helicates of different lengths when present simultaneously in a mixture.

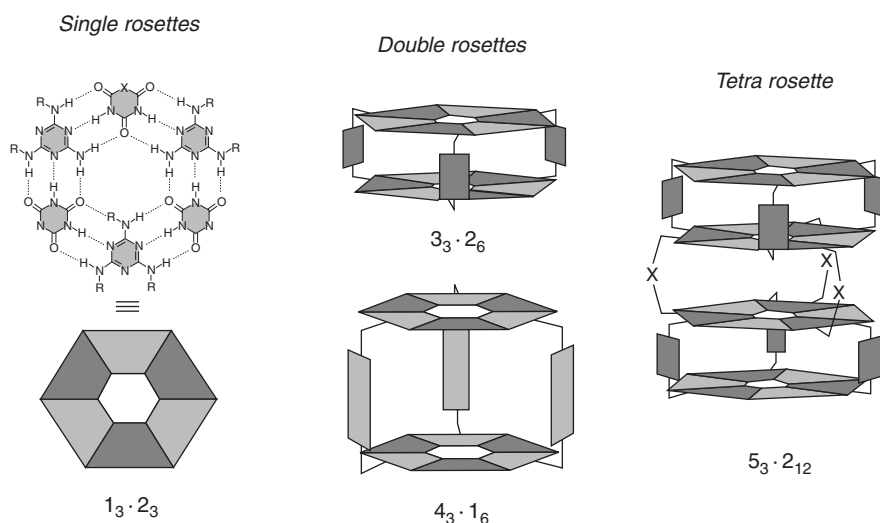
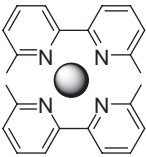
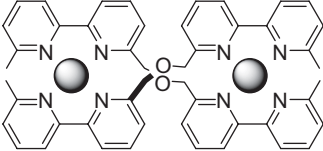
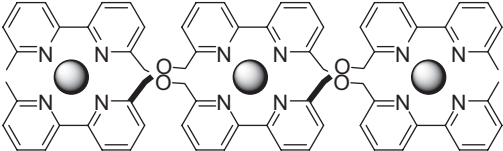
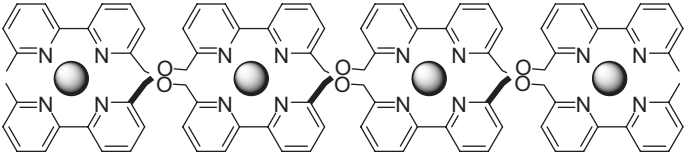
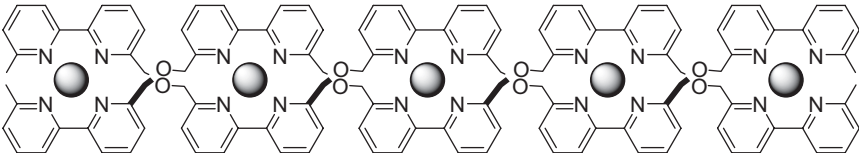


Figure 9.30. Structure and schematic representation of the single, double and tetra rosette supramolecular hydrogen-bonded assemblies characterised by diffusion NMR spectroscopy (reproduced with permission from [47], The Royal Society of Chemistry).

Table 9.9. The diffusion coefficients and calculated molecular dimensions of a series of Cu(I) helicates

Cu(I) helicate	D (10^{-10} $\text{m}^2 \text{s}^{-1}$)	r_s (Å)	Width ^a (Å)	Length (Å)
	15.5	4.1	11.0	6.5
	12.6	5.0	11.0	9.5
	10.1	6.3	11.0	16.0
	9.5	6.7	11.0	18.0
	8.3	7.6	11.0	24.5

The spheres identify the Cu(I) centres.

Adapted with permission from [48], Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

^a The widths of the assemblies were used as the reference distances for the calculation of their lengths from the hydrodynamic radii, r_s , when corrected for the ellipsoidal shapes of the structures.

9.4.6. Aggregation

The phenomenon of aggregation presents an obvious area in which one would intuitively anticipate that diffusion measurements could provide insights and indeed there have been a number of such examples. The π - π stacking of the hydrophobic herbicide Metolachlor **9.13** in D_2O has been detected by diffusion NMR measurements [49] and has been used to explain the higher than expected solubility of this compound in water through the reduced exposure of the aromatic rings to the solvent. In relatively concentrated solution, two species are evident those diffusion coefficients differ by two orders of magnitude and suggested an ordered polymeric aggregate ($D = 2.8 \times 10^{-12} \text{ m}^2 \text{s}^{-1}$) to be present in addition to the monomer ($D = 4.9 \times 10^{-10} \text{ m}^2 \text{s}^{-1}$) under conditions of slow exchange.

Both ^1H and ^7Li diffusion have been used in the discrimination of previously characterised aggregates of *n*-butyllithium in THF solvent, a widely used reagent in synthetic chemistry. Diffusion measurements at 189 K (using the double stimulated-echo sequence to overcome convection) were able to distinguish the ^1H α - CH_2 resonances of dimeric and tetrameric aggregates at -1.12 and -1.00 ppm respectively [50]. Whilst ^7Li diffusion measurements were possible for the tetramer, the broader resonance of the faster relaxing

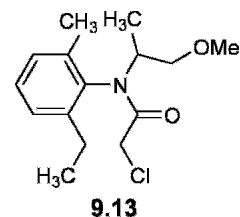
**9.13**

Table 9.10. The experimentally determined diffusion coefficients for the aggregates of n-butyllithium in THF at 189 K together with the values predicted from the solid-state structures and the gas-phase optimised structures

	Calculated D ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)		Experimental D ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)	
	X-ray	Gas-phase model	^1H	^7Li
Tetramer	0.89	0.85	0.88 ± 0.05	0.86 ± 0.01
Dimer	0.99	0.95	1.01 ± 0.03	—

The resonances of the dimer could not be observed in the diffusion experiment.

Adapted with permission from [50], Copyright 2000, American Chemical Society.

dimer prevented its observation. The experimental diffusion coefficients were in good agreement with those predicted from both the available X-ray structures and the optimised gas-phase-computed structures (Table 9.10). Organocuprates are widely used synthetic reagents for C–C bond forming reactions, and in an extensive study [51], the identification of aggregates of alkylcuprates in diethyl ether has also been achieved by diffusion NMR spectroscopy and the information used to explain the reactivity of methyl cuprates. Thus, the presence of dimethylcuprate aggregates larger than dimers (so-called higher aggregates) has been correlated with reduced reactivity and the dimer identified as the reactive species.

9.4.7. Mixture separation

Despite much of the original attention on diffusion NMR methods having focussed on the potential for mixture separation in the form of ‘NMR Chromatography’ via the DOSY presentation format, this does not appear to date to have been a widely used application of the technique in the field of organic chemistry. This is most likely due to difficulties associated with the efficient separation of species when resonance overlap occurs, a situation most likely to arise in complex mixtures where, unfortunately, the technique has greatest potential. In such cases, the use of higher magnetic fields may be beneficial owing to enhanced signal dispersion whilst the application of extended diffusion sequences to separate resonances represents an alternative or additional way forward and a number of these methods are presented in Section 9.5 below.

In one of the first applications of this sort, the concept of mixture separation based on molecular size was demonstrated with a mixture of glucose, adenosine 5'-triphosphate (ATP) and sodium dodecyl sulphate (SDS) in D_2O (Fig. 9.31), with the large differences in size providing clear separation of the constituent parts [52]. A more demanding application was the separation of the metabolite components of a perchloric acid tissue extract containing many small molecules of more similar molecular masses [53]. DOSY has been applied to the analysis of crude column fractions arising from methanol extracts of *Agelas* sponges, which were shown to contain unknown bromopyrrole alkaloids [54]. The use of DOSY in this manner to separate components of the partially purified fractions provided evidence for a novel species being present and indicated the need for additional purification and subsequent characterisation of the novel isomers Agesamides A and B **9.14**.

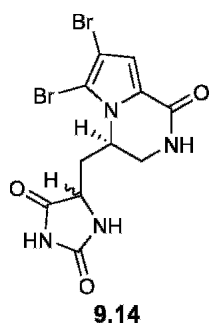
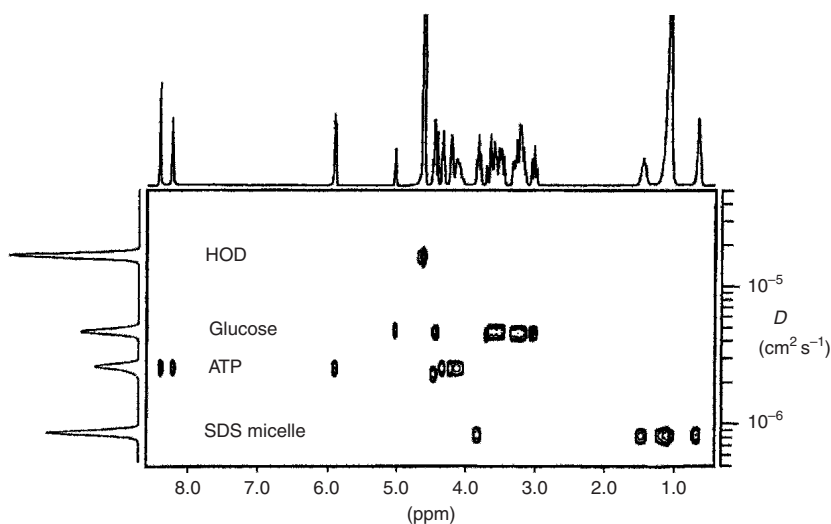


Figure 9.31. The 2D diffusion-ordered (DOSY) spectrum of a mixture of glucose, adenosine 5'-triphosphate (ATP) and sodium dodecyl sulphate (SDS) micelles in D_2O demonstrating the potential for mixture separation (reproduced with permission from [52], Copyright 1993, American Chemical Society).



9.4.8. Macromolecular characterisation

In this final section on illustrative applications, we stray into the realm of biomacromolecules with some examples of the characterisation of the molecular masses of such compounds. In the area of oligosaccharide analysis, DOSY has been proposed as a complimentary method to size exclusion chromatography for molecular weight (MW) determinations [55]. In a study of a series of N-acetyl-chitooligosaccharides (GlcNAc)_{1–6}, a linear correlation was obtained for log *MW* versus log *D* and a readily interpretable strip form of the DOSY data presented, akin to that of polyacrylamide gels in which the smaller, faster moving components appear towards the bottom of the strips (Fig. 9.32). This provided a calibration against which the molecular masses of two *Escherichia coli*-expressed chitooligosaccharides **9.15** and **9.16** were estimated. In a similar approach, uncharged water soluble polysaccharides with molecular masses in the range 5000–850,000 have also shown a similar linear log–log plot [56]. Thus, through the use of appropriate standards as diffusion calibrants, it becomes possible to use measured diffusion coefficients as direct indicators of molecular mass even for very large oligomeric or polymeric systems.

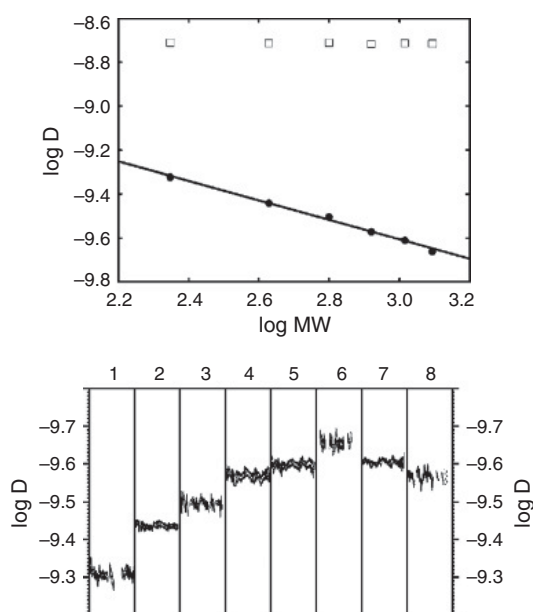
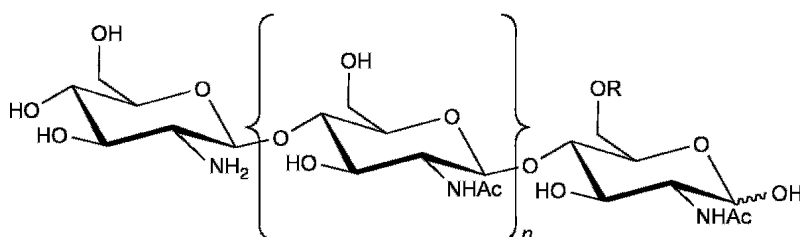
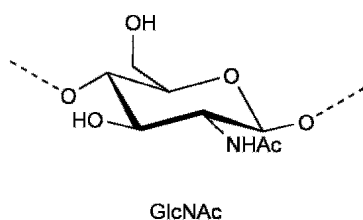


Figure 9.32. (a) A plot of log *D* versus log *MW* for a series of N-acetyl chitooligosaccharides (GlcNAc)_{1–6} and (b) an alternative presentation of DOSY strips in a format similar to gel electrophoresis. The GlcNAc oligomers appear in lanes 1 to 6 whilst lanes 7 and 8 are the traces for the chitooligosaccharides **9.15** and **9.16** (reproduced with permission from [55], Oxford University Press).



9.15: *R* = H, *n* = 3

9.16: *R* = SO₃Na, *n* = 2

9.5. HYBRID DIFFUSION SEQUENCES

Having reviewed a range of applications primarily employing the standard techniques described previously, we finish this chapter by considering alternative sequences for determining diffusion coefficients when those above prove limiting. It has been emphasised in previous discussions that an important requirement for the success of diffusion experiments is the ability to resolve the peaks of the species of interest from those of other molecules present in multi-component systems so as to accurately follow their corresponding diffusion-encoded intensity decays. Beyond using the highest available magnetic fields to maximise resonance dispersion, there have been a number of diffusion-based sequences proposed that are in essence derived from the usual array of multiple-pulse sequences used in structure elucidation to enhance the information content of spectra. This section provides a brief overview of the principal methods that have been suggested as alternatives to the basic 1D (proton) observation methods described above.

9.5.1. Sensitivity-enhanced heteronuclear methods

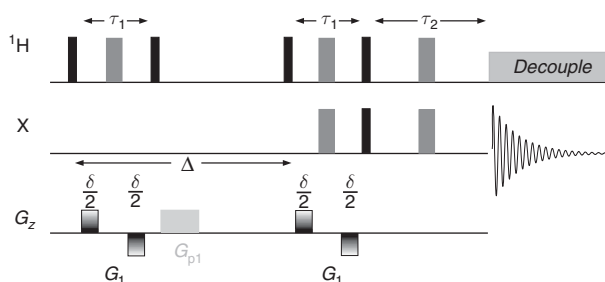
A direct approach to improving resonance dispersion is to observe a heteronucleus rather than the more typical proton, with carbon-13 an attractive alternative for organic systems since resonance overlap is rare. However, it is well known that the direct observation of low- γ nuclides suffers from reduced sensitivity, so the use of signal enhancement by polarisation transfer from protons has been demonstrated in the INEPT-DOSY sequence for both ^{13}C [57] and ^{29}Si [58]. The most direct approach is to append an INEPT transfer onto a bipolar STE sequence such that diffusion encoding is performed on proton magnetisation with signal detection on the heteroatom being combined with proton decoupling [59] (Fig. 9.33). In this, it becomes possible to include the diffusion decoding bipolar gradients within the INEPT transfer step.

An even more efficient approach, although applicable only for polarisation transfer across small, long-range heteronuclear couplings, is to use the necessarily long J -evolution delays of the INEPT sequence to encode molecular diffusion within the spin-echo [58]. This approach further benefits from convection compensation since gradient encoding-decoding can both be performed either side of the 180° pulse of the initial spin-echo. This scheme has been demonstrated for INEPT transfer using the small 2-bond ^1H – ^{29}Si couplings ($\sim 6\text{ Hz}$) in silicon-observed DOSY, and represents one example of the ‘IDOSY’ approach described in Section 9.5.3 below.

9.5.2. Spectrum-edited methods

For situations when proton sensitivity remains desirable but resonance dispersion is hampered by peak overlap, direct editing of the proton spectrum to observe selectively the signals of interest may offer a solution. An effective approach to this is to combine a selective 1D TOCSY experiment with diffusion encoding such that only magnetisation originating from the selectively excited proton, and subsequently transferred to others within the same spin system, will contribute to the final diffusion traces. The net result is a 2D DOSY spectrum of a subset of the complete proton spectrum. One implementation of this, termed STEP-DOSY [60] (selective TOCSY edited preparation–DOSY), employs excitation sculpting (Section 10.4.2) and TOCSY mixing with clean zero-quantum suppression (Section 5.7.4) as preparation for the BPP-LED diffusion experiment (Fig. 9.34). Thus, resonances that would otherwise be buried become cleanly resolved for diffusion encoding providing they share a suitable spin-coupling network with a neighbouring spin; note how this approach parallels that used for the 1D STEP-NOESY experiment described in Section 8.7.2 for the observation of NOEs originating from buried proton resonances.

Figure 9.33. The basic DOSY-INEPT sequence. The delays τ_1 are set to $1/2J_{\text{HX}}$ as required for the INEPT transfer and the delay τ_2 set as necessary for the INEPT refocusing stage to enable the use of ^1H decoupling. The diffusion decoding gradient is applied as a bipolar pair during the first part of the INEPT transfer step.



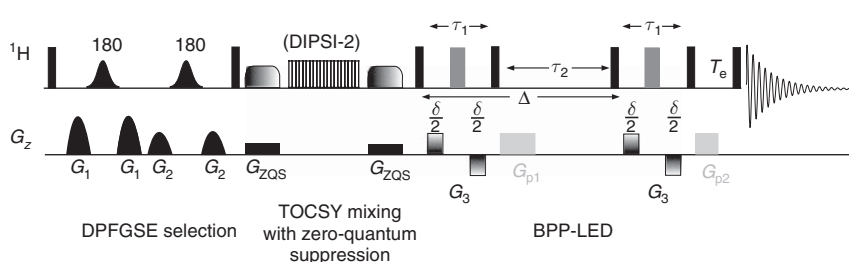


Figure 9.34. The 2D STEP-DOSY sequence for editing of ^1H diffusion spectra. The magnetisation of the DPGSE selected resonance(s) is transferred via isotropic mixing and subsequently encoded for diffusion measurements with the BPP-LED scheme. Details of each step labelled in the sequence may be found in the relevant chapter sections.

9.5.3. Diffusion-encoded 2D methods (or 3D DOSY)

A further extension to improve resonance separation is to employ the additional signal dispersion afforded by 2D NMR techniques and to combine these with diffusion encoding. In essence, there are two general approaches to this; the first is to concatenate a conventional 2D experiment with a diffusion sequence, the second is to somehow embed the diffusion encoding within the 2D experiment itself, both cases resulting in a pseudo-3D data set, that is, with one diffusion and two frequency dimensions. Planes from the 3D cube extracted for a given diffusion coefficient then present the parent 2D spectrum for only the corresponding species. Concatenation itself may take two forms in which the 2D experiment 'X' precedes the diffusion encoding (X-DOSY) or is appended to this (DOSY-X) with both approaches having been advocated. Embedding the diffusion period within the 2D method requires that suitably long periods already exist within the sequence in which to encode molecular diffusion with both J -evolution periods and TOCSY mixing times having been utilized for this purpose leading to the so-called internal-DOSY or IDOSY family of experiments that will be exemplified below.

Data acquisition demands a full 2D data set be collected for every diffusion-encoding gradient amplitude applied, and this may then lead to extended experiment times; clearly high-sensitivity 2D methods and/or relatively concentrated samples are best suited to such an approach. With one exception (mentioned below), data analysis of such experiments involves the fitting of the diffusion-encoded intensity decays of 2D crosspeak volumes to extract diffusion coefficients. Alternatively, a 3D DOSY display may be generated by direct analogy with the fitting of 1D peak areas described in Sections 9.2.4 and 9.2.5 above. Planes taken through a 3D DOSY spectrum should then contain only the signals arising from species that share the same diffusion coefficients. Examples of these experimental approaches are described briefly in the sections that follow.

Diffusion-encoded COSY

The most widely used 2D correlation experiment COSY may be combined with the BPP-LED sequence simply by replacing the final 90° read pulse of BPP-LED with the two-pulse COSY sequence to give the BPP-LED-COSY experiment [61]. However, a more direct and efficient approach is simply to incorporate the diffusion period directly into the two-pulse COSY sequence by the appropriate use of gradient pulses, as in the COSY-IDOSY sequence [62] (Fig. 9.35). The gradient pulses now cleverly serve a dual purpose providing both coherence selection, as in gradient-selected COSY, and diffusion encoding-decoding. Here, the extended Δ period employed for diffusion encoding would be expected to produce severe phase distortions in the proton traces, but these errors are masked completely with the usual absolute value (magnitude mode) processing of COSY. Note, however, that this will allow for extended J -evolution meaning the sequence is in fact more akin to the delayed or long-range COSY experiment (Section 5.6.4) and would be expected to enhance the appearance of long-range correlations arising from small proton-proton couplings. Alternatively, and advantageously, this feature can reduce the total number of t_1 increments required to resolve the COSY crosspeaks.

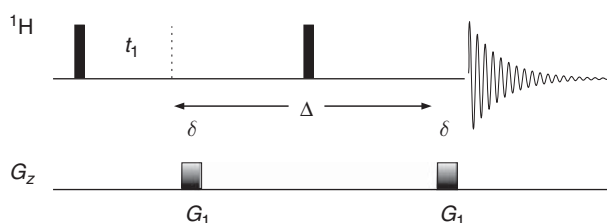


Figure 9.35. The COSY-IDOSY sequence. The gradient pulses select for the conventional N-type COSY pathway in addition to providing diffusion encoding (selection of the P-type pathway would use gradient pulses of opposite sign).

Diffusion-encoded TOCSY

By direct analogy with the COSY-based experiment above, diffusion encoding with the TOCSY experiment may be achieved by direct concatenation of the parent sequences to give the DOSY-TOCSY experiment, with most sequences reported employing the LED or BPP-LED diffusion-encoding [63–65]. However, the mixing time employed in the TOCSY step can equally lend itself to acting as the diffusion encoding period, so following the IDOSY concept [66]. Thus, encoding and decoding gradients can be placed either side of the spin-lock period, which is often many tens of milliseconds and thus potentially of sufficient duration to map diffusion. Indeed, one limitation with some early implementations of gradient-selected sequences was signal intensity losses arising from molecular diffusion when gradient pulses were placed either side of lengthy mixing or evolution periods, and many more recent sequences are designed with closely spaced gradient pulses to avoid such losses.

Diffusion-encoded *J*-resolved

Whilst the COSY- and TOCSY-derived methods described above offer the potential for crosspeak dispersion, they become less effective when analysing mixtures of similar compounds since crosspeak overlap may still be a problem. In such cases, an alternative approach may be to use the homonuclear *J*-resolved technique in an attempt to eliminate multiplet overlap by placing *J*-coupling fine structure orthogonal to the shift axis (Chapter 7). In this approach, the intensity decay of the 2D peaks may be fitted to extract diffusion coefficients or alternatively the projections of the tilted 2D data set may be treated as a homonuclear decoupled 1D spectrum and the peak intensities fitted as for conventional diffusion experiments [67].

Direct concatenation results in either the *J*-BPP-LED [68] or, alternatively, the BPP-LED-*J* [67] sequences. However, as the basic *J*-resolved experiment is itself derived from a simple spin-echo, this can be diffusion encoded directly yielding the more time-efficient and sensitive *J*-IDOSY technique [69] (Fig. 9.36). In this, the diffusion period Δ straddles the refocusing 180° pulse of the spin-echo and splits equally the t_1 evolution period. Although the minimum t_1 period is now defined by Δ this does not present a problem in the final spectrum since, as for COSY-IDOSY above, any unwelcome phase modulation this produces is masked by the absolute value presentation usually employed for *J*-resolved spectroscopy. The gradient encoding pattern avoids the need for phase cycling meaning only a single transient per t_1 increment is required, sensitivity permitting. Unfortunately the restriction to absolute value projections degrades peak resolution, and a more recent approach incorporates similar concepts to the absorption-mode *J*-resolved spectrum described in Section 7.3.3, yielding a ‘pure shift’ ^1H DOSY presentation [70]. The enhanced resolution brought about by the collapse of multiplet structure offers a significant advance over conventional ^1H DOSY experiments, and the utility of this method remains to be exploited.

Diffusion-encoded HSQC

Arguably the most effective means of avoiding resonance overlap is through the application of heteronuclear correlation methods whereby crosspeaks are dispersed by the chemical shifts of (at least) two *J*-coupled nuclei. Despite the usual penalty of sensitivity loss, heteronuclear correlation-based diffusion methods have been described based on the direct concatenation of HMQC [71] and HSQC [65] or by incorporating these with the IDOSY philosophy [58, 72]. A feature common to all of these is the need for extreme caution in the use of broadband decoupling of the heteronucleus since the relatively high powers demanded by conventional composite-pulse decoupling methods can lead to significant sample heating and may cause the onset of the dreaded convection. It is, therefore, essentially mandatory to employ lower power decoupling methods based on adiabatic frequency sweeps so as to minimise this effect (Section 10.3.1).

One example of this approach is the constant-time HSQC-IDOSY [72] experiment (Fig. 9.37) that combines the diffusion and constant-time periods and incorporates the encoding-decoding bipolar gradient pairs within the INEPT transfer steps. Additional gradients are utilised to provide coherence selection and f_1 quadrature detection according

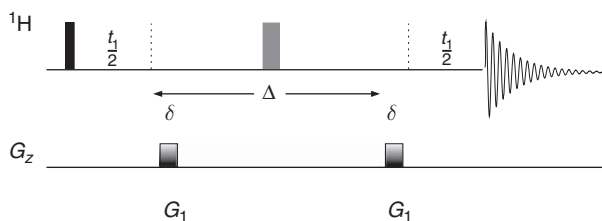


Figure 9.36. The *J*-IDOSY sequence. The diffusion period Δ sits at the centre of the incremented t_1 period and the two gradient pulses provide for both diffusion encoding and selection of the spin-echo coherence transfer pathway.

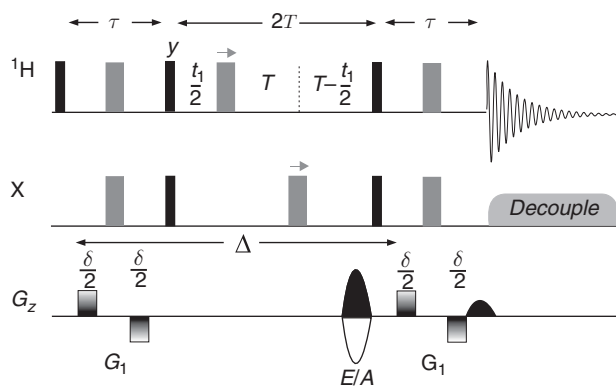


Figure 9.37. The constant-time-HSQC-IDOSY sequence. The delays τ are set to $1/2J_{\text{HX}}$ as required for the INEPT transfer and the 'constant-time' period $2T$ remains fixed, its duration being dictated by the desired diffusion time Δ . The effective t_1 evolution time is varied by moving the two 180° refocusing pulses within the constant time period (pulses shown with arrows over) and coherence selection is made with the echo/antiecho (E/A) scheme. The diffusion encoding/decoding gradients are applied as bipolar pairs during the INEPT and reverse-INEPT transfer steps.

to the echo-anti-echo approach. The total constant-time period $2T$ is defined by the required diffusion period Δ and may lead to lengthy heteronuclear evolution times and so offers the potential for high f_1 resolution at the expense of extensive t_1 sampling. This may not be practical when the 2D experiment is to be performed for each diffusion gradient amplitude, and the use of heteronuclear spectrum aliasing has been suggested as one approach to reduce this requirement [72].

REFERENCES

- [1] P. Stilbs, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1987, **19**, 1–45.
- [2] C.S. Johnson, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1999, **34**, 203–256.
- [3] Y. Cohen, L. Avram and L. Frish, *Angew. Chem. Int. Ed.*, 2005, **44**, 520–554.
- [4] K.F. Morris and C.S. Johnson, *J. Am. Chem. Soc.*, 1992, **114**, 3139–3141.
- [5] E.O. Stejskal and J.E. Tanner, *J. Chem. Phys.*, 1965, **42**, 288–292.
- [6] J.E. Tanner, *J. Chem. Phys.*, 1970, **52**, 2523–2526.
- [7] G. Wider, V. Dotsch and K. Wuthrich, *J. Magn. Reson. (A)*, 1994, **108**, 255–258.
- [8] M.D. Pelta, H. Barjat, G.A. Morris, A.L. Davis and S.J. Hammond, *Magn. Reson. Chem.*, 1998, **36**, 706–714.
- [9] S.J. Gibbs and C.S. Johnson, *J. Magn. Reson.*, 1991, **93**, 395–402.
- [10] D. Wu, A. Chen and C.S. Johnson, *J. Magn. Reson. (A)*, 1995, **115**, 260–264.
- [11] M.D. Pelta, G.A. Morris, M.J. Stchedroff and S.J. Hammond, *Magn. Reson. Chem.*, 2002, **40**, S147–S152.
- [12] M. Nilsson, M.A. Connell, A.L. Davis and G.A. Morris, *Anal. Chem.*, 2006, **78**, 3040–3045.
- [13] B. Antalek, *Concept. Magn. Reson.*, 2002, **14**, 225–258.
- [14] R. Huo, R. Wehrens, J. van Duynhoven and L.M.C. Buydens, *Anal. Chim. Acta*, 2003, **490**, 231–251.
- [15] N.M. Loening and J. Keeler, *J. Magn. Reson.*, 1999, **139**, 334–341.
- [16] N. Hedin, T.Y. Yu and I. Furo, *Langmuir*, 2000, **16**, 7548–7550.
- [17] A. Allerhand, R.E. Addleman and D. Osman, *J. Am. Chem. Soc.*, 1985, **107**, 5809–5810.
- [18] W.J. Goux, L.A. Verkruyse and S.J. Salter, *J. Magn. Reson.*, 1990, **88**, 609–614.
- [19] N. Hedin and I. Furo, *J. Magn. Reson.*, 1998, **131**, 126–130.
- [20] M. Holz and H. Weingartner, *J. Magn. Reson.*, 1991, **92**, 115–125.
- [21] E. Martinez-Viviente and P.S. Pregosin, *Helv. Chim. Acta*, 2003, **86**, 2364–2378.
- [22] K. Hayamizu and W.S. Price, *J. Magn. Reson.*, 2004, **167**, 328.
- [23] N. Esturau, F. Sánchez-Ferrando, J.A. Gavin, C. Roumestand, M.-A. Delsuc and T. Parella, *J. Magn. Reson.*, 2001, **153**, 48–55.
- [24] J. Lounila, K. Oikarinen, P. Ingman and J. Jokisaari, *J. Magn. Reson. (A)*, 1996, **118**, 50.
- [25] A. Jerschow and N. Müller, *J. Magn. Reson.*, 1997, **125**, 372–375.
- [26] M. Nilsson and G.A. Morris, *J. Magn. Reson.*, 2005, **177**, 203–211.
- [27] L.G. Longworth, *J. Phys. Chem.*, 1960, **64**, 1914–1917.
- [28] M.A. Connell, A.L. Davis, A.M. Kenwright and G.A. Morris, *Anal. Bioanal. Chem.*, 2004, **378**, 1568–1573.
- [29] H.-C. Chen and S.-H. Chen, *J. Phys. Chem.*, 1984, **88**, 5118–5121.
- [30] A.R. Waldeck, P.W. Kuchel, A.J. Lennon and B.E. Chapman, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1997, **30**, 39–68.
- [31] P.S. Pregosin, E. Martínez-Viviente and A. Kumar, *Dalton Trans.*, 2004, 4007–4014.
- [32] P.S. Pregosin, P.G.A. Kumar and I. Fernandez, *Chem. Rev.*, 2005, **105**, 2977–2998.
- [33] T. Brand, E.J. Cabrita and S. Berger, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2005, **46**, 159–196.
- [34] R. Giernoth and D. Bankmann, *Eur. J. Org. Chem.*, 2005, 4529–4532.
- [35] G.S. Kapur, E.J. Cabrita and S. Berger, *Tetrahedron Lett.*, 2000, **41**, 7181–7185.
- [36] E.M. de Carvalho, M.H.R. Velloso, L.W. Tinoco and J.D. Figueroa-Villar, *J. Magn. Reson.*, 2003, **164**, 197–204.

- [37] E.J. Cabrita and S. Berger, *Magn. Reson. Chem.*, 2001, **39**, s142–s148.
- [38] L. Fielding, *Tetrahedron*, 2000, **56**, 6151–6170.
- [39] K.S. Cameron and L. Fielding, *J. Org. Chem.*, 2001, **66**, 6891–6895.
- [40] S. Simova and S. Berger, *J. Incl. Phen. Macrocyc. Chem.*, 2005, **53**, 163–170.
- [41] L. Avram and Y. Cohen, *J. Org. Chem.*, 2002, **67**, 2639–2644.
- [42] H. Mo and T.C. Pochapsky, *J. Phys. Chem. B*, 1997, **101**, 4485–4486.
- [43] M. Valentini, H. Ruegger and P.S. Pregosin, *Helv. Chim. Acta*, 2001, **84**, 2833–2853.
- [44] E. Martinez-Viviente, P.S. Pregosin, L. Vial, C. Herse and J. Lacour, *Chem. Eur. J.*, 2004, **10**, 2912–2918.
- [45] N.G. Stahl, C. Zuccaccia, T.R. Jensen and T.J. Marks, *J. Am. Chem. Soc.*, 2003, **125**, 5256–5257.
- [46] L. Frish, S.E. Matthews, V. Böhmer and Y. Cohen, *J. Chem. Soc., Perkin Trans. 2*, 1999, 669–671.
- [47] P. Timmerman, J.-L. Weidmann, K.A. Jolliffe, L.J. Prins, D.N. Reinhoudt, S. Shinkai, L. Frish and Y. Cohen, *J. Chem. Soc., Perkin Trans. 2*, 2000, 2077–2089.
- [48] L. Allouche, A. Marquis and J.-M. Lehn, *Chem. Eur. J.*, 2006, **12**, 7520–7525.
- [49] S. Viel, L. Mannina and A. Segre, *Tetrahedron Lett.*, 2002, **43**, 2515–2519.
- [50] I. Keresztes and P.G. Williard, *J. Am. Chem. Soc.*, 2000, **122**, 10228–10229.
- [51] X. Xie, C. Auel, W. Henze and R.M. Gschwind, *J. Am. Chem. Soc.*, 2003, **125**, 1595–1601.
- [52] K.F. Morris and C.S. Johnson Jr, *J. Am. Chem. Soc.*, 1993, **115**, 4291–4299.
- [53] H. Barjat, G.A. Morris, S. Smart, A.G. Swanson and S.C.R. Williams, *J. Magn. Reson. (B)*, 1995, **108**, 170–172.
- [54] M. Tsuda, T. Yasuda, E. Fukushi, J. Kawabata, M. Sekiguchi, J. Fromont and J. Kobayashi, *Org. Lett.*, 2006, **8**, 4235–4238.
- [55] P. Groves, M.O. Rasmussen, M.D. Molero, E. Samain, F.J. Canada, H. Driguez and J. Jimenez-Barbero, *Glycobiology*, 2004, **14**, 451–456.
- [56] S. Viel, D. Capitani, L. Mannina and A. Segre, *Biomacromolecules*, 2003, **4**, 1843–1847.
- [57] N.E. Schlörer, E.J. Cabrita and S. Berger, *Angew. Chem. Int. Ed.*, 2002, **41**, 107–109.
- [58] M.J. Stchedroff, A.M. Kenwright, G.A. Morris, M. Nilsson and R.K. Harris, *Phys. Chem. Chem. Phys.*, 2004, **6**, 3221–3227.
- [59] D. Wu, A. Chen and C.S. Johnson Jr, *J. Magn. Reson. (A)*, 1996, **123**, 215–218.
- [60] S.A. Bradley, K. Krishnamurthy and H. Hu, *J. Magn. Reson.*, 2005, **172**, 110–117.
- [61] D. Wu, A. Chen and C.S. Johnson Jr, *J. Magn. Reson. (A)*, 1996, **121**, 88–91.
- [62] M. Nilsson, A.M. Gil, I. Delgadillo and G.A. Morris, *Chem. Commun.*, 2005, 1737–1739.
- [63] A. Jerschow and N. Muller, *J. Magn. Reson. (A)*, 1996, **123**, 222–225.
- [64] M. Lin and M.J. Shapiro, *J. Org. Chem.*, 1996, **61**, 7617–7619.
- [65] R.T. Williamson, E.L. Chapin, A.W. Carr, J.R. Gilbert, P.R. Graupner, P. Lewer, P. McKamey, J.R. Carney and W.H. Gerwick, *Org. Lett.*, 2000, **2**, 289–292.
- [66] N. Birlirakis and E. Guittet, *J. Am. Chem. Soc.*, 1996, **118**, 13083–13084.
- [67] J.C. Cobas and M. Martín-Pastor, *J. Magn. Reson.*, 2004, **171**, 20–24.
- [68] L.H. Lucas, W.H. Otto and C.K. Larive, *J. Magn. Reson.*, 2002, **156**, 138–145.
- [69] M. Nilsson, A.M. Gil, I. Delgadillo and G.A. Morris, *Anal. Chem.*, 2004, **76**, 5418–5422.
- [70] M. Nilsson and G.A. Morris, *Chem. Commun.*, 2007, 933–935.
- [71] H. Barjat, G.A. Morris and A.G. Swanson, *J. Magn. Reson.*, 1998, **131**, 131–138.
- [72] B. Vitorge and D. Jeanneat, *Anal. Chem.*, 2006, **78**, 5601–5606.

Chapter 10

Experimental methods

This final chapter principally considers a collection of experimental methods that find widespread use in modern high-resolution NMR yet cannot be considered as individual techniques in their own right. Rather they are sequence segments that are used within, or may be added to, the techniques already encountered to enhance their information content or to overcome a range of experimental limitations; in addition to the familiar simple rf pulses, they are the components used to construct modern NMR experiments. Depending on context, they may be considered as essential to the correct execution of the desired experiment or viewed as an optional extra to enhance performance. For example, the broadband decoupling of protons during carbon acquisition routinely makes use of the so-called ‘composite-pulse decoupling’ (CPD) schemes to achieve efficient removal of all proton couplings and is nowadays considered essential. Pulsed field gradients (PFGs) could equally well have been included in this chapter since in many cases they serve as an alternative means of signal selection to traditional phase-cycling procedures. The fact that PFGs have been described in a previous chapter lays testament to the manner in which these pervade modern NMR sequences and may now be considered routine for many experiments. The chapter concludes with a brief introduction to some newer experimental methods that have not yet found widespread use but have the potential to become more widely employed in the future.

10.1. COMPOSITE PULSES

The plethora of NMR multi-pulse sequences used throughout modern chemical research all depend critically on nuclear spins experiencing rf pulses of precise flip angles for their successful execution. Careful pulse width calibration is essential in this context; yet despite this, a number of factors invariably conspire against the experimentalist and produce pulses that deviate from these ideals, thus leading to degraded experimental performance. Beyond the limitations imposed by the capabilities of the spectrometer itself with regard to the accuracy of pulse timing, powers, rf phase shifts and so on (which the operator can do little about), there are two notable contributions to pulse imperfections that can be addressed experimentally, namely rf (B_1) inhomogeneity and off-resonance effects.

Inhomogeneity in the applied rf field means not all nuclei within the sample volume experience the desired pulse flip angle (Fig. 10.1b), notably those at the sample periphery. This is similar in effect to the (localised) poor calibration of pulse widths and references to ‘rf (or B_1) inhomogeneity’ below could equally read ‘pulse width miscalibration’. Modifications that make sequences more tolerant of rf inhomogeneity therefore also provide the experimentalist with some leeway when setting up pulse experiments. Off-resonance excitation arises when the transmitter frequency does not exactly match the Larmor frequency of the spin and has already been introduced in Section 3.2. To recap briefly, off-resonance magnetisation vectors experience an effective rf field in the rotating frame that is tilted out of the x - y plane, rather than the applied B_1 field itself (Fig. 10.2). These vectors are driven about this effective field and thus do not follow the trajectory of the ideal on-resonance case (see Fig. 10.1c). The problem stems from the fact that pulse NMR instruments use monochromatic rf radiation when polychromatic excitation is required.

Combating the effects of B_1 inhomogeneity lies very much with the design of the rf coil, and this is in the hands of probe manufacturers who have made considerable progress. Overcoming off-resonance effects directly requires the use of higher-power transmitters that are able to excite over wider bandwidths, but there are two principal problems with this. First is the inability of probe circuitry to sustain such high powers without being damaged and second is the insidious effect of rf sample heating, which may damage precious samples. Improvements in probe circuitry that minimise this heating have largely been paralleled by increases in static field strengths, so despite technical developments the problem remains significant. This has greater relevance at higher field strengths and for those nuclei that exhibit a wide chemical shift range, such as ^{13}C or ^{19}F . Consider data acquisition with a simple 90° pulse that has a uniform excitation bandwidth of around $\pm \gamma B_1$ Hz or $2\gamma B_1$ Hz in total, where γB_1 is the rf field strength (and where γB_1 symbolises

Figure 10.1. Imperfections in pulse excitation. (a) A perfect 90°_x pulse applied at equilibrium, (b) the effect of B_1 inhomogeneity and (c) the effect of off-resonance excitation.

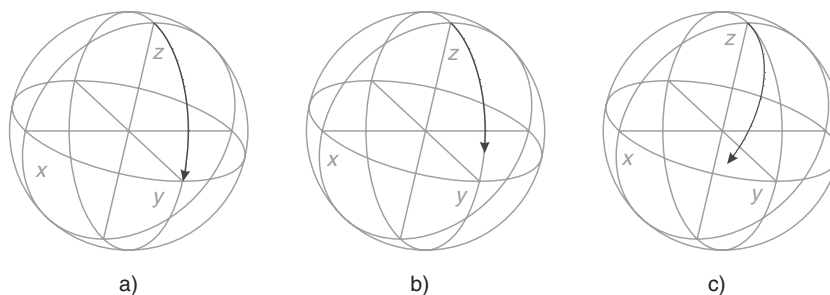


Figure 10.2. Off-resonance excitation causes the bulk vector to rotate about an effective rf field, tipped out of the transverse plane.

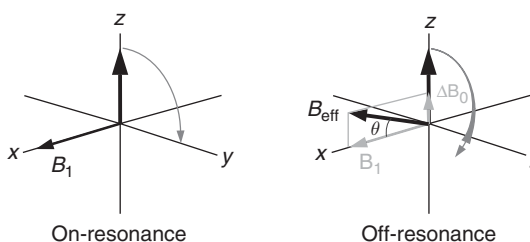
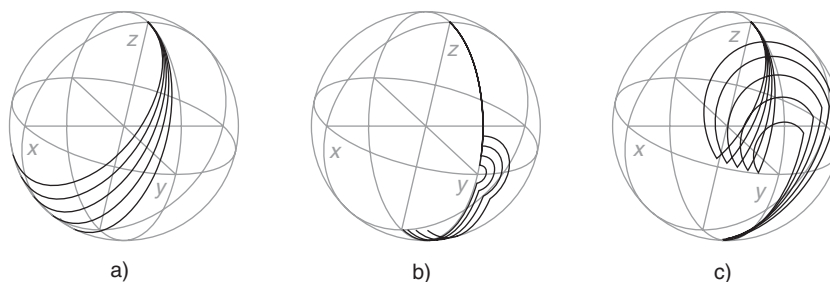


Figure 10.3. The simulated effect of (a) resonance offset (of $0.2\text{--}0.6\gamma B_1$) on the inversion properties of a single 180°_x pulse, and the compensating effect of the $90_x180_y90_x$ composite 180° pulse on (b) B_1 inhomogeneity and (c) resonance offset.



$\gamma B_1/2\pi$; Section 3.5). Taking a typical value of $10\text{ }\mu\text{s}$ for the 90° pulse, this corresponds to a γB_1 of 25 kHz. For ^1H excitation at 400 MHz, the total excited bandwidth is thus 125 ppm, which is clearly ample, and for ^{13}C , it is a satisfactory 500 ppm. In contrast, a 180° inversion pulse has a total bandwidth (here taken to mean $> 90\%$ effective) of only $\sim 0.4\gamma B_1$, which in this example corresponds to 25 ppm for proton, again acceptable, but only 100 ppm for ^{13}C . Carbon nuclei that resonate outside this bandwidth will experience reduced inversion efficiency. Off-resonance errors for a 180° inversion pulse are illustrated in Fig. 10.3a, in which it is clear that the trajectories of the magnetisation vectors are far from ideal and terminate further from the ‘South Pole’ as the offset increases, thus generating unwanted transverse magnetisation. The combined effects of such pulse imperfections in multi-pulse sequences are to degrade sensitivity and to potentially introduce spurious artefacts, and so it is advantageous to somehow compensate for these.

An elegant approach to compensating deficiencies arising from B_1 inhomogeneity or resonance offset is the use of a *composite pulse* [1]. This is, in fact, a cluster of pulses of varying duration and phase that are used in place of a single pulse and have a net rotation angle equal to this but have greater overall tolerance to these errors. The first composite pulse [2] is the three-pulse cluster $90_x180_y90_x$, equivalent to a 180°_y pulse. The self-compensating abilities of this sequence for spin inversion are illustrated by the trajectories of Fig. 10.3. For both B_1 inhomogeneity (see Fig. 10.3b) and for resonance offset (see Fig. 10.3c), the improved performance is apparent from the clustering of vectors close to the South Pole. The effective bandwidth of the $90_x180_y90_x$ composite pulse is $\sim 2\gamma B_1$, which is five times greater than the simple 180° pulse, a considerable improvement from such a simple modification. A single 90° excitation pulse, in contrast, is effective over $\sim 2\gamma B_1$ owing to a degree of ‘in-built’ self-compensation for off-resonance effects when judged by its ability to generate transverse magnetisation. Here, the increased effective field experienced by off-resonance spins tends to drive the vectors further towards the transverse plane (as described in Section 3.2.1) at the expense of frequency-dependent phase errors (Fig. 10.4). For high-power pulses, these errors are approximately a linear function of frequency and are readily removed through phase correction of the spectrum. Compensation for B_1 inhomogeneity can be achieved with the composite 90_x90_y (or still better 90_x110_y [1]) sequence, which places the magnetisation vectors closer to the transverse

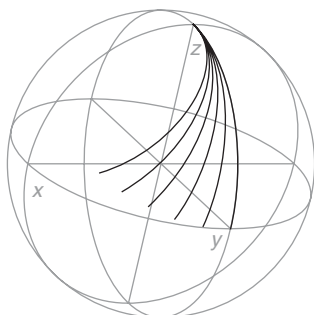


Figure 10.4. The 90° pulse is self-compensating with respect to resonance offset in its ability to generate transverse magnetisation, although there is no compensation for phase errors.

plane than a single pulse (Fig. 10.5). This may prove beneficial in situations where the elimination of z -magnetisation is of utmost importance, such as the NOE difference experiment (see discussions in Section 8.6.1; the $270_x360_{-x}90_y$ composite 90° pulse has been suggested as a better alternative in this case [3]).

A composite pulse can be included within a pulse sequence directly in place of a single pulse. The *relative* phase relationships of pulses within each cluster *must* be maintained but are otherwise stepped according to the phase cycling associated with the single pulse they have replaced. The selection of a suitable composite pulse is not always a trivial process, and they are now increasingly superseded by adiabatic pulses that offer a number of advantages as described in the following section. Composite pulses however still find routine use within broadband decoupling schemes such as those discussed in Section 10.3, and composite adiabatic pulses follow similar principles of self-compensation.

10.1.1. A myriad of pulses

The design of composite pulses has been a major area of research in NMR for many years, and a huge variety of sequences have been published and many reviewed [4]. In reality, only a rather small subset of these has found widespread use in routine NMR applications, some of which are summarised in Table 10.1. One general limitation is that the sequences that provide the best compensation tend to be the longest and the most complex, often many times longer than the simple pulse they have replaced, and as such may not be readily implemented or are unsuitable for use within a pulse sequence. Furthermore, most composite pulses are effective against *either* B_1 inhomogeneity *or* offset effects, but not both simultaneously, so some compromise must be made, usually with the emphasis on offset compensation in high-resolution NMR (in other applications, such as *in-vivo* spectroscopy, B_1 compensation has far greater importance because of the heterogeneous nature of the samples studied). In some cases, dual compensation has been included [5], although again at the expense of longer sequences (see Table 10.1). Numerical optimisation methods have also been applied to the design of more advanced sequences that aim at keeping the total duration acceptably small, most of which also make use of small rf phase shifts, that is, those other than 90° . A comparison of the performance of some composite inversion pulses is illustrated in Fig. 10.6 and clearly demonstrates the offset compensation they provide relative to a single 180° pulse. Nevertheless, their performance is still inferior to that attainable with frequency-swept pulses, which are now available on modern spectrometers; see Section 10.2.

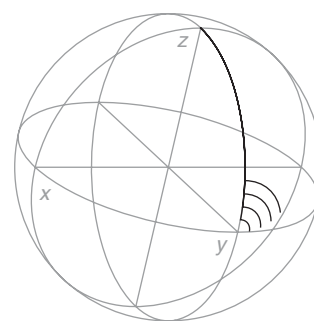


Figure 10.5. The 90_x90_y composite 90° pulse compensates for B_1 inhomogeneity by placing the vector closer to the transverse plane.

Table 10.1. Properties of a single 180° pulse and some composite 180° pulses

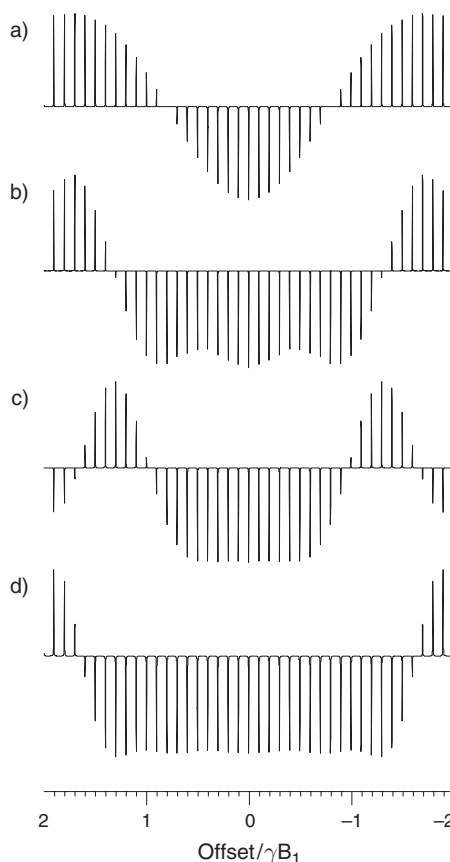
Composite pulse	Duration (x 180°)	Bandwidth ^a (γB_1)	Properties	References
<i>Inversion</i>				
180_x	1	0.4		
$90_y180_x90_y$	2	2.0		[2]
$90_y240_x90_y$	2.3	1.2	More uniform inversion profile than $90_y180_x90_y$	[1]
$360_x270_{-x}90_y360_{-y}270_y90_x$	8	2.0	Compensated for B_1 inhomogeneity ($\pm 25\%$) and resonance offset	[5]
$38_x111_{-x}159_x250_{-x}$	3.1	2.6		[6]
$151_{247}342_{182}180_{320}342_{182}151_{247}$	6.5	2.0	Requires small rf phase shifts ^b	[7, 8]
<i>Refocusing</i>				
180_x	1	0.5		
$90_y180_x90_y$	2	0.6	Introduces phase errors to spin-echoes	
$90_y240_x90_y$	2.3	1.0	Introduces phase errors to spin-echoes	[9]
$360_x270_{-x}90_y360_{-y}270_y90_x$	8	1.0	Compensated for B_1 inhomogeneity ($\pm 25\%$) and resonance offset	[5]
$336_x246_{-x}10_y74_{-y}10_y246_{-x}336_x$	7	1.2	Phase-distortionless spin-echoes	[10]
$151_{247}342_{182}180_{320}342_{182}151_{247}$	6.5	2.0	Phase-distortionless spin-echoes, requires small rf phase shifts ^b	[7, 8]

Only a selection of available pulses is presented with the emphasis on compensation for resonance offset effects and on sequences of short total duration. Individual pulses are presented in the form XX_{yy} , where XX represents the nominal pulse flip angle and yy its relative phase, either in units of 90° ($x, y, -x, -y$) or directly in degrees where appropriate. Sequences are split into those suitable for (A) population inversion (act on M_z) or (B) spin-echo generation (act on M_{xy}).

^a Bandwidths are given as fractions of the rf field strength and represent the total region over which the pulses have $\sim 90\%$ efficacy.

^b Small rf phase shifts refer to those other than multiples of 90° .

Figure 10.6. Simulated inversion profiles for (a) 180_x , (b) $90_y180_x90_y$, (c) $90_y240_x90_y$, and (d) $38_x111_{-x}159_x250_{-x}$ (see Fig. 10.3 and Table 10.1).



10.1.2. Inversion versus refocusing

One complicating factor associated with the implementation of composite pulses arises from the fact that many composite sequences have been designed with a particular initial magnetisation state in mind and may not perform well, or give the expected result, when the pulses act on other states. Two principal applications are the use of composite 180° pulses for population *inversion*, for example, in the inversion-recovery experiment, and in the ubiquitous spin-echo for *refocusing*, in which they act on longitudinal and transverse magnetisation, respectively. Thus, for example, the $90_y180_x90_y$ sequence provides little offset compensation when used as a refocusing pulse (see Table 10.1). Although it offers compensation for B_1 inhomogeneity and thus makes the magnitude of the echoes less sensitive to this by returning vectors closer to the x - y plane, it introduces errors in the *phase* of the echoes that may be detrimental to the overall performance of the experiment. The $90_y240_x90_y$ sequence has been proposed as a better refocusing element for offset compensation [9], and more sophisticated sequences that exhibit low phase distortion have been generated [10, 11]. Sequences designed by numerical optimisation methods have been developed without a pre-defined initial state and are therefore equally effective as inversion or phase-distortionless refocusing elements [7, 8].

The moral of all this is that considerable care must be taken when composite pulses are introduced into pulse sequences. It is wise to test the compensated pulse sequences *vs* the original uncompensated sequences on a known sample to see if improvements are achieved in reality. This offers a check on whether the composite elements have been correctly introduced and on whether the spectrometer is capable of correctly executing the desired sequences, many of which demand accurate control of rf amplitudes and phases for extended periods. The experimental performance of the composite pulse itself is best tested with a simple experiment, such as an inversion sequence ($180_{x,-x}-90_x$ -FID $_x$) or a spin-echo sequence ($90_x-180_{x,y,-x,-y}$ -FID $_{x,-x}$) with a composite 180° pulse, and by analysing the results for a single resonance. Offset compensation may be investigated by stepping the transmitter frequency for the 180° composite pulse away from the initial on-resonance position (but setting it to be on-resonance for the 90° pulse), and B_1 inhomogeneity may be simulated by reducing pulse flip angles.

10.2. ADIABATIC AND BROADBAND PULSES

An alternative and highly efficient approach to spin inversion is offered by the so-called adiabatic pulses [12, 13]. Rather than pulses applied at a single frequency, these employ a

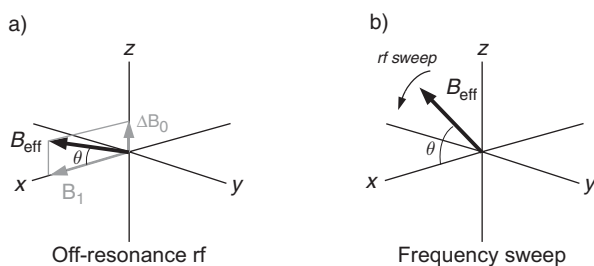


Figure 10.7. The adiabatic inversion pulse: (a) the effective field B_{eff} resulting from an rf pulse applied off-resonance and (b) the rf frequency sweep during the adiabatic pulse causes the effective field experienced by the spins to trace an arc from the $+z$ to the $-z$ axis, dragging with it the bulk magnetisation vector.

frequency sweep *during* the pulse that begins far from resonance, for example, at a negative offset, passes through the resonance condition and finally terminates far from resonance with, in this case, a positive offset. During this, the effective rf field B_{eff} experienced by the spins (see Section 3.2.1 for discussions of off-resonance effects) begins along the $+z$ axis, traces an arc that passes through the x - y plane at the on-resonance condition and finishes along the $-z$ axis (Fig. 10.7). If the sweep is sufficiently slow, magnetisation vectors initially at equilibrium will continually circle about B_{eff} during the pulse and will be ‘dragged along’ by the effective field, thus also terminating at the South Pole and experiencing the desired inversion. More formally, the sweep must be slow enough to satisfy the adiabatic condition

$$\left| \frac{d\theta}{dt} \right| < \omega_{\text{eff}} = \gamma B_{\text{eff}} \quad (10.1)$$

(where θ represents the angle between B_{eff} and the x axis) but should be fast with respect to spin relaxation (hence it is referred to as *adiabatic fast passage*). So long as the adiabatic condition is met, precise inversion is achieved regardless of the magnitude of the applied rf field and hence these pulses are very tolerant of both B_1 inhomogeneities and power mis-settings. In practice, this simply requires that the pulse power is set at or above the required minimum for the inversion to be effective and that accurate pulse power calibrations are not essential. Furthermore, they are effective over very wide bandwidths and are thus well suited to broadband inversion or refocusing and also have application to ultra-broadband decoupling (Section 10.3.1). The ability to manipulate spins over a very wide frequency window makes these pulses especially desirable for the inversion and refocusing of heteronuclear spins that exhibit large chemical shift ranges on high-field spectrometers where conventional 180° pulses have poor offset performance, and the application of adiabatic pulses in this context has been mentioned in numerous sections of preceding chapters, notably in the context of manipulating carbon-13 spins.

Adiabatic pulses are described by their frequency sweep and amplitude profile, which, when combined with the peak rf amplitude $\omega_{1(\text{max})}$, defines the total power of the pulse. The total frequency range, ΔF , over which the pulse sweeps is commonly many tens of kilohertz and pulse durations, T , are typically of the order of 1 ms, corresponding to frequency sweep rates of 10–100 MHz/s. The degree to which the adiabatic condition is satisfied for the pulse is quantified by the adiabaticity factor Q

$$Q = \frac{\omega_{\text{eff}}}{|d\theta/dt|} \quad (10.2)$$

this being most critical when the pulse passes through resonance as it is here when ω_{eff} is smallest since there is no contribution to this from $\Delta\omega$ when on-resonance. As the rf field passes through resonance, the below expression can be written for the on-resonance adiabaticity factor Q_0 [14, 15]

$$Q_0 = \frac{\omega_1^2}{|d\Delta\omega/dt|} \quad (10.3)$$

In the case of the commonly employed linear (i.e. constant rate) frequency sweep, the term $d\Delta\omega/dt$ may be equated with the sweep rate of the pulse, $\Delta F/T$, and the on-resonance rf field defines the maximum amplitude for the pulse $\omega_{1(\text{max})}$ rad/s, which one can write more usefully in terms of $\gamma B_{1(\text{max})}$ as

$$(\gamma B_{1(\text{max})})^2 \text{ (Hz)} = Q_0 \frac{\Delta F}{2\pi T} \quad (10.4)$$

From this expression, one calculates the maximum peak amplitude of the adiabatic pulse $\gamma B_{1(\text{max})}$ for a chosen value of Q_0 , which must usually be significantly larger than unity, and is typically set to 3–5 for most applications. Thus, a 0.5-ms adiabatic pulse sweeping over a

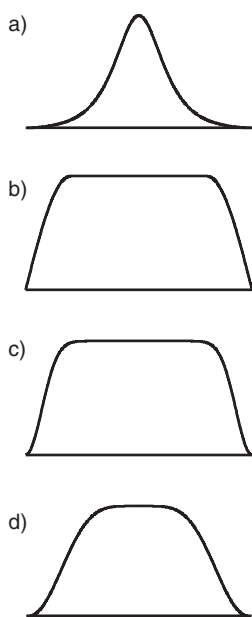


Figure 10.8. The amplitude profiles for adiabatic pulses: (a) hyperbolic secant, (b) CHIRP with smoothing of the first and last 20%, (c) WURST with $n = 20$ (WURST-20) and (d) WURST with $n = 5$ (WURST-5).

60-kHz window requires a peak amplitude of 9.77 kHz with Q_0 of 5, which corresponds to the power of a 25.6- μ s-hard 90° pulse. Such calculations are performed most conveniently within the software tools provided with spectrometer software, and their experimental implementation is most readily achieved on instruments with linear amplifiers where the necessary rf attenuation may be calculated directly from hard-pulse calibrations (see Section 3.5.1).

Eq. (10.4) also demonstrates that the frequency window of an adiabatic pulse scales with the *square* of the applied rf amplitude whereas simple hard pulses exhibit only a linear dependence on this, and it is this feature that explains the efficiency of the swept pulses as broadband inversion pulses.

10.2.1. Common adiabatic pulses

The development of efficient adiabatic pulses has been an active area of research within the NMR community for some time with the classical hyperbolic secant pulse leading the way. This makes use of modulation of both the rf amplitude (Fig. 10.8a) and the frequency but, whilst providing an excellent inversion profile, has limited application due to its high peak pulse amplitude, making it unsuitable for application over wide bandwidths. Nowadays two adiabatic pulses find most widespread use in high-resolution NMR and hence we shall limit discussions to these. The simplest pulse has a rectangular amplitude profile and makes use of a linear frequency sweep and has been termed a CHIRP pulse [16]. The performance of this at the edges of the frequency sweep can be improved by gently truncating the rectangular amplitude envelope such as with a half-sine profile, so that the rf amplitude begins and ends at zero, leading to the smoothed CHIRP profile of Fig. 10.8b. This smoothing ensures that B_{eff} genuinely begins and ends along the $\pm z$ axis at the extremities of the sweep. The second widely used but independently developed pulse has a rather similar amplitude profile to the smoothed CHIRP and again defines a linear frequency sweep having the name WURST (wideband uniform rate and smooth truncation) [14, 15, 17]. The shape of the amplitude profile in this case is defined by the function

$$\varpi_1(t) = \varpi_{1(\text{max})} (1 - |\sin(\beta t)|^n) \quad (10.5)$$

where $-\pi/2 \leq \beta t \leq +\pi/2$ and defines a sausage-shaped profile suggestive of its name (see Fig. 10.8c and d). The exponent n defines the steepness of the truncation with larger values leading to a more rapid power attenuation at the edges; typically $n = 20$ –40. The inversion trajectory for a WURST-20 pulse is shown in Fig. 10.9 and shows the swooping paths followed by the magnetisation vectors as they are pulled down towards the South Pole, terminating along the $-z$ axis as required. Initially the vectors circle the North Pole when the rf field is far from resonance, pass through the x - y plane at the on-resonance condition and are then swept southwards as the rf field moves away.

Clearly it can be seen from Fig. 10.8 that the CHIRP and WURST profiles are very similar and not surprisingly their performance characteristics are likewise closely matched. Figure 10.10 illustrates the inversion profiles for a CHIRP smoothed at 20% and the WURST-20 both simulated for a 60-kHz sweep over 0.5 ms under similar adiabatic conditions ($Q = 5.24$, $\gamma B_{1(\text{max})} = 10$ kHz). The total bandwidth over which any inversion occurs, ± 25 kHz, is $\sim 80\%$ of the total sweep range although the bandwidth over which the pulses have $>90\%$ inversion efficiency is somewhat less than this at ± 17 kHz. Nevertheless, this corresponds to a total 270-ppm window for ^{13}C at 125 MHz ($^1\text{H} = 500$ MHz), clearly ample for complete inversion over the whole chemical shift range one typically encounters. In contrast, a conventional hard 180° pulse of 25 μ s ($\gamma B_{1(\text{max})} = 20$ kHz, typical for ^{13}C) exhibits a *total* bandwidth of only 32 kHz and, still worse, displays non-uniform intensity over the whole of this region, despite employing twice the rf amplitude relative to the adiabatic pulses (see Fig. 10.10c). This ability to use the adiabatic pulses at low powers yet retain broadband inversion efficiency makes them especially attractive for broadband decoupling (see Section 10.3.1).

The single adiabatic pulses presented so far are suitable for use as inversion pulses (when acting on longitudinal magnetisation) but are less well behaved for refocusing in spin-echoes (when acting on transverse magnetisation) due to the introduction of an offset-dependent phase role across the spectrum. Transverse magnetisation vectors may not experience the expected 180° flip but instead effectively undergo rotations in the transverse plane giving rise to the phase errors. One solution to this phase problem is to further elaborate a single pulse to a *composite adiabatic pulse* of sequential sweeps of relative duration 1:2:1 wherein the central pulse flips the effective field B_{eff} , enabling the third pulse to refocus the phase errors created by the first [18]. Thus, the 0.5-ms inversion pulse extends to a 2-ms adiabatic pulse of sequential sweeps of 0.5–1.0–0.5 ms durations, somewhat reminiscent of the 90° – 180° – 90° composite hard pulses. Thus, single adiabatic pulses may be used for inversion whereas composite adiabatic pulses may be used for refocusing. The inclusion of adiabatic pulses

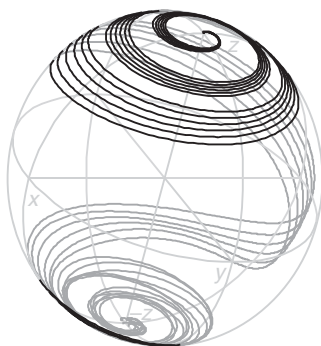


Figure 10.9. Inversion trajectories for a 0.5-ms WURST-20 pulse swept over 60 kHz with $\gamma B_{1(\text{max})}/2\pi = 10$ kHz ($Q = 5.24$). Trajectories are shown for offsets of 0–5 kHz in 1-kHz steps.

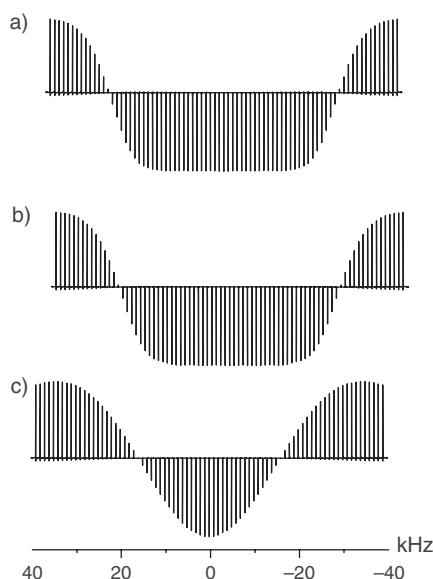


Figure 10.10. The simulated inversion profiles of (a) 20% smoothed CHIRP, (b) WURST-20 and (c) 25- μ s hard 180° pulse. For both (a) and (b), a 60 kHz sweep was employed over 0.5 ms pulse with $\gamma B_{1(\text{max})}/2\pi = 10$ kHz ($Q = 5.24$). The profiles were generated with a $180^\circ_{x,-x}-90^\circ_{(\text{hard})x}$ sequence over two transients to reveal the inverted M_z component.

offers an effective means for extending the operating bandwidth for 180° inversion and refocusing pulses for nuclei with wide shift ranges and especially at higher magnetic fields, and they are finding increasing use within 2D experiments. Their inclusion can present additional complications, however, because of the long duration of these elements, in the order of milliseconds. This means that spins with different chemical shift offsets will experience inversion at differing times, and this can in turn degrade the refocusing efficiency of these pulses and give rise to phase errors in spectra. These errors may not prove too problematic for many experiments and are unlikely to prevent the use of these pulses, but various approaches have been proposed to compensate for these. For multiplicity-edited HSQC experiment, one recommendation is to synchronise the frequency sweep with the magnitude of the $^1J_{\text{CH}}$ coupling constant to improve refocusing efficiency on the editing step [19]. The use of matched pairs of adiabatic pulses in HSQC has also been suggested [20].

Finally, we note that in the execution of these swept pulses, spectrometers actually encode the necessary modulation not as a variation of the applied frequency but as a ramp in the *phase* of the applied rf. This is more readily implemented with the very accurate phase control found within NMR instruments. To see why this equality holds, one could imagine the behaviour of a rf sine wave of constant frequency passing by for which the phase of the wave is steadily increased as it does so. The appearance would be to accelerate the passage of the passing wave and thus make it behave as if it is of higher frequency. In effect, the frequency of the rf pulse has been increased by incrementing its phase, which then causes excitation at a frequency away from that of the applied transmitter rf; this is how spectrometers generate all shaped pulses at arbitrary offsets from the transmitter frequency, such as those described in Section 10.4. Extending this idea to a pulse whose phase is incremented in a non-linear fashion leads to this having the equivalent effect as one in which the frequency is swept. To exemplify this, the amplitude and phase profile of a WURST-20 pulse swept through 60 kHz in 500 μ s is illustrated in Fig. 10.11.

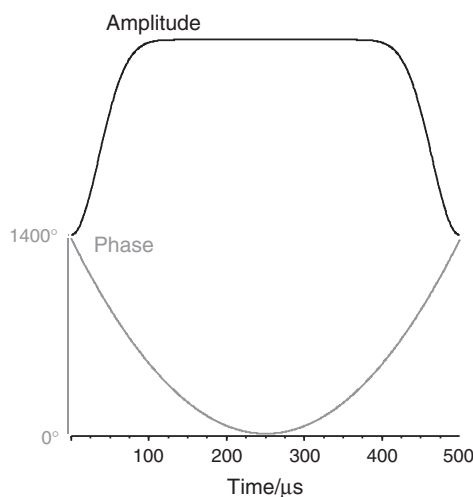


Figure 10.11. The amplitude and phase modulation of a WURST-20 pulse (60 kHz sweep in 500 μ s).

10.2.2. Broadband inversion pulses: (BIPs)

An alternative family of pulses effective over very wide bandwidths are the so-called BIPs (Broadband Inversion Pulses) [21]. These have been designed through numerical optimisation to offer highly effective inversion pulses of higher power and shorter duration than the adiabatic pulses described above and are aimed specifically for use as single pulses within multi-dimensional sequences rather than for repeated, sequential application as required in broadband decoupling. Surprisingly, it turns out that the resultant pulses do not require shaping of the rf amplitude profile (and in this respect are reminiscent of the early CHIRP pulses) and their properties are dictated by the phase ramping alone. They are defined, and named, according to their individual performance characteristics, which encompass the following:

- *Pulse duration*: defined as the length (in degrees) of a hard pulse executed at the same rf amplitude.
- *Inversion bandwidth*: expressed as a percentage of the applied rf field γB_1 over which the pulse has >95% efficacy.
- *Tolerance to B_1 inhomogeneity*: expressed as a percentage of the inhomogeneity.

Thus, the BIP-720-50-20 (Fig. 10.12) has an equivalent duration as would a 720° hard pulse, has an effective bandwidth of $\pm 0.5 \gamma B_1$ and is tolerant to pulse inhomogeneity, or miscalibration, of $0.2 \gamma B_1$. Therefore, applying this with a γB_1 of 20 kHz (which equates to a 25- μ s hard 180° pulse) defines a 100- μ s pulse ($720^\circ/180^\circ \times 25 \mu$ s) which has >95% inversion efficiency over a total 20 kHz bandwidth. This equates to a 40 or 160-ppm window for ^1H and ^{13}C , respectively, at 500 MHz. The inversion profile of this pulse is shown in Fig. 10.13 and compared to a 180° hard pulse applied at the same rf amplitude. Not only does the BIP easily outperform the hard pulse but also its useable bandwidth considerably exceeds the $\pm 0.5 \gamma B_1$ range defined for this pulse when applying the stringent 95% inversion requirement, having good efficacy over approximately twice this range. Still greater bandwidths are available with other members of the BIP family; the 192- μ s BIP-1382-250-15 provides complete inversion over a 100 kHz bandwidth.

The BIPs therefore provide for broadband pulses of shorter duration and higher power than do the adiabatic pulses and may be better suited when longer pulses can be problematic, as mentioned above. They are suitable as single inversion pulses in

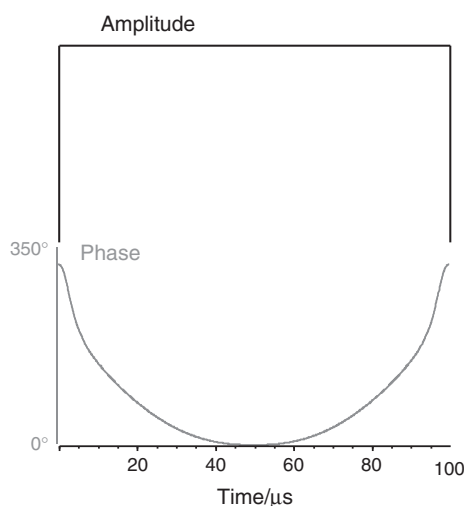


Figure 10.12. The amplitude and phase profiles of the BIP-720-50-20 of 100 μ s duration ($\gamma B_1 = 20$ kHz).

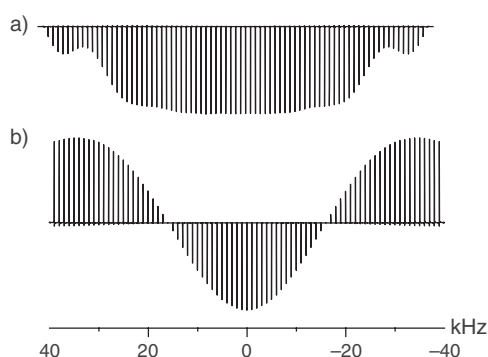


Figure 10.13. The simulated inversion profiles of (a) the 100- μ s BIP-720-50-20 and (b) 25- μ s hard 180° pulse. Both pulses use $\gamma B_1 = 20$ kHz. The profiles were generated with a $180^\circ_x - x - 90^\circ_{(\text{hard})x}$ sequence over two transients to reveal the inverted M_z component.

multi-dimensional NMR experiments and can also be used for refocusing of spin-echoes whereby they must be used in pairs to cancel phase errors generated by a single pulse. In a separate development, constant-amplitude, phase-modulated pulses have also been introduced for broadband *excitation*, the so-called BEBOP pulses (*broadband excitation by optimized pulses*) [22], which have relevance to excitation of, say, ^{13}C at very high fields when even conventional 90° pulses show inadequate bandwidths.

10.3. BROADBAND DECOUPLING AND SPIN LOCKING

An area in which composite pulses have been applied routinely and continue to be used with great success is broadband heteronuclear decoupling. Their use in this area arises from the realisation that heteronuclear decoupling may be achieved by the continuous application of a train of 180° inversion pulses on the decoupled spin. This is illustrated in Fig. 10.14 for a X–H pair in the presence of proton decoupling. Following excitation of X, doublet vectors diverge for a period τ according to J_{XH} . A 180° pulse applied to protons at this time inverts the proton α and β states, thus reversing the sense of precession of the X spin vectors that refocus after a further period τ . If this procedure is repeated during the observation of X at a fast rate relative to the magnitude of J_{XH} , the action of the coupling is suppressed and hence the spectrum appears decoupled.

From discussions in the previous section, it should be apparent that the application of a sequence of simple 180° pulses is unlikely to give effective decoupling over a wide bandwidth or in the presence of B_2 inhomogeneity or pulse miscalibration (note the use of the symbol B_2 with reference to a *decoupling* rf field). Further, by reducing the period τ to be infinitely small, the sequence becomes a single, continuous decoupler pulse (so-called *continuous-wave* decoupling), and by the same arguments, this is also a poor decoupling sequence, particularly off-resonance. One solution to achieving decoupling over wider bandwidths is to employ composite inversion pulses that display superior off-resonance performance, and modern CPD (composite pulse decoupling) has evolved from the original $90_x180_y90_x$ and $90_x240_y90_x$ sequences.

However, simply inserting these in place of single 180° pulses does not turn out to be the full answer. Defining the composite inversion cluster $90_x180_y90_x$ as an element R , we can see that the sequence RR (two sequential composite pulses) should return spin vectors back to the $+z$ axis for the rotation sequence to begin again. However, these elements themselves are not perfect (see Fig. 10.3), leaving small errors, which will accumulate if the sequence is simply repeated. The trick is to repeat the process but in reverse, with all rf phases inverted so as to counteract these errors, giving rise to the element $\bar{R}(90_x180_y90_x)$ and the so-called ‘magic cycle’ $RR\bar{R}\bar{R}$. This original decoupling sequence is termed MLEV-4. It was subsequently realised that small residual errors from the magic cycle could be compensated by further nesting of these elements to produce ‘super cycles’, such as $RR\bar{R}\bar{R}RR\bar{R}\bar{R}RR\bar{R}\bar{R}$, giving, in this case, MLEV-16. These cycles prove more effective over greater bandwidths without the requirement of excessive rf powers.

Numerous such CPD sequences have been developed along these lines over the years (Table 10.2), the most widely used being WALTZ-16 whose basic inversion element is $90_x180_{-x}270_x$ (or more succinctly $1\bar{2}3$, hence the name). This provides very effective decoupling over a bandwidth of $2\gamma B_2$ leaving only small residual splittings (<0.1 Hz) and is thus favoured in high-resolution applications such as broadband proton decoupling of heteronuclear spectra. This bandwidth can, however, be limiting when decoupling nuclei with greater chemical shift dispersion, for example, ^{13}C or ^{31}P , as is required in proton-detected heteronuclear shift correlation sequences such as HMQC or HSQC. For such cases, other CPD sequences have been developed, through computational optimisation, which have less-stringent requirements for residual linewidths (<1.5 Hz). The most popular of these has been GARP (see Table 10.2), which is effective over $4.8\gamma B_2$ (Fig. 10.15). With the

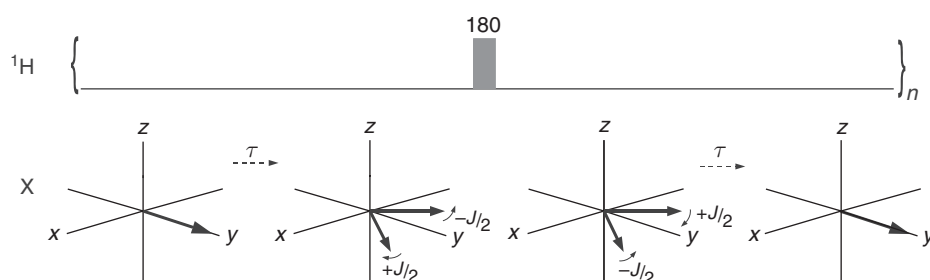


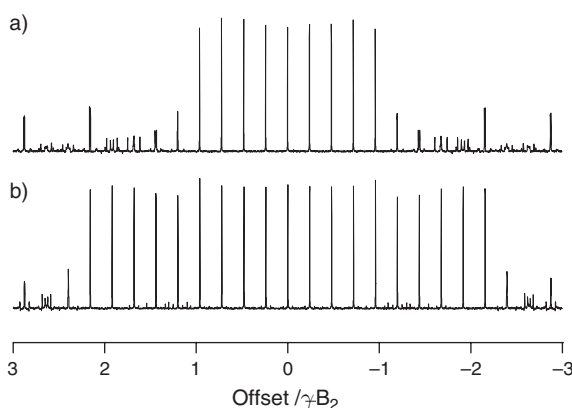
Figure 10.14. Heteronuclear broadband proton decoupling with a sequence of 180° proton pulses.

Table 10.2. Selected composite-pulse sequences for broadband decoupling and spin locking

Sequence	Bandwidth (γB_2)	Application	References
<i>Decoupling</i>			
Continuous wave	< 0.1	Selective decoupling only	
MLEV-16	1.5	^1H decoupling	[29]
WALTZ-16	2.0	High-resolution ^1H decoupling	[30, 31]
DIPSI-2	1.2	Very high-resolution ^1H decoupling	[32]
GARP	4.8	X-nucleus decoupling	[33]
SUSAN	6.2	X-nucleus decoupling	[34]
<i>Spin-locking</i>			
MLEV-17	0.6	TOCSY mixing scheme	[35]
DIPSI-2	1.2	TOCSY mixing scheme	[36]
FLOPSY-8	1.9	TOCSY mixing scheme	[37]

The technicalities of broadband decoupling are extensively discussed in [38].

Figure 10.15. Experimental comparison of the decoupling offset profiles of (a) WALTZ-16 and (b) GARP relative to the decoupling rf field strength γB_2 . The sample was carbon-13-labelled methanoic acid in D_2O ($^1J_{\text{CH}} = 195 \text{ Hz}$).

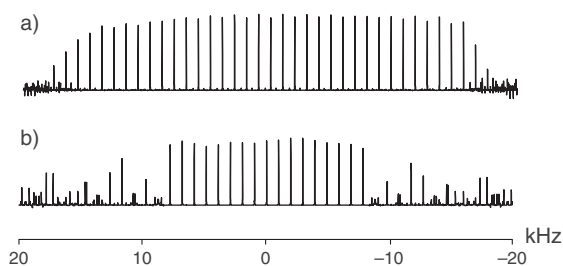


continued increase in static fields, even greater decoupling bandwidths become necessary and the most effective approach so far appears to be the use of adiabatic decoupling methods.

10.3.1. Broadband adiabatic decoupling

As broadband decoupling relies upon the repeated application of 180° inversion pulses, it is rational to suppose that greater decoupling bandwidths can be achieved through the use of adiabatic inversion pulses within decoupling schemes. Indeed, such methods have resulted in decoupling schemes that are effective over far greater bandwidths ($>20 \gamma B_2$) than those attainable with conventional composite-pulse sequences [14, 23, 24]. As for CPD, these schemes benefit from the use of suitable supercycles, one common scheme employing a five-step $0^\circ, 150^\circ, 60^\circ, 150^\circ, 0^\circ$ phase cycle [25] nested with the MLEV-4 cycle described above. It is also common practice to employ adiabatic pulses with lower adiabaticity factor Q in order to reduce power requirements and thus avoid sample heating; typically a Q of 2–3 is suitable for decoupling applications. The use of such a decoupling scheme is illustrated in Fig. 10.16 for ^{13}C decoupling using a 42-kHz CHIRP sweep of 1.5 ms duration (20% smoothing), with Q reduced to 2.5 and $\gamma B_{2(\text{max})} = 3.34 \text{ kHz}$, corresponding to a hard 90° pulse of $75 \mu\text{s}$. The decoupling is effective over $\sim 30 \text{ kHz}$ (around 70% of the total sweep range), equivalent to a 240 ppm bandwidth for ^{13}C at 500 MHz

Figure 10.16. Experimental $^1\text{H}\{^{13}\text{C}\}$ broadband decoupling profiles of (a) smoothed CHIRP (20%, 42 kHz, 1.5 ms, $Q = 2.5$) and (b) GARP. In both cases, the $\gamma B_{2(\text{max})}$ was 3.34 kHz. The sample was carbon-13-labelled methanoic acid in D_2O ($^1J_{\text{CH}} = 195 \text{ Hz}$).



and ample for most applications. This is contrasted with the decoupling bandwidth afforded by GARP CPD applied at the same rf amplitude, a typical power for heteronuclear decoupling. Clearly the adiabatic decoupling has a useable bandwidth that is, in this case, approximately twice that of GARP.

One of the more significant drawbacks to the use of adiabatic decoupling schemes is the appearance of unwelcome cycling sidebands, modulation sidebands that flank the main decoupled resonance, and may give rise to interfering peaks in multi-dimensional spectra [26, 27]. These can be minimised by employing a so-called *bi-level decoupling* scheme that starts initially with a brief high-power spin lock applied prior to the adiabatic decoupling itself to momentarily delay the onset of the modulation that gives rise to the sidebands. The duration of the spin lock is varied according to the number of transients collected such that addition of all transients acts to attenuate the sidebands responses.

The use of adiabatic schemes has most significance for the decoupling of heteronuclear X spins at higher fields, although they may still have relevance to decoupling at more modest fields, for example, in the decoupling of ^{19}F over a large chemical shift range. They have also been suggested for improved ^1H decoupling when very accurate quantification of ^{13}C spectra is required [28].

10.3.2. Spin-locking

The ability to ‘spin-lock’ magnetisation along a predefined axis plays an essential role in the TOCSY experiment, and Section 5.7.1 has already introduced the idea that a single continuous pulse or a series of closely spaced 180° pulses can, in principle, be used in this context to repeatedly refocus chemical shift evolution whilst allowing *homonuclear* couplings to evolve. Following from the above discussions, a better approach is clearly to use a series of 180° composite pulses, which offer compensation for resonance offset and rf inhomogeneity, and many of the sequences originally designed as heteronuclear decoupling sequences (which require repeated spin inversions) have since been applied as spin-lock sequences (which require repeated spin refocusing). The original and still widely used mixing scheme is based on the MLEV-16 sequence, in which each cycle is followed by a 60° pulse to compensate errors that would otherwise accrue during extended mixing, producing the popular MLEV-17 spin lock (see Table 10.1). More sophisticated sequences that allow for the influence of homonuclear couplings have since been developed, a factor not considered during the design of the early heteronuclear decoupling sequences. In particular, the DIPSI-2 sequence has better performance than MLEV or WALTZ in these circumstances and is thus widely used for isotropic mixing in the TOCSY experiment.

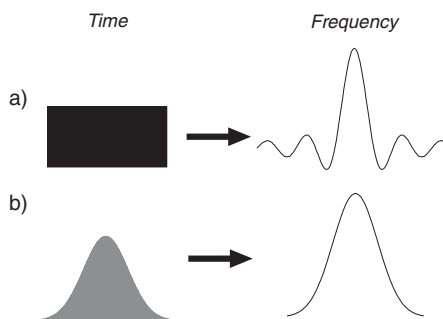
10.4. SELECTIVE EXCITATION AND SOFT PULSES

So far discussions in this chapter have dealt with the use of the so-called *hard* pulses, that is, pulses that (ideally) are equally effective over the whole chemical shift range, or with *adiabatic* pulses that have similar goals. We have also seen composite pulses that help one approach these ideals more closely. In some instances, however, it can be a distinct advantage if only a selected region of the spectrum is influenced by a so-called *soft* pulse, and a number of examples utilising such *selective excitation* have been presented in Chapters 5–8. Principal applications in a chemical context include the following:

- Reduced dimensionality of $n\text{D}$ sequences, for example, 1D analogues of 2D experiments;
- Selective removal of unwanted resonances, for example, solvent suppression;
- Extraction of specific pieces of information, for example, the measurement of specific long-range ^1H – ^{13}C coupling constants;
- 1D sequences that intrinsically require the selection of a single resonance, for example, inversion (saturation) transfer experiments in studies of chemical dynamics.

Replacing hard pulses with their selective counterparts within a multi-dimensional sequence leads to a spectrum of lower dimensionality having a number of advantages over its fully fledged cousin [39, 40]. One-dimensional analogues of more conventional 2D experiments allow greater digital resolution and thus a more detailed insight into fine structure; these equate to high-resolution slices through the related 2D experiment at the shift of the selected spin, for example, the 1D TOCSY experiment of Section 5.7.4 or the 1D NOESY of Section 8.7.2. They may also benefit by being quicker to acquire and analyse. In short, such methods may provide faster and more detailed answers when a specific structural question is being addressed, and discussions on the use of selective pulses in high-resolution NMR have featured in a number of extensive reviews [39, 41–43].

Figure 10.17. Schematic excitation profiles for (a) a low-power rectangular pulse and (b) a smoothly truncated-shaped pulse.



The basic approach to producing a *selective* pulse is to reduce the rf power and increase the duration of the pulse. By reducing B_1 , the frequency spread over which the pulse is effective likewise diminishes. At the same time, the speed of rotation of vectors being driven by the rf also decreases, meaning longer pulses are required to achieve the desired tip angle. The simplest selective pulse is therefore a long, weak rectangular pulse, by analogy with the usual rectangular hard pulse. Unfortunately, this has a rather undesirable excitation profile in the form of sidelobes that extend far from the principal excitation window (Fig. 10.17a), resulting in rather poor selectivity. The origin of the undesirable sidelobes lies in the sharp edges of the pulse that introduce a sinc-like oscillation to the profile; note the similarity with the ‘sinc wiggles’ in spectra caused by the premature truncation of an FID (Section 3.2.3). These may be suppressed by smoothing the edges of the pulse (see Fig. 10.17b), just as apodisation of the truncated FID reduces the wiggles, and such *pulse shaping* has given rise to a multitude of selective pulses with various characteristics that find use in modern NMR. A small selection of these is considered below, which have been chosen for their general applicability and/or otherwise desirable properties for high-resolution spectroscopy.

10.4.1. Shaped soft pulses

Associated with the use of selective pulses are a number of experimental factors that have a considerable bearing on the selection (and design) of a soft pulse. In short, the key features are the following:

- duration;
- frequency profile; and
- phase behaviour.

Soft pulses are typically 1–100 ms long, three orders of magnitude longer than hard pulses, which may have implications with regard to chemical shift and coupling evolution and to relaxation losses during the pulse. Therefore, it is desirable to have a soft pulse that is as short as possible but still able to deliver the desired selection. This selection is defined by the frequency bandwidth over which the pulse is effective. Ideally, the bandwidth profile should be rectangular, with everything outside the desired window insensitive to the pulse; such a profile is sometimes referred to as the ‘top-hat’ for obvious reasons [44]. The phase of the excited resonances should also be uniform over the whole excitation window, although in reality this can be difficult to achieve since off-resonance effects are severe with the weak B_1 fields used. Unless corrected, this results in considerable phase distortion away from exact resonance, which can be detrimental to multi-pulse sequences where the precise control of magnetisation is often required. These primary considerations are addressed below for some commonly encountered shaped pulses, whilst their practical implementation is described in Section 10.4.4.

Gaussian pulses

The original and experimentally simplest shaped pulse has the smooth Gaussian envelope [45] (Fig. 10.18). The (absolute-value) frequency excitation profile is also Gaussian, tailing away rapidly with offset, and, although not an ideal top-hat profile, is clearly superior to the rectangular pulse (Fig. 10.19). Just outside the principal excitation window, magnetisation vectors also feel the effect of the pulse but tend to be driven back towards the starting position, so experience no *net* effect, as can be seen in the trajectories of Fig. 10.20a. This figure also illustrates the problem of phase dispersion across the bandwidth for different offsets. For a pulse of phase x , both the desired M_y and unwanted M_x components (absorptive and dispersive responses, respectively) are created by the pulse, producing a significant phase gradient, which can be corrected in the simplest of

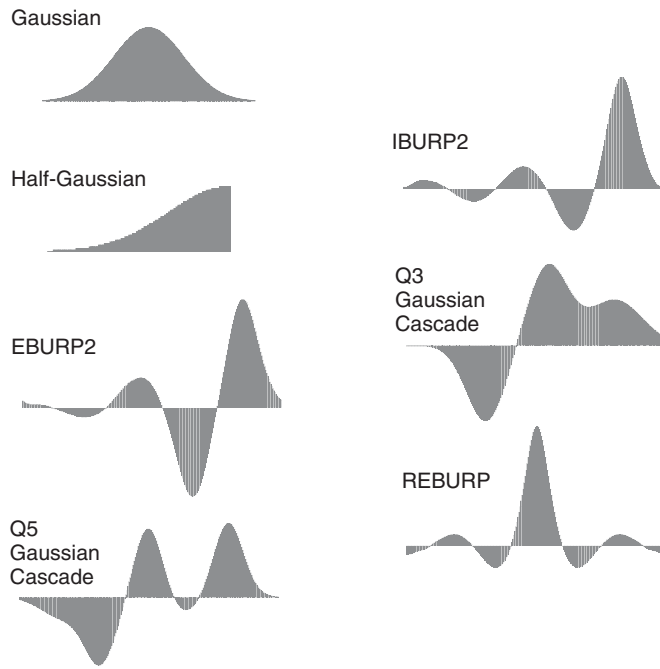


Figure 10.18. Time-domain profiles of some common shaped, selective excitation pulses.

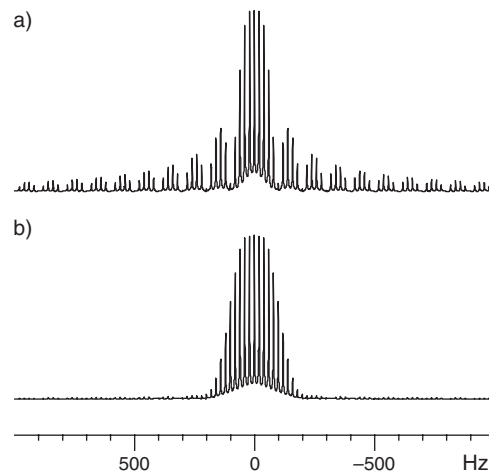


Figure 10.19. Absolute-value frequency-domain excitation profiles for (a) a rectangular pulse and (b) a Gaussian-shaped pulse.

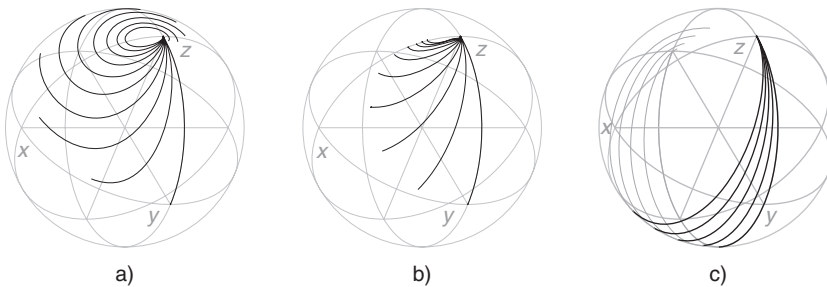
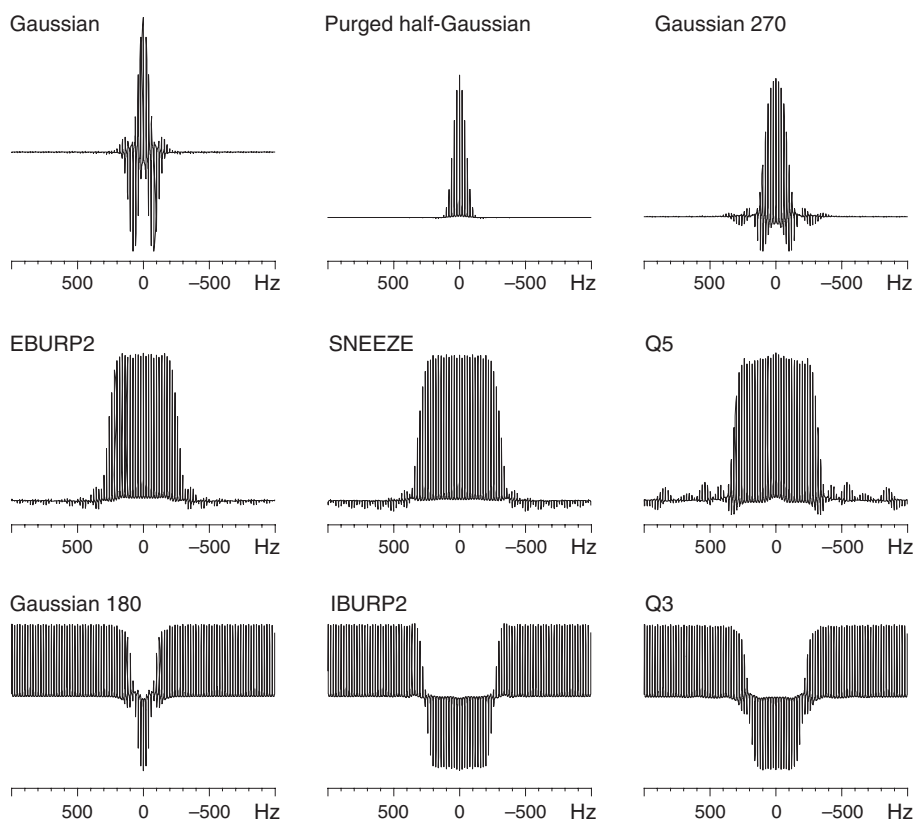


Figure 10.20. Simulated excitation trajectories as a function of resonance offset for (a) the Gaussian, (b) the half-Gaussian and (c) the Gaussian-270.

experiments, but in general may be problematic. In addition, inverted responses are also generated (those terminating in the $-y$ hemisphere) and thus the profile retains some undesirable oscillatory behaviour (this is hidden in Fig. 10.19 because an absolute-value display was used but can be seen in Fig. 10.21).

Better alternatives for the excitation of longitudinal magnetisation are the half-Gaussian [46], which, as the name suggests, is simply a Gaussian profile terminated at the midpoint, and the 270° Gaussian [47]. The half-Gaussian pulse does not produce negative sidelobes because vectors never reach the $-y$ hemisphere, although it still generates a considerable

Figure 10.21. Simulated excitation profiles of selected shaped pulses (of 10 ms duration); see Table 10.3. The inversion profiles (lower trace) were simulated with a 180(soft)–90(hard) sequence.



dispersive component, M_x , as is apparent from the trajectories of Fig. 10.20b. The dispersive responses may be removed by a phase-alternated hard ‘purge’ pulse applied orthogonally to and immediately after the half-Gaussian. This cancels any M_x components, retaining only the desired M_y components and thus removing the phase gradient. The improved phase properties of this scheme give rise to the name ‘purged half-Gaussian’ [48] (see Fig. 10.21). The 270° Gaussian pulse equates to a net –90° rotation and is identical to the 90° pulse except for a three-fold amplitude difference. The interesting feature of this pulse is that for spins close to resonance, it has a self-refocusing effect on both chemical shifts and coupling constants (see Fig. 10.20c) and thus has better phase properties than the 90° Gaussian pulse (see Fig. 10.21). As it does not require purging, it is simple to implement and is the excitation pulse of choice when high selectivity is not critical.

Pure-phase pulses

More elaborate pulse shapes that aim at producing a near top-hat profile yet retain uniform phase for all excited resonances within a predefined frequency window have been developed over the years. These operate without the need for purging pulses or further modifications, allowing them to be used directly in place of hard pulses. They are typically generated by computerised procedures, which result in more exotic pulse envelopes (see Fig. 10.18, and acronyms!) that drive magnetisation vectors along rather more tortuous trajectories than the simpler Gaussian-shaped cousins. Trajectories are shown in Fig. 10.22 for two members of the BURP family of pulses (*B*and-selective, *U*niform Response,

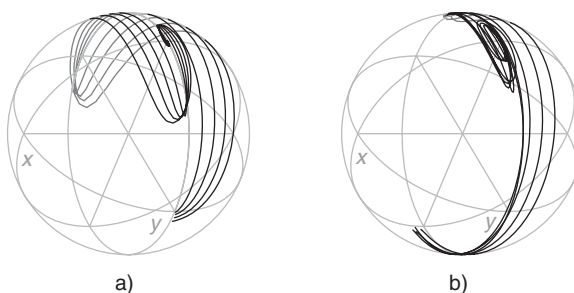


Figure 10.22. Simulated trajectories of nuclear spin vectors as a function of resonance offset during the (a) EBURP2 90° excitation pulse and (b) the IBURP2 180° inversion pulse.

Table 10.3. Properties of some shaped soft pulses

Shaped pulse	Application	Bandwidth factor	Attenuation factor (dB)	References
Rectangular	Universal	1.1	–	–
<i>90° pulses</i>				
Gaussian	Universal	2.1	7.7	[45]
Purged half-Gaussian	Excitation only	0.8	7.7	[46, 48]
Gaussian-270	Excitation only	1.3	16.7	[47]
EBURP2	Excitation only, pure phase	4.9	24.3	[50]
SNEEZE ^a	Excitation only, pure phase	5.8	26.6	[65]
UBURP	Universal, pure phase	4.7	32.2	[50]
E-SNOB ^b	Excitation only, pure phase	1.9	7.0	[56]
G4 Gaussian cascade	Excitation only, pure phase	7.8	25.4	[51]
Q5 Gaussian cascade	Universal, pure phase	6.2	25.3	[52]
<i>180° pulses</i>				
Gaussian-180	Universal	0.7	13.7	[45]
IBURP2	Inversion only, pure phase	4.5	25.9	[50]
REBURP	Universal, pure phase	4.6	28.0	[50]
I-SNOB2 ^b	Inversion only, pure phase	1.6	13.4	[56]
R-SNOB ^b	Universal, pure phase	2.3	13.4	[56]
G3 Gaussian cascade	Inversion only, pure phase	3.6	23.1	[51]
Q3 Gaussian cascade	Universal, pure phase	3.4	22.4	[52]

Universal pulses act equally on any initial magnetisation state whereas excitation and inversion pulses are designed to act on longitudinal magnetisation only. The bandwidth factor is the product of the pulse duration, Δt , and the excitation bandwidth, Δf , which is here defined as the excitation window over which the pulse is at least 70% effective (net pulse amplitude within 3 dB of the maximum; other publications may define this value for higher levels and so quote smaller bandwidth factors). This factor is used to estimate the appropriate pulse duration for the desired bandwidth. The attenuation factor is used for approximate power calibration and represents the amount by which the transmitter output should be increased over that of a soft rectangular pulse of equal duration. The Gaussian-based profiles are truncated at the 1% level.

^aThe SNEEZE pulse produces more uniform excitation of M_z than EBURP2.

^bThe SNOB family are designed to have short pulse durations.

Pure-phase pulses) [49, 50] namely the EBURP2 excitation pulse and the IBURP2 inversion pulse (see Section 10.4.4 and Table 10.3). Despite the globetrotting journeys undertaken, trajectories for all offsets within the effective window terminate close to the ideal endpoint with only small intensity or phase errors. The corresponding profiles for these pulses are also shown in Fig. 10.21 and illustrate the uniform, pure-phase behaviour (although experimentally these depend *critically* on accurate pulse calibrations). An experimental demonstration of selective excitation with the EBURP2 pulse is shown in Fig. 10.23 for two different excitation bandwidths. Interestingly, a different design approach has led to a similar family of pulses with largely similar properties, the so-called Gaussian cascades [51, 52] (see Table 10.3 and Fig. 10.18). As the name implies, these are composed of clusters of Gaussian pulses whose net effect is closer to the ideal than any single element, reminiscent of the composite hard pulses of Section 10.1.

One feature of these more elaborate envelopes is that they have often been designed with a particular function in mind and may not perform well at anything else. For example, the EBURP pulses are 90° *excitation pulses* designed to act on longitudinal magnetisation and will not perform as desired on transverse magnetisation. Likewise, selective 180° inversion pulses may not work as refocusing elements in the generation of spin-echoes. Those designed to act on *any* initial magnetisation state are referred to as *universal pulses*. More complex profiles also tend to be longer than their simpler counterparts for a given excitation bandwidth and tip angle, meaning relaxation effects may be problematic, particularly for the case of

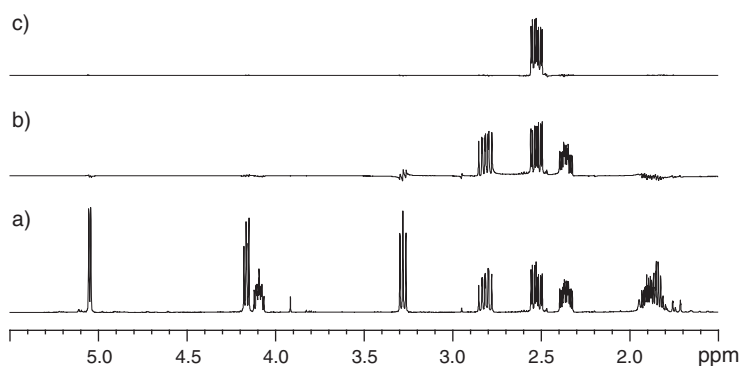


Figure 10.23. Selective excitation with the EBURP2 band-selective pulse, using a pulse duration of (b) 14 ms and (c) 100 ms, shown above the conventional 1D spectrum (a).

larger molecules or for very long, highly selective pulses. The influence of relaxation on their performance has been addressed [53, 54] with more tolerant profiles suggested (SLURPs [55]) and shorter pulses developed with larger molecules in mind (SNOBs [56]).

Implementing shaped pulses

In modern high-resolution instruments, the control of pulse amplitudes ‘on-the-fly’ is a standard feature. Typically, the pulse envelope is defined in a series of discrete steps as a histogram, with each element having a defined amplitude and phase. To provide a close match to the smooth theoretical pulse shape, a sufficient number of elements must be defined, typically 1000 or more (but is instrument-dependent). Many pulse envelopes come predefined on modern instruments, allowing their direct implementation.

10.4.2. Excitation sculpting

Modern methods of selective excitation now routinely combine shaped pulses with pulsed field gradients to produce experimentally robust excitation sequences with a number of desirable properties [42]. These sequences are based on either single pulsed field gradient spin-echoes (SPFGSEs) or double PFG spin-echoes (DPFGSEs) (Fig. 10.24), which may be understood with reference to the single echo sequence (see Fig. 10.24a). This may be represented G_1-S-G_1 where S represents *any* selective 180° pulse (or pulse train) and the bracketing gradients G_1 are identical. For those spins that experience the selective inversion pulse, the two gradients act in opposition and thus refocus this selected magnetisation, hence this is known as a gradient echo. Spins that do not experience this pulse, that is, those outside its effective bandwidth, feel only the cumulative effect of both gradients, so they remain fully dephased in the transverse plane and thus unobservable. This single gradient echo therefore achieves clean resonance selection according to the profile of pulse S .

The *phase* profile of the selected resonance(s) is also dictated by the phase properties of the selective pulse S , which may not be ideal. Repeating the gradient echo once again with a different gradient strength G_2 (to avoid accidental refocusing of the previously dephased unwanted magnetisation, Fig. 10.24b) exactly cancels any remaining phase errors, and the resulting *pure-phase* excitation profile depends only on the *inversion* properties of the selective pulse. Experimentally this is an enormous benefit because it makes implementation of the selective sequence straightforward and because the field gradients ensure excellent suppression of unwanted resonances. It is also very much easier to select (and design) a pulse with a desirable ‘top-hat’ inversion profile when its phase behaviour is of no concern. The resulting excitation profile of the double PFG spin-echo (DPFGSE) sequence is dictated by the cumulative effect of the repeated inversion pulses, resulting in a ‘chipping away’ of magnetisation by the series of gradient echoes, hence the term *excitation sculpting* [57, 58]. An example of the clean, pure-phase selective excitation that can be achieved with this sequence is illustrated in Fig. 10.25. This could represent the starting point for various selective 1D experiments, including TOCSY [59, 60] and NOESY [58, 61]; see, for example, the 1D gradient TOCSY or NOESY experiments of Sections 5.7.4 and 8.7.2, respectively. The use of a second DPFGSE sequence after one transfer step leads to more elaborate ‘doubly selective’ experiments also described in Section 8.7.2, whilst a slight modification produces an extremely effective solvent suppression scheme, described below in Section 10.5. Alternatively, the shorter single-echo excitation may be more appropriate

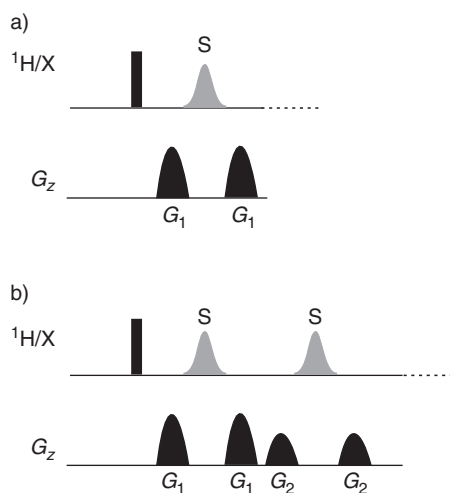


Figure 10.24. Selective excitation sequences based on (a) a single and (b) a double pulsed field gradient spin-echo. The element S represents any selective 180° inversion pulse or pulse cluster.

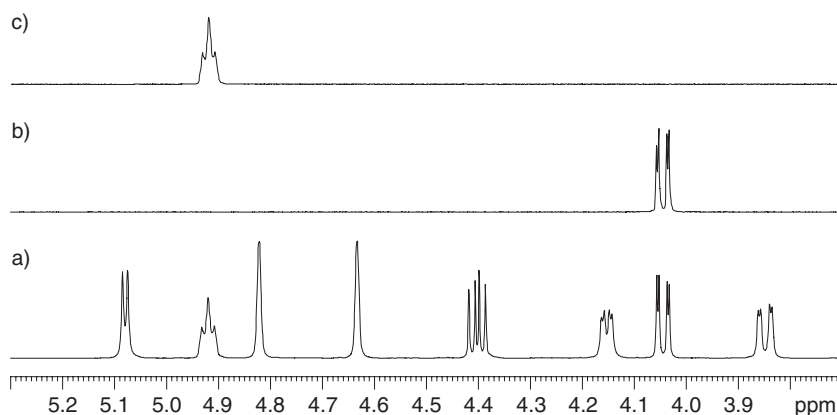


Figure 10.25. Clean selective excitation with the double pulsed field gradient spin-echo sequence using a 40-ms Gaussian 180° pulse and gradients of 0.07:0.07:0.03:0.03 T/m.

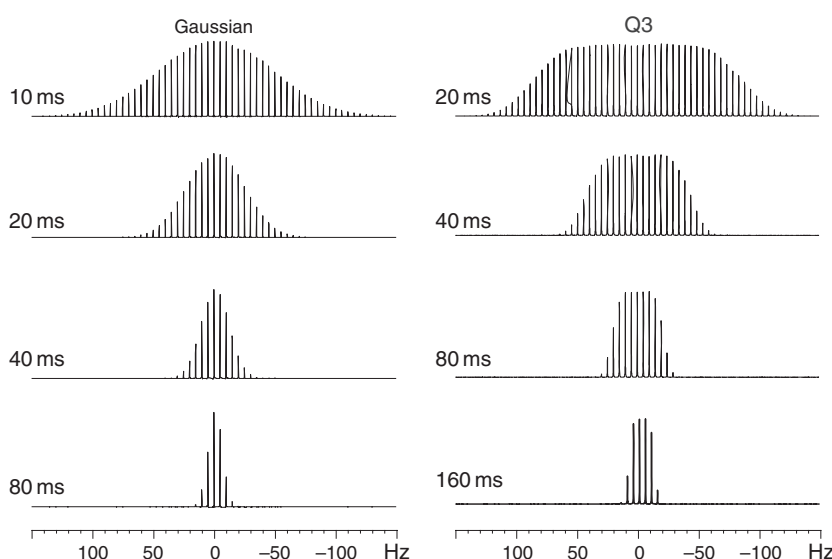


Figure 10.26. Experimental excitation profiles for the double pulsed field gradient spin-echo excitation sequences in which the element S is either a 180° Gaussian pulse (truncated at 1%) or a 180° Q3 pulse of the durations shown. Line spacings are 5 Hz.

for the study of larger molecules where T_2 relaxation losses can be significant and more generally may be preferable when dealing with broader resonances or fast relaxing spins.

For use in the laboratory, it is convenient to choose a simple, robust inversion pulse as the element S , and the Gaussian pulse is well suited to routine use. Example excitation profiles for this are illustrated in Fig. 10.26 and offer guidance on selection of pulse duration for a desired excitation window. For proton spectroscopy, a Gaussian pulse of around 40 ms proves suitable for many applications. With increasing ease of implementation of shaped pulses on spectrometers with linearised amplifier outputs, the use of more sophisticated shapes becomes more attractive since their power attenuations may be computed and low-power pulse calibrations are not required. The excitation profile of the Q3 Gaussian cascade is compared with the simpler Gaussian pulse in Fig. 10.26. This demonstrates the more uniform profile one would expect but at the expense of greater pulse duration, as predicted by the corresponding pulse bandwidth factors (see Table 10.3).

10.4.3. DANTE sequences

In the early days of selective excitation, spectrometers were not equipped to generate amplitude-modulated rf pulses and the DANTE method (Delays Alternating with Nutation for Tailored Excitation) was devised [62] requiring only short, hard pulses. Although largely superseded by the amplitude-modulated soft pulses, DANTE may still be the method of choice in older instrumentation.

The basic DANTE sequence is composed of a series of N short, hard pulses of tip angle α where $\alpha \ll 90^\circ$, interspersed with fixed delays, τ , for free precession

$$\text{DANTE: } [\alpha - \tau]_N \text{ or more correctly } [\tau/2 - \alpha - \tau/2]_N$$

The total length of the selective pulse is the product $N\tau t_p$ where t_p is the duration of each hard pulse, and the net on-resonance tip angle is the sum of the individual pulses. The effect of such a sequence is illustrated in Fig. 10.27. Following each hard pulse, spins are able to

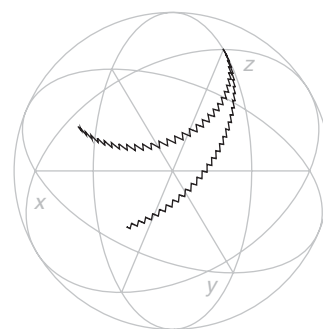


Figure 10.27. Simulated zigzag excitation trajectories of nuclear spin vectors at different resonance offsets for the DANTE sequence. Thirty pulses of 3° each were separated by delays of 0.33 ms, producing an effective 10-ms selective pulse.

precess during the short delay τ according to their offset from resonance before being pulsed once again. On-resonance trajectories show no precession and are thus driven directly towards the $+y$ axis whilst those close to resonance follow a zigzag path. If N is sufficiently large (typically ≥ 20), the result closely resembles the smooth path taken under the influence of a soft rectangular pulse of equivalent total duration and similar selectivity results.

Pulse ‘shaping’ in the DANTE approach is achieved by keeping the pulse *amplitude* constant but varying the *duration* of each hard pulse throughout the sequence to match the desired envelop. Thus, for example, a Gaussian envelope may be emulated by varying pulse durations according to a Gaussian profile. Limitations with this approach arise when very small pulse durations are required ($< 1 \mu\text{s}$), since pulse transmitters may then be unable to deliver the necessary precision. In such cases it may be necessary to add fixed attenuation to the transmitter output to allow the use of longer but more accurate hard pulses.

The major difference between soft-shaped pulses and DANTE methods is the occurrence of strong sideband excitation windows on either side of the principal window with DANTE. These occur at offsets from the transmitter at multiples of the hard-pulse frequency $1/\tau$. They arise from magnetisation vectors that are far from resonance and that precess full circle during the τ period. Since this behaviour is precisely equivalent to no precession, they are excited as if on-resonance. Further, sidebands at $\pm 2/\tau$, $3/\tau$ and so on also occur by virtue of trajectories completing multiple full circles during τ . Such multi-site excitation can at times be desirable [63, 64] but if only a single excitation window is required, the hard-pulse repetition frequency must be adjusted by varying τ to ensure that the sideband excitations do not coincide with other resonances.

10.4.4. Practical considerations

This section addresses some of the practical consequences of employing soft pulses, which, in general, demand rather more care and attention in their implementation than hard pulses and so typically require operator intervention for their success. The general rule for soft-pulse selection is to choose the simplest pulse that will provide the performance characteristics required. More complex pulse shapes, although providing improved profiles, tend to require greatest care in calibration and are thus rather more awkward to use. For example, the BURP pulses are extremely sensitive to pulse width miscalibration, which leads to significant distortion of otherwise ‘uniform’ profiles [50]. From a purely practical perspective, the simple Gaussian pulses are most robust and easiest to employ and represent a suitable initial choice for many applications [39] or for novice users. If very high selectivity is required, the pure-phase pulses (BURP or Gaussian cascade families) prove more suitable, and the choice of a specific or a universal pulse (see Table 10.3) becomes significant, according to the application. The linearised amplifiers found on modern instruments make implementation of all shaped pulses a more straightforward process since their power settings can be derived by calculation from hard-pulse calibrations, and tedious low-power calibrations can be avoided.

The excitation profile of soft pulses is defined by the duration of the pulse, these two factors sharing an inverse proportionality. More precisely, pulse shapes have associated with them a dimensionless *bandwidth factor*, which is the product of the pulse duration, Δt , and its effective excitation bandwidth, Δf , for a correctly calibrated pulse. This is fixed for any given pulse envelope and represents its time efficiency. It is used to estimate the required pulse duration for a desired effective bandwidth; Table 10.3 summarises these factors for some common pulse envelopes. Thus, an excitation bandwidth of 100 Hz requires a 21-ms 90° Gaussian pulse but a 49-ms EBURP2 pulse; clearly the Gaussian pulse is more time-efficient but has a poorer excitation profile.

Having determined the necessary pulse duration, the transmitter *power* must be calibrated so that the pulse delivers the appropriate tip angle. As already alluded to, this can be avoided on instruments with linearised amplifier outputs, *provided accurate hard-pulse calibrations are known*. The calibration of soft pulses differs from that for hard pulses where one uses a fixed-pulse amplitude but varies its duration. For practical convenience, amplitude calibration is usually based on previously recorded calibrations for a soft rectangular pulse (as described below), from which an estimate of the required power change is calculated. Table 10.3 also summarises the necessary changes in transmitter attenuation for various envelopes of equivalent duration, with the more elaborate pulse shapes invariably requiring increased rf peak amplitudes (*decreased* attenuation of transmitter output).

As an illustration, suppose one wished to excite a window of 100 Hz with a SNEEZE pulse and had previously determined that a soft 90° rectangular pulse of 10 ms required an

attenuation of 60 dB. From the bandwidth factor, one can determine the pulse duration must be 58 ms. From the power ratio equations given in Section 3.5.1, one may calculate that a soft *rectangular* pulse of this duration requires 15.3 dB *greater attenuation* than the 10-ms pulse ($20 \log 5.8$), simply because it is longer. Table 10.3 shows that the SNEEZE envelop requires 26.6 dB *less* attenuation than a rectangular pulse of equal duration. The SNEEZE pulse therefore requires 11.3 dB ($26.6 - 15.3$) *less attenuation* than that of the reference 10-ms soft rectangular pulse, and the transmitter amplitude setting becomes 48.7 dB. Fine tuning may then be required for optimum results without linearised amplifiers, but when these are available, the power may be determined directly from the high-power, 90° pulse calibration. In this example, with a 90° pulse of 10 μ s at 0 dB attenuation, the calculation becomes $20(\log 58000/10) - 26.6 = 48.7$ dB.

Amplitude calibration

In the absence of linearised amplifiers, the amplitude calibration of a soft pulse essentially follows the procedures introduced for hard pulses in Section 3.5.1 and the descriptions below assume familiarity with these. For soft pulses on the observe channel, the transmitter frequency should be placed on-resonance for the target spin and the 90° or 180° condition sought directly by variation of transmitter power, starting with very low values (high attenuations) and progressively increasing (note there is a logarithmic not linear dependence on dB). If calibrations must be performed indirectly, for example, on the decoupler channel of older instruments, a slight variation in the method of Fig. 3.62 (Chapter 3) for a two-spin AX pair is used (Fig. 10.28a). In this, an additional spin-lock pulse is applied to the observe channel during the soft pulse to decouple the AX interaction [66]. This collapse of the AX doublet means that the soft pulse can be applied to the centre of the A spin doublet. Variation of the transmitter power produces results similar to those of Fig. 3.63 (Chapter 3) when the 90° or 180° condition is satisfied. With modern instrumentation, however, calibrations determined directly on the observe channel are equally valid if this is later used as the indirect (decoupler) channel in subsequent experiments, thus bypassing the need for such indirect calibrations.

Phase calibration

Precise control of the *relative* phase between pulses is crucial to the success of many multi-pulse NMR experiments, and some correction to the phase of a soft pulse may be required to maintain these relationships when both hard and soft pulses are to be applied to the same nucleus. When soft pulses are used on the observe channel, the phase difference (which may arise because of the potentially different rf paths used for high- and low-power pulses) may be determined by direct inspection of two separate 1D pulse-acquire spectra recorded with high- and low-power pulses but under otherwise identical conditions. Using only zero-order (frequency-independent) phase correction of each spectrum, the difference in the resulting phase constants (soft *minus* hard) represents the phase difference between the high- and low-power rf routes. Adding this as a constant offset to the soft pulse phase should yield spectra of phase identical to that of the hard-pulse spectrum when processed identically, and this correction can be used in all subsequent experiments, *provided the soft-pulse power remains unchanged*.

If phase differences must be determined indirectly, the sequence of Fig. 10.28b is suitable in which an additional hard pulse is used on the indirect channel prior to the soft. When two 90° pulses are of the same phase, their effect is additive and the sequence behaves as if a net 180° pulse has been applied to the selected spin, resulting in an inversion of the antiphase AX doublet. Similarly, two 180° pulses of similar phase behave as a net 360° pulse and have no effect on the doublet.

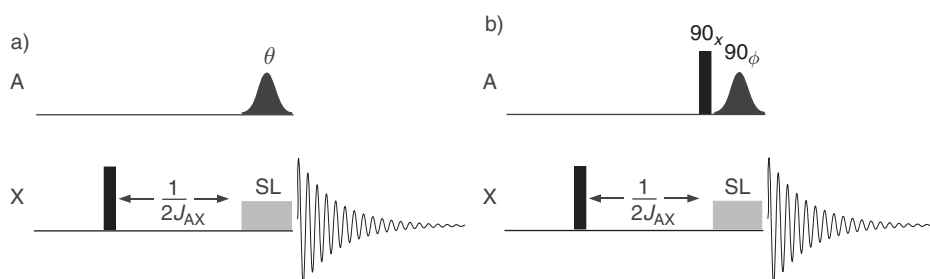


Figure 10.28. Soft-pulse calibration sequences for (a) amplitude and (b) hard/soft phases differences for selective pulses applied on the indirect (decoupler) channel. SL is a spin lock applied to decouple A and X during the soft pulse.

10.5. SOLVENT SUPPRESSION

Fortunately the majority of solvents used in organic NMR spectroscopy are readily available in the deuterated form. For proton spectroscopy in particular, this allows the chemist to focus on the solute spectrum, undisturbed by the solvent that is present in vast excess. Unfortunately, most molecules of biochemical or medicinal interest, notably biological macromolecules, must be studied in water and in order to observe all protons of interest within these molecules, including the often vitally important labile protons, protonated water must be employed as the solvent (containing 10% D₂O to maintain the field-frequency lock). Although H₂O is 110 M in protons, solute concentrations are more typically in the millimolar region and the 10⁴–10⁵ concentration difference imposes severe experimental difficulties, which demand the attenuation of the solvent resonance. In addition, LC-NMR may favour the use of protonated solvents for reasons of economy, also making efficient and robust solvent suppression essential. At a more mundane level, when using D₂O or CD₃OD for routine spectroscopy, a considerable residual HDO resonance may remain that may limit the useable receiver amplification and appear aesthetically unappealing.

The principal reason to suppress a large solvent resonance in the presence of far smaller solute resonances is to ensure that the dynamic range of the NMR signals lies within the dynamic range of the receiver and ADC (Section 3.2.6). Further concerns include the baseline distortions, *t*₁ noise in 2D experiments, radiation damping and potential spurious responses that are associated with very intense signals. Radiation damping leads to severe and undesirable broadening of the water resonance, which may mask the solute resonances. It arises because the intense NMR signal produced on excitation of the water generates a strong rf current in the detection coil (which ultimately produces the observed resonance). This current in turn generates its own rf field, which drives the water magnetisation back towards the equilibrium position; if you like, it acts as a water-selective pulse. The loss of transverse magnetisation is therefore accelerated, producing a reduced apparent *T*₂ and hence a broadened resonance.¹ In general, good lineshape is a prerequisite for successful solvent suppression and whilst efforts to optimise shimming pay dividends, the intense water singlet of H₂O is ideally suited to gradient shimming (Section 3.4.5). Improved results have also been reported when microscopic air bubbles have been removed from the sample by ultrasonic mixing.

The goal of solvent suppression is therefore to reduce the magnitude of the solvent resonance *before* the NMR signal reaches the receiver. This seemingly simple requirement has generated an enormous research area [67, 68], which is still on-going, emphasising the fact that this is by no means a trivial exercise. The more widely used approaches can be broadly classified into three areas:

- methods that saturate the water resonance;
- methods that produce zero net excitation of the water resonance; and
- methods that destroy the water resonance with pulsed field gradients.

The following sections illustrate examples from these areas that have proved most popular but represent only a small subsection of available methods (see reference [69]). All schemes inevitably involve some loss of signal intensity for those resonances close to that of the solvent, and careful adjustment of solution conditions, the simplest being sample temperature, can prove useful in avoiding signal losses by shifting the water resonance relative to solute signals (Fig. 10.29).

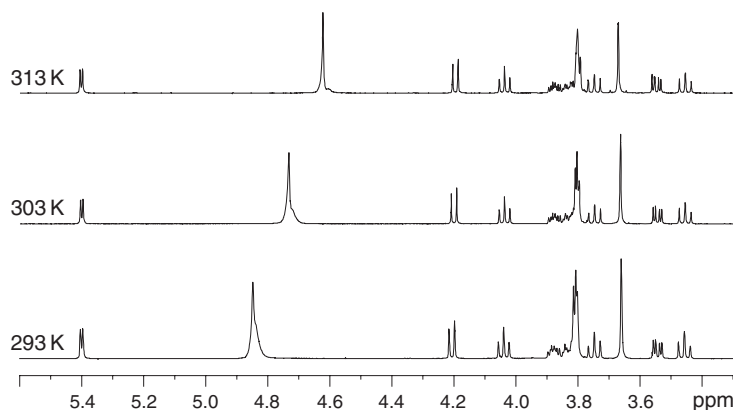


Figure 10.29. The temperature dependence of HDO (here partially suppressed). The shift corresponds to approx. 5 Hz/K at 500 MHz. Spectra are referenced to internal TSP.

¹ A test for radiation damping involves detuning the proton coil and reacquiring the proton spectrum. This degrades the *rf* coupling, thus reducing the back electromotive force (emf) and sharpening the water resonance.

10.5.1. Presaturation

The simplest and the most robust technique is presaturation of the solvent [70]. This is simple to implement, may be readily added to existing experiments and leaves (non-exchangeable) resonances away from the presaturation frequency unperturbed. It involves the application of continuous, weak rf irradiation at the solvent frequency prior to excitation and acquisition (Fig. 10.30a), rendering the solvent spins saturated and therefore unobservable (Fig. 10.31). Resonances invariably close to the solvent frequency also experience some loss in intensity, with weaker irradiation leading to less spillover but reduced saturation of the solvent. Longer presaturation periods improve the suppression at the expense of extended experiments, so a compromise is required and typically 1–3 s are used; trial and error usually represents the best approach to optimisation. Wherever possible, the same rf channel should be used for both presaturation and subsequent proton pulsing, with appropriate transmitter power switching.

A feature of simple presaturation in protonated solutions (particularly with older probes, which may also lack appropriate shielding of the coil leads [71]) is a residual ‘hump’ in the 1D spectrum that originates from peripheral regions of the sample that suffer B_0 and B_1 inhomogeneity. An effective means of suppressing this for 1D acquisitions is the so-called NOESY-presat sequence, which simply employs a *non-selective* 1D NOESY with short mixing time, or in other words, the first increment of a 2D NOESY experiment with mixing times of around 0–10 ms (see Fig. 10.30b). The usual NOESY phase cycle suppresses the hump, but the sequence is restricted to acquisition with 90° pulses, which is not optimal for signal averaging or spectrum integration. A variation, termed FLIPSY [72] (flip-angle-adjustable one-dimensional NOESY, Fig. 10.30c), allows the use of an arbitrary excitation pulse tip angle θ and is thus better suited to routine use.

A more recent approach [73] to cancelling the solvent signal arising from peripheral regions of the sample that minimises the loss of solute signal intensity employs an

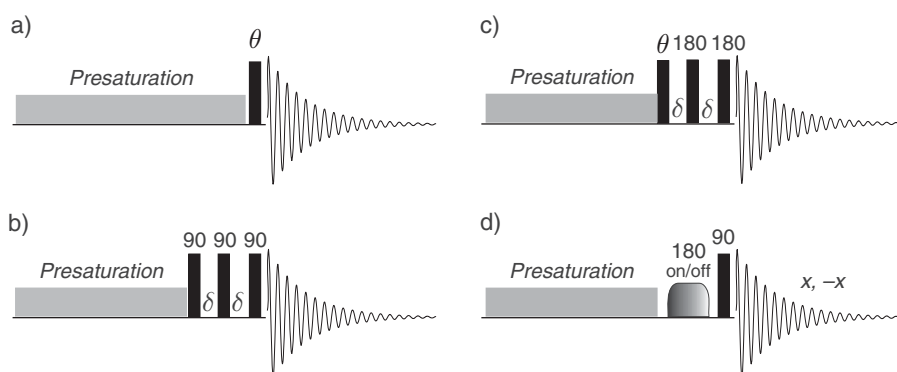


Figure 10.30. Solvent suppression schemes based on presaturation; (a) presaturation alone, (b) 1D NOESY, (c) FLIPSY and (d) Pre-SAT180. Sequence (b) makes use of the conventional NOESY phase cycle whereas FLIPSY uses EXORCYCLE on one (or both) of the 180° pulses (i.e. pulse = $x, y, -x, -y$, receiver = $x, -x, x, -x$). Sequence (d) applies the 180° adiabatic sweep on alternate scans combined with receiver inversion; optional opposing purging gradients may be applied on either side of the 180° pulse.

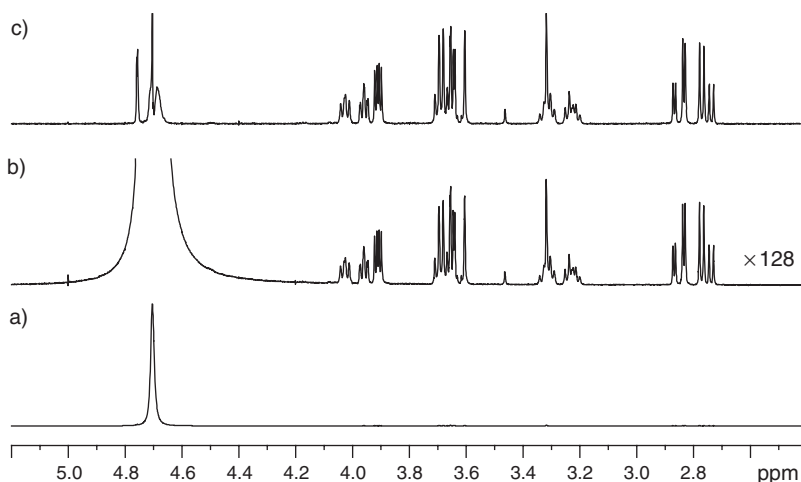


Figure 10.31. Solvent presaturation (2 s) reveals a resonance at 4.75 ppm in (c) previously masked by the H₂O resonance in (a) and its expansion (b).

alternating (on/off) 180° adiabatic sweep after simple presaturation but immediately prior to the observe pulse (see Fig. 10.30d). Subtraction of the data sets acquired with and without the adiabatic pulse leads to the co-addition of all signals in the bulk of the sample as these are inverted by the swept pulse but cancellation of the peripheral solvent signal, which does not sense the inversion. The so-called Pre-SAT180 method may also be incorporated within standard 2D sequences as an alternative to simple presaturation alone.

The principal disadvantage of all presaturation schemes is that they also lead to the suppression of exchangeable protons by the process of saturation transfer. In favourable cases, solution conditions may be altered (temperature and pH) to slow the exchange sufficiently to reduce signal attenuation, but this approach has limited applicability. The methods presented below largely avoid such losses.

10.5.2. Zero excitation

The second general approach strives to produce no net excitation of the solvent resonance. In other words, the sequence ultimately returns the solvent magnetisation to the $+z$ axis whilst at the same time placing all other magnetisation in the transverse plane prior to acquisition. Section 10.5.3 describes a related approach used in conjunction with pulsed field gradients.

Jump-return

To appreciate the principle behind zero excitation, consider the simplest example, the 'jump-return' sequence [74] $90_x-\tau-90_{-x}$, which subsequently spawned many others. The transmitter frequency is placed on the solvent resonance and all magnetisation is tipped into the transverse plane by the first 90° hard pulse. During the subsequent delay τ (typically a few hundred microseconds long), all vectors fan out in the transverse plane according to their offsets, except that of the solvent, which, being on-resonance, has zero frequency in the rotating frame. Thus, the solvent resonance is tipped back to the $+z$ axis as the second 90° pulse rotates the xy plane into the xz plane. The only remaining transverse magnetisation is the $\pm x$ -component of all vectors prior to the second pulse. Hence, this produces a sine-shaped excitation profile (Fig. 10.32a) with maximum amplitude at offsets from the transmitter frequency of $\pm 1/4\tau$ Hz and with resonances on either side of the transmitter displaying opposite phase (owing to their $\pm x$ orientations) but without additional phase dispersion. In practice, this simple scheme produces a rather narrow null at the transmitter offset and a significant solvent resonance typically remains, so more sophisticated sequences have been investigated.

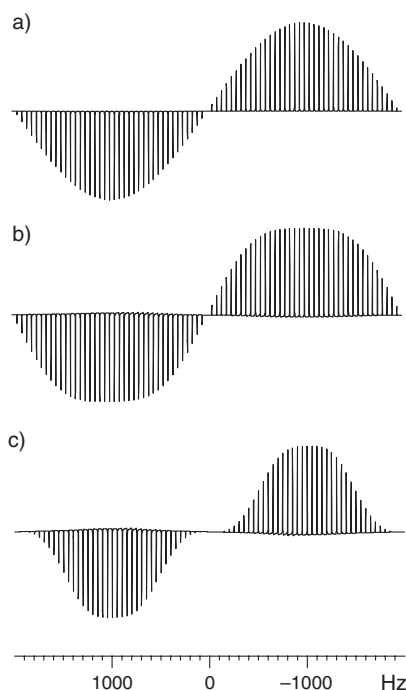


Figure 10.32. Simulated excitation profiles for (a) the jump-return sequence, (b) the $1-\bar{1}$ and (c) $1-\bar{3}-3-\bar{1}$ binomial sequences. The jump-return sequence used $\tau = 250 \mu\text{s}$ and the binomial sequences used $\tau = 500 \mu\text{s}$.

Binomial sequences

The binomial sequences aim at improving the zero excitation profile and provide schemes that are less sensitive to spectrometer imperfections. The series may be written $1\bar{1}$, $1\bar{2}-1$, $1\bar{3}-3\bar{1}$ and so on, where the numbers indicate the relative pulse widths, each separated by a delay τ , and the overbar indicates phase inversion of the pulse. For *off-resonance spins*, the pulse elements are *additive* at the excitation maximum, so, for example, should one require 90° off-resonance excitation, $1\bar{1}$ corresponds to the sequence $45_x-\tau-45_{-x}$. Of this binomial series, it turns out that the $1\bar{3}-3\bar{1}$ sequence [75] has good performance and is most tolerant of pulse imperfections by virtue of its symmetry [76]. The trajectory of spins with frequency offset $1/2\tau$ from the transmitter for a net 90° pulse ($1 \equiv 11.25^\circ$) is shown in Fig. 10.33. During each τ period, the spins precess in the rotating frame by half a revolution, so the effect of the phase-inverted pulses is additive and the magnetisation vector is driven stepwise into the transverse plane. As before, the on-resonance solvent vector shows no precession, so it is simply tipped back and forth, finally terminating at the North Pole.

Although maximum excitation occurs at $\pm 1/2\tau$ Hz from the transmitter offset, further nulls occur at offsets of $\pm n/\tau$ ($n = 1, 2, 3, \dots$ corresponding to complete revolutions during each τ), so a judicious choice of τ is required to provide excitation over the desired bandwidth. The excitation profiles of the $1\bar{1}$ and $1\bar{3}-3\bar{1}$ sequences are shown in Fig. 10.32b and c. Clearly the excitation is non-uniform, so places limits on quantitative measurements, and once again there exists a phase inversion on either side of the solvent. Both provide an effective null at the transmitter offset and suppression ratios in excess of 1000-fold can be achieved.

When implementing this sequence, it may be necessary to attenuate the transmitter output to increase the duration of each pulse so that the shorter elements do not demand very short ($<1\ \mu\text{s}$) pulses (note the similarity with the requirements for the DANTE hard-pulse selective excitation described above). The binomial sequences can be adjusted to provide an arbitrary overall tip angle by suitable adjustment of the tip angles for each element. For example, *inversion* of all off-resonance signals can be achieved by doubling all elements relative to the net 90° condition. Exactly this approach has been exploited in the gradient echo methods described below.

10.5.3. PFGs

The most effective approach to date to solvent suppression is the destruction of the net solvent magnetisation by pulsed field gradients, thus ensuring nothing of this remains observable immediately prior to acquisition. The gradient schemes described here act on transverse magnetisation, so they are readily appended to existing 1D and multi-dimensional sequences [77].

One popular approach, termed WATERGATE [78, 79] (water suppression by gradient-tailored excitation), makes use of a single PFG spin-echo, G_1-S-G_1 (Fig. 10.34a), in which both gradients are applied in the same sense. The element S is chosen to provide zero net rotation of the solvent resonance but to provide a 180° *inversion* to all others. This results in the solvent magnetisation experiencing a cumulative dephasing by the two gradients leading to its destruction whilst all others are refocused in the spin-echo by the second gradient and are therefore retained.

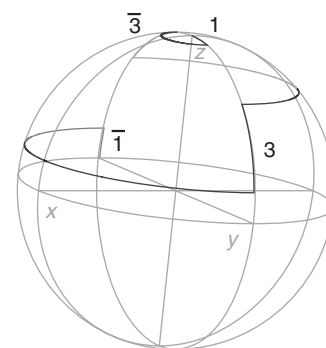
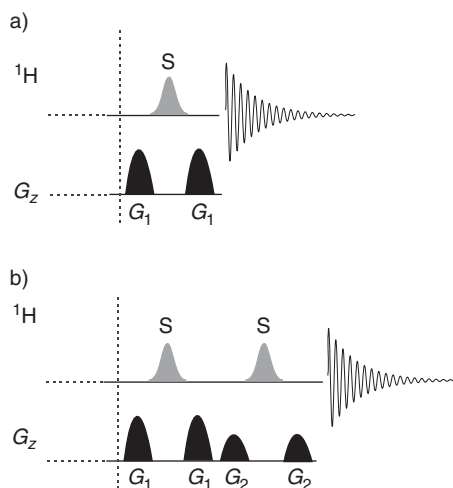


Figure 10.33. The trajectory of a magnetisation vector during the $1\bar{3}-3\bar{1}$ binomial sequence. The trace is shown for a spin with an offset from the transmitter of $+1/2\tau$ Hz corresponding to the excitation maximum.

Figure 10.34. Solvent suppression schemes employing pulsed field gradients based on (a) WATERGATE (single-echo) and (b) excitation sculpting (double-echo) principles. The pulse element S has zero net effect on the solvent resonance but inverts all others.

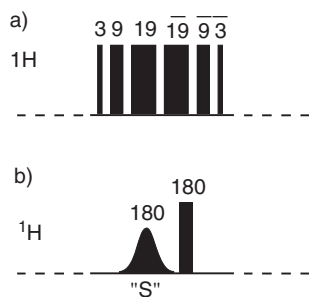


Figure 10.35. Two possible versions of the off-resonance inversion element *S* based on (a) a binomial-type hard-pulse sequence and (b) a combination of soft and hard pulses.

As apparent from the previous section, a binomial sequence has a suitably tailored profile for the element *S*, and the series $3\alpha-9\alpha-19\alpha-19\alpha-9\alpha-3\alpha$ (Fig. 10.35a, with $26\alpha = 180^\circ$ and a delay τ between pulses, here termed W3) [80] has a desirable off-resonance inversion profile for this purpose. The WATERGATE excitation profile for this is shown in Fig. 10.36a. Once again characteristic nulls also occur at offsets of $\pm n/\tau$ Hz, but between these the excitation is quite uniform and does not suffer the phase inversion of the unaccompanied 90° binomials. Extended binomial sequences have been shown to provide a narrower notch at the transmitter offset, thus reducing the attenuation of solute signals close to the solvent, and have a wider region of uniform excitation [80]. The improved profile of the so-called W5 sequence is shown in Fig. 10.36b. This sequence alas lacks the elegant shorthand form of its predecessor and is thus represented 7.8-18.5-37.2-70.0-134.2-134.2-70.0-37.2-18.5-7.8, where each number represents the pulse tip angle in degrees.

The element *S* may also be applied as a combination of hard and soft pulses [57, 78] (see Fig. 10.35b). Here, the soft pulse acts only on the water resonance causing it to experience a net 360° rotation whilst all others undergo the desired inversion by virtue of the hard pulse. In this approach, the suppression profile is dictated by the inversion properties of the soft-pulse combination, and off-resonance nulls no longer occur (although correction for any phase difference between the hard and soft pulses may be required). Typically, the soft 180° pulse has a short duration ($\sim 2-8$ ms) and a rectangular or, still better, smoothed profile. This method has the notable advantage that the suppression may be applied away from the centre of the spectrum simply by shifting the location of the selective pulse and allows for optimal transmitter positioning for multi-dimensional experiments.

An improved approach to gradient suppression employs the method of ‘excitation sculpting’ described in Section 10.4.2 and applies a double PFG spin-echo instead of one [57]. This has the advantage of refocusing the evolution of homonuclear couplings and can produce spectra with improved phase properties and less baseline distortion. The elements *S* are exactly as above, except with the double-echo the suppression notch is wider since it is applied twice. The water suppression spectrum of the 2 mM sucrose in 90% H_2O using this approach is shown in Fig. 10.37a and illustrates the impressive results now routinely available. The residual solvent signal can be further removed (see Fig. 10.37b) by subtracting low-frequency components from the FID prior to FT [81], which requires that the transmitter frequency be placed on the solvent resonance. Alternatively, the spectrometer software may be able to remove user-defined, higher-frequency components of the FID such as those corresponding to the residual solvent resonance, allowing arbitrary positioning of the transmitter. The excitation sculpting sequence can also be readily tailored to achieve multi-site suppression [82, 83].

Finally, we note that the PURGE method (presaturation-utilizing relaxation gradients and echoes) [84] offers excellent baseline and phase properties whilst providing efficient and highly selective suppression. This combines solvent presaturation with gradient-purged echo periods prior to acquisition and has been recommended for when quantitative or statistical analyses of 1D spectra are required. Its implementation within multi-dimensional experiments is possible but potentially limited by the requirements for phase cycling.

One final point regarding PFGs is that their use in certain experiments for coherence selection also leads to solvent suppression without further modification. For example, the DQF-COSY experiment inherently filters out uncoupled spins (i.e. singlets), and when PFGs are used to provide this filtering, the singlet water resonance is also removed at source. In fact, the original publication that stimulated widespread use of field gradients in high-resolution NMR impressively demonstrated such suppression [85]. Similarly, heteronuclear correlation experiments such as HMQC and HSQC intrinsically select protons

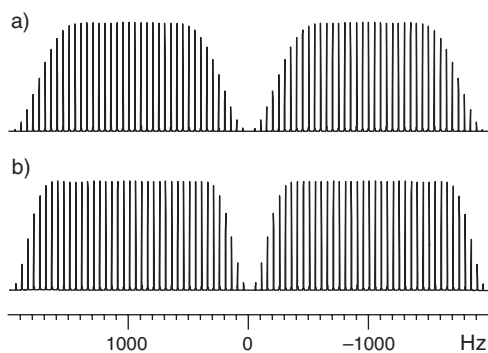


Figure 10.36. Simulated excitation profiles for WATERGATE using (a) the W3 and (b) W5 binomial-type sequences to provide a null at the solvent shift ($\tau = 500 \mu s$).

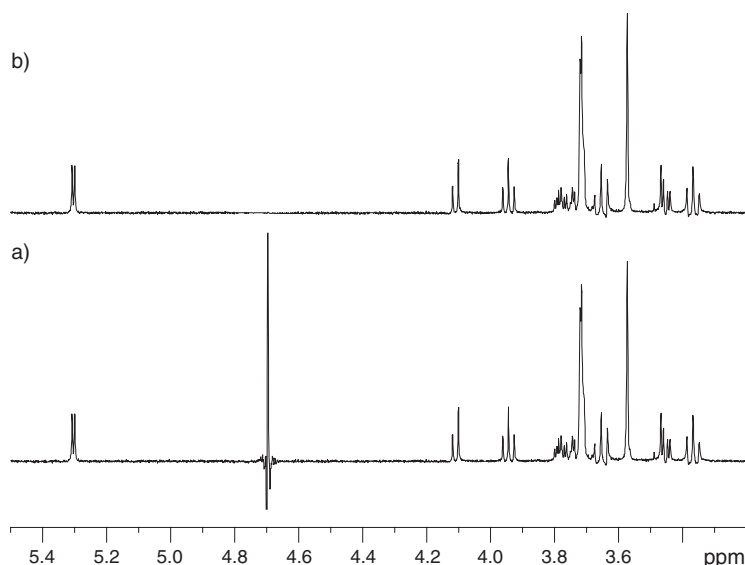


Figure 10.37. Solvent suppression with the excitation sculpting scheme using the approach of Fig. 10.35b with a 8-ms soft pulse (Gaussian) and gradients of 0.1:0.1:0.03:0.03 T/m. The sample is 2 mM sucrose in 9:1 H₂O:D₂O. In (b) the small residual solvent signal has been completely removed through additional processing of the FID (see text).

bound to, say, ^{13}C or ^{15}N and thus also reject water. Although these methods on their own are unlikely to prove sufficient for 90% H₂O solutions (in which case the above gradient echo methods may be appended), they are often sufficient for ‘wet’ D₂O samples.

10.6. SUPPRESSION OF ZERO-QUANTUM COHERENCES

Zero-quantum coherence (ZQC) was described in Section 5.4 as a form of multiple-quantum coherence that may arise from J -coupled spins and is often generated in multiple-pulse NMR sequences. As with all forms of multiple-quantum coherence, it does not give rise to observable signals but can nevertheless be reconverted into observable single-quantum coherence and so give rise to responses that are often considered undesirable artefacts in an NMR spectrum. Commonly employed experiments in which this can be a problem include NOESY and TOCSY in which the zero-quantum contributions give rise to anti-phase dispersive signals that complicate or even mask the appearance of the desired peaks; see, for example, Fig. 8.38. Unfortunately, since the ZQC has a coherence order $p = 0$, as the name implies, it is insensitive to both phase cycling and pulsed field gradients and so cannot be suppressed by either of these methods alone. In this section, we review a number of approaches to zero-quantum suppression that can be applied to a wide range of pulse techniques, as has been demonstrated in previous chapters. All methods rely on the fact that zero-quantum coherence is one manifestation of the general notion of transverse magnetisation and that this can evolve during periods of free precession according to the frequency *differences* of the chemical shifts of the coupled spins A and X ($|\nu_A - \nu_X|$; expressed in Hz). The manipulation of the ZQC evolution provides the key to its suppression.

10.6.1. The variable-delay z -filter

The idea behind the z -filter is that only desired in-phase magnetisation present during a pulse sequence can pass through the filter and return unaffected whilst all other components are suppressed. The basic filter is illustrated in Fig. 10.38a and works by temporarily ‘storing’ the desired magnetisation along the z axis whilst other components

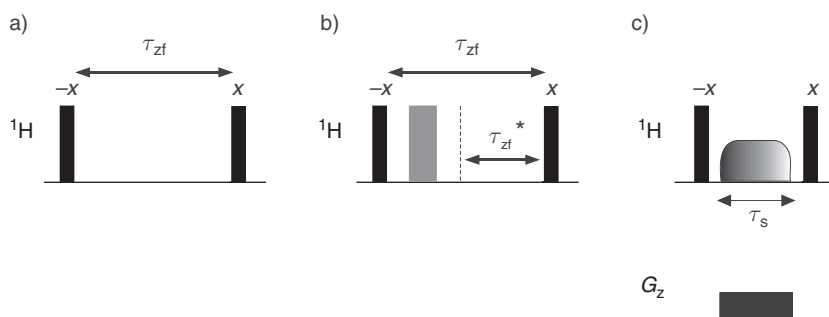


Figure 10.38. Approaches to suppressing zero-quantum coherences: (a) the basic z -filter, (b) the spin-echo z -filter and (c) the zero-quantum dephasing element employing an rf frequency sweep/gradient pulse combination; see text for details.

are somehow destroyed (by phase cycling or PFGs) and then returning this to the transverse plane for detection free from undesirable contributions. Considering the case of zero-quantum coherences, the interpulse delay τ_{zf} allows any coherence generated to evolve as described above and to obtain a phase dictated by the zero-quantum evolution frequency. If one were to repeat the experiment but this time use a different filter delay $\tau_{zf(2)}$, then the extent of evolution and hence the phase acquired will be different from that in the first experiment using delay $\tau_{zf(1)}$. If the filter timings could be set such that the phase difference between the two experiments was exactly 180° , that is, when $\tau_{zf(1)} - \tau_{zf(2)} = (2|\nu_A - \nu_X|)^{-1}$, then addition of the two data sets would lead to cancellation of the zero-quantum contributions. In reality, there will exist a range of zero-quantum frequencies evolving arising from different coupled spin pairs, so it is usually necessary to repeat the experiment many times using systematic variation of the τ_{zf} delays and co-add all the data sets to achieve sufficient artefact suppression [86, 87]. The amount of variation required clearly depends on the shift differences between coupled spins whereby large differences demand a smaller spread in τ_{zf} . Since higher field magnets also give rise to larger shift separations (expressed in Hz), correspondingly smaller variations are again required. Typical values of τ_{zf} will range from 0 to 20 ms, which should suppress all zero-quantum contributions for J -coupled spins with $\nu_A - \nu_X > 25$ Hz. The need to repeat experiments to achieve suppression can dictate lengthy acquisitions and represents the principal limitation to this approach, leading to the development of more efficient alternatives.

10.6.2. Zero-quantum dephasing

The most recent, successful and widely applicable approach to suppressing zero-quantum contributions employs a pulsed field gradient method for the net dephasing of these coherences *in a single scan* with no requirement for experiment repetition [88]. Since PFGs alone cannot manipulate ZQCs, the gradients must be applied in a rather ingenious manner to be effective, the operation of which may be understood as follows.

To begin we again consider the approach described above for generating differential evolution of ZQCs by variation of the total duration of the z -filter delay, τ_{zf} . An alternative approach to this would be to maintain τ_{zf} at a fixed duration and to place within this a 180° pulse that moves throughout the delay on each repetition of the experiment [89] (see Fig. 10.38b). This pulse introduces a spin-echo to the z -filter in which the ZQ evolution is refocused such that the net evolution is restricted only to the residual period τ_{zf}^* . Relocation of the 180° pulse for each experiment thus leads to differential evolution between them.

Now consider the action of the zero-quantum dephasing sequence of Fig. 10.38c. This employs a frequency-swept (adiabatic) 180° pulse simultaneously with a field gradient applied along the z axis of the sample. The gradient imposes a frequency spread across the length of the sample tube and so imparts the usual spatial encoding such that responses from spins at the top of the tube would correspond to, say, the high-frequency end and those at the bottom to the lower-frequency end. At the same time, the 180° adiabatic pulse sweeps through this frequency window (Section 10.2), meaning it inverts spins in different spatial regions of the sample *at different times*. Assuming that this sweeps from high to low frequency, this would in the above scenario, corresponds to the application of a refocusing pulse initially to the top of the sample tube followed by a steady progression down the tube, finally refocusing at the bottom as the swept pulse ends. In a manner similar to that described above for the scheme of Fig. 10.38b, this results in differential net evolution of the ZQCs but now in *different spatial regions* of the sample (Fig. 10.39). The detected NMR response is clearly the sum of all these regions along the length of the NMR sample, leading to a destructive summation for all zero-quantum coherences *on every scan* and hence their removal from the final spectrum.

The degree of attenuation A_{ZQC} depends upon the zero-quantum frequency Ω_{ZQC} (here in rad/s) and the duration of the frequency sweep τ_s according to the following equation [88]:

$$A_{ZQC} = \frac{\sin \Omega_{ZQC} \tau_s}{\Omega_{ZQC} \tau_s} \quad (10.6)$$

This approximates to $1/\Omega_{ZQC} \tau_s$ when the sine term oscillations are ignored and allows an estimate of the sweep durations that need to be employed. Thus, in the case of a spectrum recorded at 600 MHz with J -coupled spins separated by 0.5 ppm (that is,

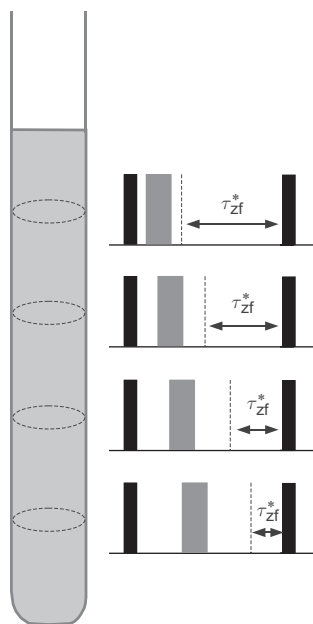


Figure 10.39. Schematic representation of events during the zero-quantum dephasing element. The effective timing of the 180° refocusing pulse and hence the net zero-quantum evolution time τ_{zf}^* becomes dependent upon spatial location along the z axis, meaning the zero-quantum coherences evolve to differing extents according to the position of the participating spins in the sample.

ZQC frequencies equivalent to 300 Hz or approximately 2000 rad/s), a 50-ms sweep will attenuate ZQC by $\sim 99\%$ ($A_{\text{ZQC}} \sim 0.01$). For spins separated by 1 ppm at this field strength, a 25-ms sweep would be sufficient to produce similar attenuation. In general, the larger the frequency separation between the coupled spins, due to greater chemical shift differences or the use of higher B_0 fields, the shorter the sweep durations that need to be employed. Conversely, coupled spins with similar chemical shifts will present the most demanding cases for suppression.

It has also been shown that greater degrees of attenuation can be achieved without the need for very long dephasing elements by cascading two or more discrete elements that employ orthogonal pulsed field gradient pulses [90] such that the first uses a z -gradient, as above, and the second an x -gradient, and so on. The resulting attenuation is the product of that given by each element and hence is time-efficient, but as this demands probes with triple-axis (x, y, z) gradient capabilities, it has less general applicability. An earlier approach employed the simultaneous application of a field gradient pulse and a short spin lock to dephase ZQC, relying upon the rf (B_1) inhomogeneity of the probe to produce the dephasing [91]. Despite some success, this method is likely to become less useful as probe developments lead to improved B_1 homogeneity.

Implementing zero-quantum dephasing

The application of the zero-quantum dephasing element has been illustrated in earlier chapters within sequences such as TOCSY (Section 5.7.4) and NOESY (Section 8.7.2), so here we consider only the steps necessary for the calibration of the element itself. For the dephasing to perform correctly, one needs to match the frequency profile of the swept pulse with the frequency spread imposed on the spectrum by the simultaneous field gradient, and the calibration sequence of Fig. 10.40 is designed to achieve this.

The process begins with the selection of a suitable dephasing duration for the swept pulse τ_s according to Eq. (10.6). Since all zero-quantum frequencies may not be known, this is likely to provide a guideline only but typical values for the sweep duration would be 20–50 ms. Next the total frequency sweep width for the rf pulse is defined, which, as a rule of thumb, may be set to ~ 10 times the normal spectrum window typically yielding sweep widths of 40–60 kHz. From these figures, the radiofrequency field strength for the chosen pulse profile must be determined such that the adiabatic condition is satisfied, a procedure most conveniently performed by direct calculation (see Section 10.2 on adiabatic pulses). Suitable pulse profiles include WURST and the smoothed CHIRP. Initially, the sequence of Fig. 10.40 is executed with the gradient G_s and the swept-pulse rf field strength both set to zero and the imaging gradient G_i set to a low level such that the resonance lines are broadened slightly (Fig. 10.41b). Applying this gradient during the acquisition time of the FID imposes spatial encoding on the sample effectively providing a 1D image of this along the z axis. Now the sequence is repeated with the adiabatic frequency sweep operational, which should lead to complete inversion of the whole spectrum (see Fig. 10.41c). The final calibration procedure then involves increasing the filter gradient strength G_s in stages aiming to make this as large as possible whilst retaining full inversion of all resonances. Should G_s become too high, the very edges of each resonance are no longer inverted (see Fig. 10.41f); under these conditions, the frequency spread imposed by the gradient is now larger than that being covered by the frequency-swept pulse and the ends of the sample no longer experience the desired 180° inversion.

This calibration process must be performed for all dephasing elements of different durations, but the optimum parameters so determined may then be used directly within appropriate sequences containing this element.

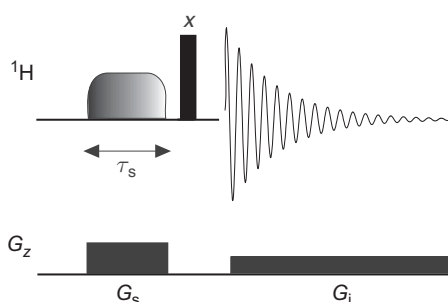
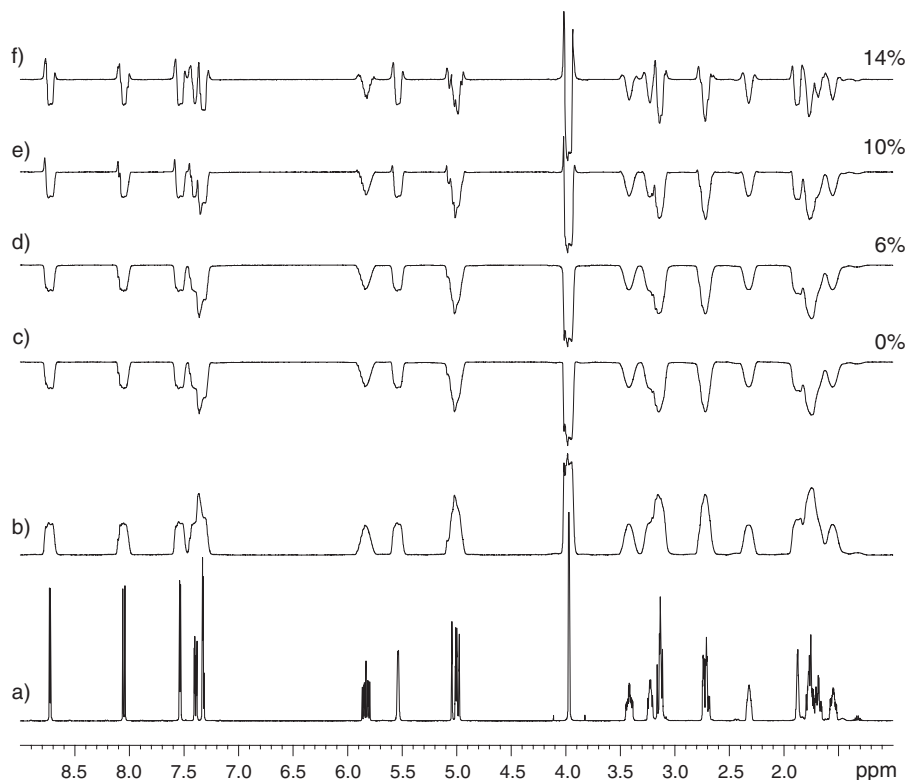


Figure 10.40. The zero-quantum dephasing calibration sequence.

Figure 10.41. Execution of the zero-quantum dephasing calibration routine; see text for discussions. (a) 1D spectrum, (b) with imaging gradient only applied, (c) with additional application of adiabatic inversion pulse, and then with the filter gradient applied at (d) 6%, (e) 10% and (f) 14% of maximum. A filter gradient a little under 10% represents the optimum setting.



10.7. HETEROGENEOUS SAMPLES AND MAS

Despite the fact that most synthetic organic chemistry takes place in solution, interest remains in solid-phase organic synthesis protocols for the production of combinatorial libraries of new molecules [92, 93]. A challenging factor in this approach is the analysis of the newly synthesised materials for which, ideally, direct structural analysis of the material would be carried out whilst still attached to the solid support and thus still available for further chemistry. The direct NMR analysis of such materials, even when solvated, is complicated by two principal factors, which can severely degrade spectrum resolution:

- restricted motion of the tethered analyte, and
- physical heterogeneity within the sample.

The restricted motion of the tethered analytes relative to that of free molecules in solution may mean that dipolar couplings, D , are not fully averaged to zero. These have a time-averaged angular dependence

$$D \propto r^{-3}(3 \cos^2 \theta - 1) \quad (10.7)$$

where r is the internuclear separation and θ the angle between the static field and the internuclear vector. For isotropically tumbling molecules in low-viscosity solutions, the angular term is averaged to zero as the molecule freely rotates, so dipolar couplings are not observed. When motion is sufficiently restricted, some residual dipolar coupling may be reintroduced, which will contribute to broadening of the NMR resonances. The use of long linker chains allows greater motion of the terminal analyte in solution and can therefore reduce this effect. A potentially greater problem lies in the sample heterogeneity. Solutions containing solid-phase resins, usually in the form of beads, suffer from local-field inhomogeneity at the bead-solvent interfaces due to magnetic susceptibility differences, and this leads to severe line broadening (Fig. 10.42a). Although some success has been reported with gel-phase NMR of carbon-13 and other nuclei [94, 95], these deleterious effects mean high-resolution spectra of solvated resins cannot be obtained with conventional solutions probes, a particular problem for proton spectroscopy.

Magnetic susceptibility has a similar angular dependence to that above, and line broadening from both dipolar couplings and susceptibility discontinuities can be eliminated by rapid rotation of the sample about the so-called 'magic angle' $\theta = 54.7^\circ$, such that the above

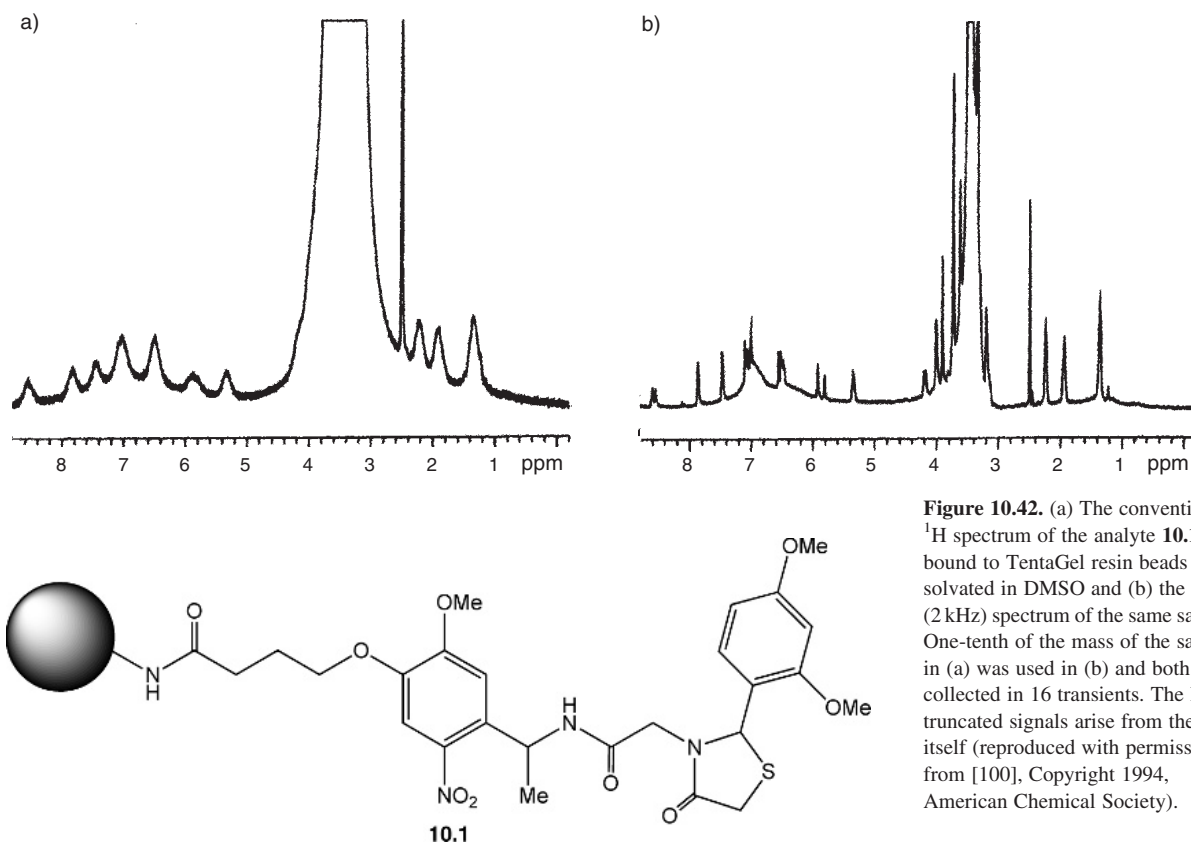


Figure 10.42. (a) The conventional ^1H spectrum of the analyte **10.1** bound to TentaGel resin beads solvated in DMSO and (b) the MAS (2 kHz) spectrum of the same sample. One-tenth of the mass of the sample in (a) was used in (b) and both were collected in 16 transients. The large truncated signals arise from the resin itself (reproduced with permission from [100], Copyright 1994, American Chemical Society).

angular term becomes zero and the overall molecular motion emulates that in solution. This MAS (Magic Angle Spinning) (Fig. 10.43) has long been used in solid-state NMR spectroscopy to average the effects of chemical shift anisotropy [96] and has now become a standard technique for the direct analysis of resin-bound entities [97, 98] and has also found application in the analysis of biomedical tissue extracts [99], an area beyond the scope of this text. The substantial gain in resolution and hence sensitivity provided by MAS over a conventional solutions probe is illustrated in Fig. 10.42 [100]. The MAS spectrum shows a three-fold signal-to-noise gain yet uses one tenth of the sample relative to the conventional solution spectrum and has considerably greater information content. To achieve such results, samples are typically spun at rates of 2–5 kHz, in purpose-built rotors requiring access to the appropriate high-resolution MAS probe head [101] and spin regulation hardware. The full range of high-resolution 1D and 2D techniques can now be applied to resin-bound samples under MAS conditions for the complete, non-destructive identification of new synthetic products [102–106]. The influence of various solvents and resin combinations has also been investigated and shown to have considerable bearing on the quality of spectra one obtains [107]. Various sequences have also been optimised for the investigation of these heterogeneous samples. The TOCSY experiment benefits from the use of mixing schemes based on adiabatic pulses owing to their greater tolerance of rf inhomogeneity and those based on WURST-2 pulses have been suggested as optimum [108, 109]. Variants of the inverse ^1H – ^{13}C correlation experiments that avoid the need for gradient pulses, HMQC and HMBC, have also been presented since PFGs are not always available on HR-MAS probes [110].

10.8. EMERGING METHODS

This final section briefly considers some more recent developments that are not established methods but, at least at the time of writing, show interesting potential for future development and may yet impact upon the range of techniques employed in the research laboratory in the context of high-resolution NMR spectroscopy. The first of these can provide ultra-fast routes to the collection of 2D data sets, the second offers the potential for boosting detection sensitivity by orders of magnitude, the third presents an alternative approach to the definition of stereochemistry through dipolar couplings and the last a route to faster data collection. All these areas represent active research topics and it remains

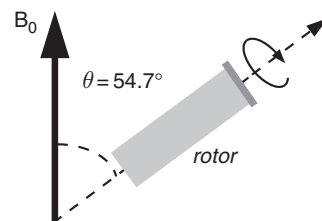


Figure 10.43. Magic angle spinning involves rapid rotation of a sample inclined at the magic angle of 54.7° to the static field.

to be seen how they develop and to what extent they influence the application of NMR to structural chemistry.

10.8.1. Fast data acquisition: single-scan 2D NMR

In recent years, a number of approaches have been developed that aim at reducing the time required for recording multi-dimensional data sets, notably when detection sensitivity is not limiting. Many of these methods are tailored towards high-dimensionality experiments ($\geq 3D$) or to spin systems associated with the common amino acids, so they are of most relevance to biomolecular NMR spectroscopy [111]. However, one ingenious method stands out as having wide applicability and considerable potential for very fast data acquisition and will be considered here. It makes use of spatial encoding of spins with pulsed field gradients, borrowing from methods developed for magnetic resonance imaging, and produces complete 2D spectra *in a single scan* [112, 113].

As described in previous chapters, the collection of conventional 2D data sets relies upon the *sequential* acquisition of experiments collected as a series of 1D experiments of increasing t_1 evolution times according to the preparation–evolution–mixing–detection paradigm. In contrast, the single-scan approach collects these *simultaneously* and it is this parallelisation that affords significant time savings. The approach taken is to excite different ‘slices’ through a sample at slightly different times such that the start point of the t_1 evolution period is shifted in time for each one. At the start of the 2D mixing period, the total evolution time experienced for spins in different slices will therefore vary such that responses from each slice correspond to experiments recorded with differing t_1 periods. The ‘slicing’ of the sample is achieved by the imposition of a field gradient along the z axis so that resonant frequencies vary along the sample due to differences in the local magnetic field. A sequence of selective pulses is then applied with each progressively stepped in frequency so as to cause excitation of only those spins within a discrete slice of the sample, this being referred to as *spatially selective excitation*. Since these pulses are applied sequentially, the start of t_1 evolution is correspondingly stepped in time between each slice, resulting in parallel evolution periods (Fig. 10.44).² The number of t_1 increments is therefore equal to the number of selective pulses applied. To refocus chemical shift evolution caused by the gradient pulse, a second must be applied in the opposite sense so that only chemical shifts imposed by the external B_0 field give rise to net evolution during t_1 . This gives rise to the general preparation–evolution (encode) segment of Fig. 10.45, following which any standard mixing element may be applied according to the correlations being sought.

Data acquisition requires that the spatial encoding used to define the discrete t_1 frequency-labelled segments is now decoded and this may be achieved through the application of a series of read gradients during the acquisition period (see Fig. 10.45). The action of the decoding module may be understood by considering the results from a sample containing only two resonances of chemical shift offsets ν_A and ν_B . The net chemical shift evolution experienced during t_1 for spins with offset ν_A imposes a helical dispersion of magnetization vectors along the sample length due to the differing effective t_1 periods experienced within each slice. The application of the read gradient causes these vectors to refocus and produce

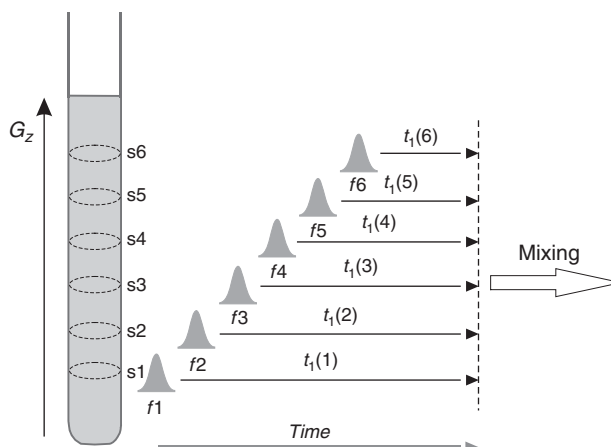


Figure 10.44. Spatially selective excitation for single-scan 2D spectra showing a hypothetical experiment of six t_1 increments, each initiated by selective excitation applied at frequencies f_1 – f_6 corresponding to ‘slices’ s_1 – s_6 .

² This idea is similar to that used in the spatially dependent refocusing of ZQCs caused by the application of a frequency-swept pulse in the presence of a field gradient (Section 10.6.2).

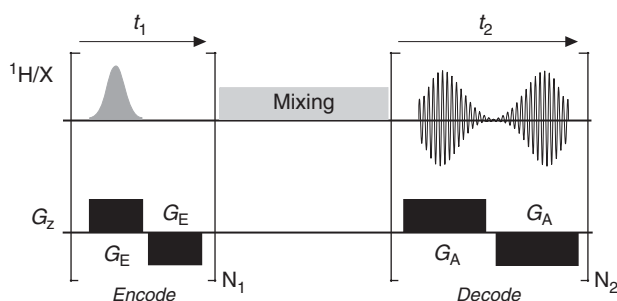


Figure 10.45. A generalised scheme for the collection of single-scan 2D spectra.

an echo within the acquisition period at a time dictated by how rapidly the helix unwinds and thus by the magnitude of ν_A . Spins with greater offset ν_B will refocus more slowly and hence will generate an echo at a later time. In each case, the initial chemical shift evolution occurring during t_1 has been forcibly refocused by the applied read gradient, and the position of each echo reflects the corresponding chemical shifts in f_1 . A second read gradient is also applied in the opposite sense to refocus the echoes once more, and the corresponding data sets generated for the positive and negative gradients are stored separately. These may be considered as the echo and antiecho counterparts giving rise to data sets reflected in f_1 and can be added in a manner similar to the conventional protocol to improve signal-to-noise. The read gradient module is repeated N_2 times to define the total t_2 acquisition period.

Surprisingly, it turns out that the complete 2D spectrum arises from FT of the t_2 dimension *only* since the locations of the detected echo peaks define the f_1 peak positions directly. Most significantly, the whole 2D data set is acquired in only a single transient, and data collection is complete within a fraction of a second. This is exemplified by the single-scan 2D TOCSY spectrum of *n*-butyl chloride recorded in a mere 200 ms (Fig. 10.46).

Beyond the obvious requirement for good signal-to-noise in a single transient, the principal limitations with this approach lie in the need for high-power gradients with fast switching times that can be applied repetitively without intensity loss or imbalance between positive and negative pulses. Current gradient systems do not achieve all these goals and further technical developments are required before these methods gain wider application, but if these can be overcome, the impact of such methods may be profound.

10.8.2. Hyperpolarisation: DNP

It is undeniably the case that NMR experiments suffer from poor sensitivity when compared to most other spectrometric methods, and this arises in large part from the rather small energy differences and hence miniscule population differences across the associated

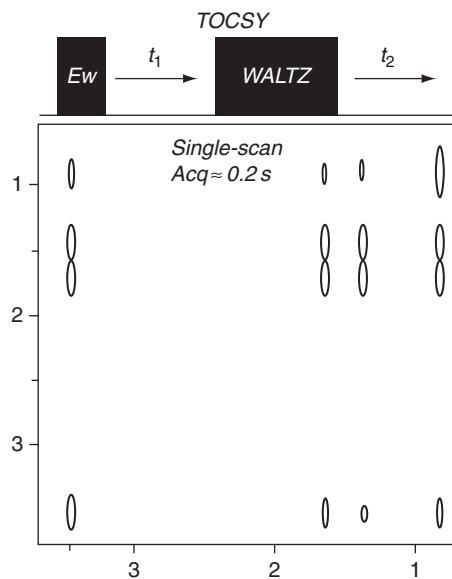


Figure 10.46. A single-scan 2D TOCSY spectrum of *n*-butyl chloride recorded with a 74 ms mixing period and a total data collection time of ~ 200 ms (reproduced with permission from [113], Copyright 2003, American Chemical Society).

spin transitions. These correspond to less than 1 part in 10^4 with conventional spectrometers, or in other words, to polarisation levels of $<0.01\%$. A direct approach to boosting the sensitivity of an NMR experiment is therefore to enhance the polarisation levels of the observed nuclei, with increases in excess of 10,000-fold theoretically possible. A number of approaches to generating hyperpolarised samples have been proposed and demonstrated of which two have relevance to high-resolution NMR measurements. One of these, *para*-hydrogen-induced polarisation (PHIP), uses the large population differences associated with the *para*-H₂ molecule and transfers this onto a substrate molecule via catalytic hydrogenation. The need for a chemical reaction step places significant limitations on the applicability of the method, so will not be considered further; the method has been comprehensively reviewed [114].

An alternative method, termed DNP (Dynamic Nuclear Polarisation), relies on the transfer of polarisation from unpaired electrons onto nuclear spins in the solid state but has been further developed to significantly enhance the sensitivity of solution-state NMR, as described briefly below. The technique has been applied to boost the polarisation levels for low- γ heteronuclear spins in particular and shows most promise to date for ^{13}C and ^{15}N observation; the initial report on the technique described a gain in signal-to-noise in excess of 10,000-fold for both these elements when compared to data collected at thermal equilibrium [115]. The hyperpolarisation of ^{31}P and ^{29}Si has also been demonstrated.

The method requires a sample to be doped with a suitable radical (organic trityl radicals have proved especially effective) and dispersed homogeneously within an amorphous frozen matrix to allow intimate contact between the analyte and radical molecules. The electron spins are then polarised by placing the sample in a suitable magnetic field whilst at low temperature, typically only 1–2 K, and the DNP process initiated by irradiation of the sample with microwaves. The microwave frequency is chosen to match the difference between the electron and nuclear Larmor frequencies and nuclear polarisation levels build over some hours during the irradiation according to the solid-state T_1 relaxation time constants. Carbon-13 polarisation levels in the solid state can typically reach 20–40%. The mechanisms involved in the polarisation transfer are complex [116] but may be thought of as solid-state Overhauser effects; the original reports of Overhauser were in fact for electron–nuclear transfers. The large solid-state polarisation that develops can be exploited directly in solid-state NMR measurements [116, 117], but to be useful for high-resolution NMR, these must be transferred into the solution state. A number of approaches may be envisaged, including melting and dissolution, and it is the second of these that receives greater application at present. In a technically remarkable process [118], the frozen, hyperpolarised pellet is sequentially dissolved in a super-heated solvent, typically methanol, and passed under pressure through a transfer line directly into an NMR tube held within a conventional NMR magnet (Fig. 10.47). This whole process occurs very rapidly and must be fast on the relaxation timescale since polarisation is lost through the usual spin relaxation processes as soon as the sample enters the solution state. The sample must therefore pass from the solid state at $\sim 1\text{ K}$ into solution at $\sim 300\text{ K}$ and equilibrate sufficiently for a

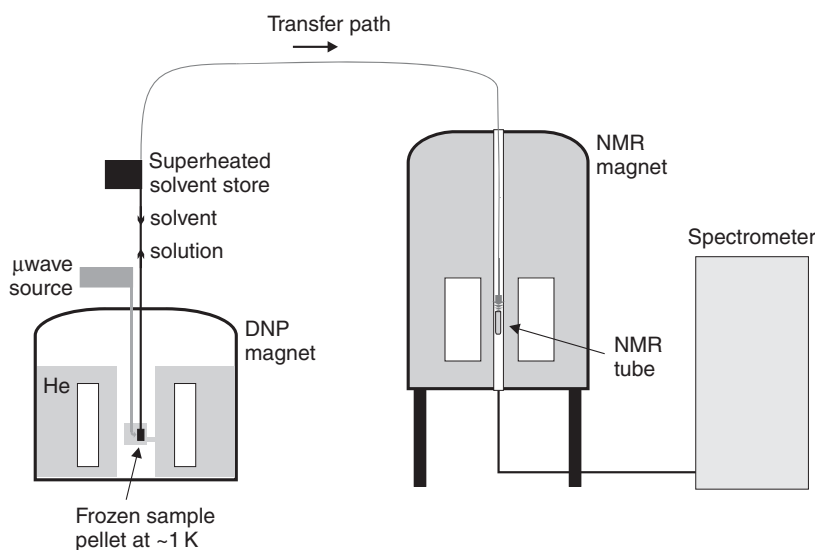


Figure 10.47. A schematic illustration of the DNP-NMR spectrometer assembly.

high-resolution experiment within a matter of seconds! Once in the NMR tube the hyperpolarised sample may be subject to investigation by pulse techniques but again within the constraint that the large polarisation levels will be lost through normal spin-relaxation processes. This means, in essence, that the technique is limited in most part to single-scan acquisitions, typical examples being 1D proton-decoupled heteronuclear spectra. A pulse sequence encapsulating the complete DNP acquisition process is illustrated in Fig. 10.48. Although signal averaging is not possible, the high initial polarisation levels means detection sensitivity can be sufficient to observe all resonances in a single scan even on very small sample quantities. Figure 10.49 compares the conventional ^{13}C spectrum of 60 μmol ethyl acetoacetate **10.2** collected as 3000 transients over 3 hours with the single-scan DNP-enhanced spectrum at the same concentration recorded after 3 hours of polarisation (the labelled resonance at ~ 39 ppm is missing in trace (a) as the carbon centre becomes deuterated in CD_3OD through *keto–enol* tautomerism whereas this does not occur for the DNP-enhanced sample, which used CH_3OH as the dissolution solvent; the minor *enol* form gives rise to the smaller resonances apparent only in the DNP-enhanced spectrum). One notable feature of DNP-enhanced spectra that is apparent in Fig. 10.49 is that, in contrast to conventional heteronuclear spectra, the resonances of non-protonated centres appear with high intensity, often greater than those of proton-bearing atoms. This is most apparent for the carbonyl resonances above 160 ppm and is a direct consequence of the slower relaxation associated with non-protonated centres (since attached protons act as dominant relaxation sources), meaning these retain more of the initial polarisation during the dissolution and transfer process.

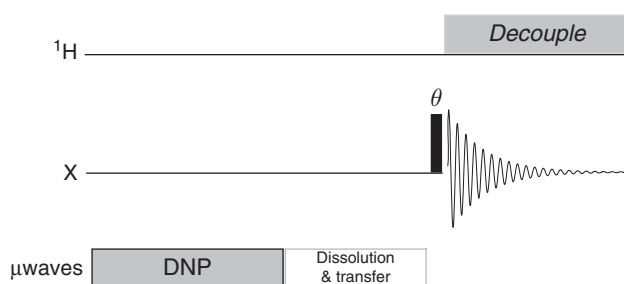


Figure 10.48. A pulse sequence suitable for acquiring 1D heteronuclear spectra from DNP-NMR.

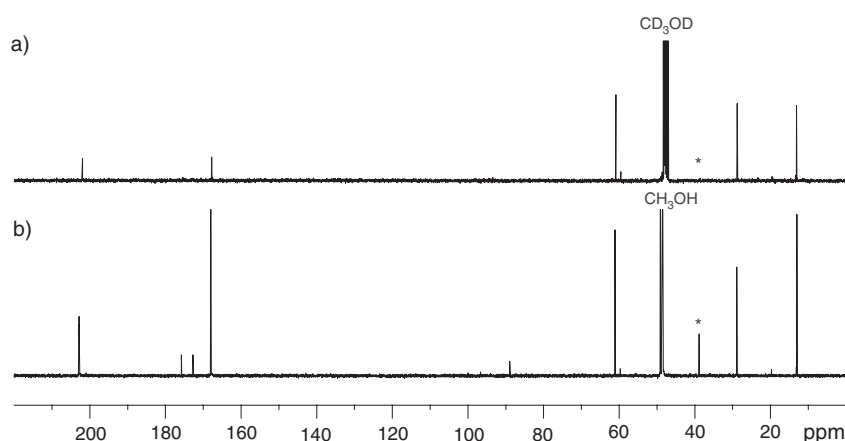


Figure 10.49. A comparison of (a) the conventional 1D ^{13}C spectrum of ethyl acetoacetate (60 μmol , 3000 scans, 400 MHz) with (b) the DNP-enhanced ^{13}C spectrum (60 μmol , single scan, 400 MHz). The resonance labelled with an asterisk is missing in trace (a) due to deuteration of the centre as a result of *keto–enol* tautomerism; see text (data courtesy of Oxford Instruments Molecular Biotools).

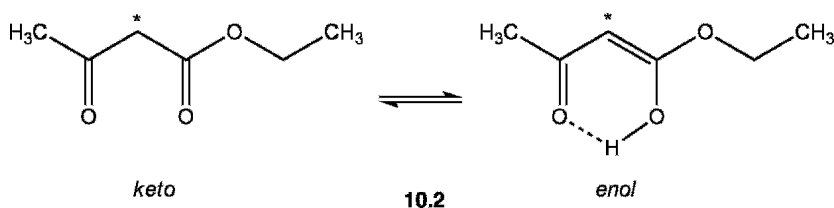
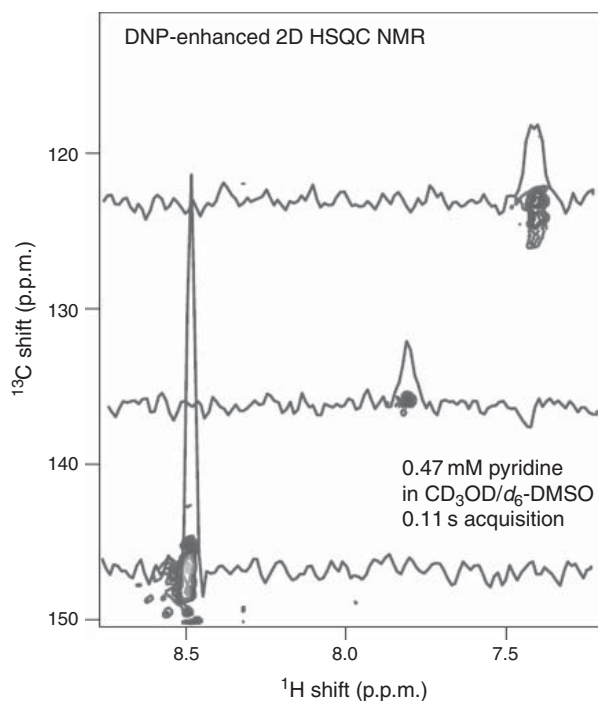


Figure 10.50. The 2D single-scan HSQC spectrum of 0.47 mM ^{13}C -hyperpolarised pyridine (reproduced with permission from [121], Copyright 2007, Nature Publishing Group).



The initial motivation for developing the solution-state DNP technology was for *in-vivo* studies employing ^{13}C magnetic resonance imaging [119], and applications of DNP in high-resolution NMR are only recently emerging. The detection of samples of limited availability and perhaps lifetime offers one potential area of application. The simultaneous polarisation of both ^{13}C and ^{15}N has also been demonstrated, with the two nuclei excited and detected sequentially to yield independent DNP-enhanced spectra [120]. A more exciting development that shows interesting potential is to marry the DNP method with the single-scan acquisition methods introduced in the previous section and thus opens the way to two- (or greater) dimensional NMR experiments on hyperpolarised samples. This has been demonstrated for single-scan ^1H - ^{13}C heteronuclear correlation spectrum of 0.47 mM hyperpolarised pyridine [121] (Fig. 10.50). Data were collected with a modified HSQC experiment that exploits the initial ^{13}C polarisation, and the total experiment time was a fleeting 110 ms. With current technology, one limiting factor in the use of DNP-NMR is the loss of polarisation on transfer from the polarizer to the NMR magnet, restricting the method to more slowly relaxing samples, usually meaning smaller molecules. This also means signal-to-noise gains of around two orders of magnitude relative to conventional single-scan acquisitions appear to reflect more realistic expectations for small molecules at present. Future technological developments that reduced these restrictions and extend the scope of DNP-NMR may be anticipated.

10.8.3. Residual dipolar couplings (RDCs)

This section presents an alternative approach to defining relative internuclear relationships that potentially compliment the use of scalar couplings and NOEs [122–124]. The methodology involves the direct measurement of dipolar couplings between nuclei, an approach that has become well-established in recent years in structural elucidation protocols of biological macromolecules. Although these couplings provide the basis for the NOE, they are not usually observed directly in solution spectra owing to the time averaging to zero of the dipolar splittings as molecules tumble rapidly and isotropically in solution. In contrast, such couplings can reach many hundreds of kilohertz in the absence of motional averaging and may dominate the appearance of solid-state spectra. It is, however, possible to reveal dipolar couplings in solution spectra if the molecules are constrained such that they no longer tumble isotropically but instead have some orientational preference imposed upon them. This effect has been known for many decades notably in the study of organic liquid crystals that produce very large dipolar couplings and prove complex to analyse. More recent developments have employed weakly aligning media that introduce only small RDCs (residual dipolar couplings); in effect these media very gently ‘squeeze’ the solute molecules such that their motion becomes weakly anisotropic. In such cases the RDCs are typically less than 50 Hz and do not complicate spectra excessively. They can be measured with conventional NMR experiments as any additional coupling arising from dipole–dipole

interactions sums with the scalar J -coupling present. Thus, in the case of a one-bond carbon–proton dipole coupling $^1D_{\text{CH}}$, the total observed splitting $^1T_{\text{CH}}$ will be

$$^1T_{\text{CH}} = ^1J_{\text{CH}} + 2^1D_{\text{CH}} \quad (10.8)$$

Splittings can be measured in a proton-coupled HSQC, for example, and comparison with the equivalent experiment performed on a conventional isotropic sample (revealing $^1J_{\text{CH}}$ couplings) yields the magnitude of the RDC.

The value of these couplings lies in their ability to provide information on the relative orientations of bond vectors within a molecule. This can, in principle, indicate the relative stereochemistry at different centres within the molecule. The magnitudes of the dipolar splittings D_{IS} are defined for two isolated spins I and S as

$$D_{\text{IS}} = \frac{b_{\text{IS}}}{2\pi} \left\langle \frac{3 \cos^2 \theta - 1}{2} \right\rangle \quad (10.9)$$

The angle θ defines the angle between the IS internuclear vector and the static magnetic field (Fig. 10.51a) and is time-dependent, the brackets indicating the time average over all molecular orientations as the molecule tumbles. The term b_{IS} is the dipole–dipole coupling constant

$$b_{\text{IS}} = -\frac{\mu_0 \gamma_{\text{I}} \gamma_{\text{S}} \hbar}{4\pi r_{\text{IS}}^3} \quad (10.10)$$

where μ_0 is the vacuum permeability, γ_{I} and γ_{S} are the magnetogyric ratios of the spins I and S, respectively, and r_{IS} is the internuclear distance between them. If this distance is known (the bond length for directly attached spins), the size of the dipolar interaction depends on the unknown angle θ . The r_{IS}^{-3} dependence also suggests that dipole–dipole couplings can be measured over greater distances than NOEs (which have a r_{IS}^{-6} dependence) and may provide for the investigation of longer-range interactions.

In order to relate the RDCs determined experimentally to intramolecular orientations, it is necessary to define an arbitrary molecular coordinate system that is fixed within the framework of the molecule and rotates with this, against which the angular relationships of internuclear vectors can be measured (angles α_x , α_y , and α_z in Fig. 10.51b). This framework is then related to the coordinates of the static magnetic field (angles β_x , β_y , and β_z) so as to define an alignment (or order) tensor for the molecule, that is, a measure of the extent and direction of ordering of the molecular coordinate system imposed by the alignment media. The alignment tensor may be calculated from a sufficient number of experimentally derived RDCs with at least five independent values required to determine this. Independent values in this context mean RDCs from vectors with different (non-parallel) orientations within the molecule. When combined with a proposed structural model, knowledge of the alignment tensor enables the prediction of RDCs, which may be compared with experimental values and the model structure refined to optimise the match between predicted and experimental data.

A simple conceptual illustration of the application of RDCs is in the differentiation of axial and equatorial protons in pyranose sugars **10.3** through the consideration of one-bond $^1D_{\text{CH}}$ values [125]. Within the molecular framework, all axial protons share the same parallel orientation (bond vectors in black) and thus exhibit RDCs of the same size, whereas the equatorial protons point in different directions (vectors in red) and so display different values, enabling their identification. A similar analysis allows the definition of the relative configuration of a dihydropyridone **10.4** [126]. In the *trans* isomer, the highlighted C–H

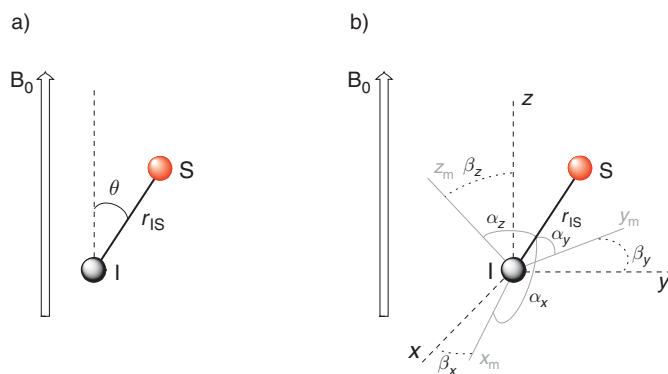
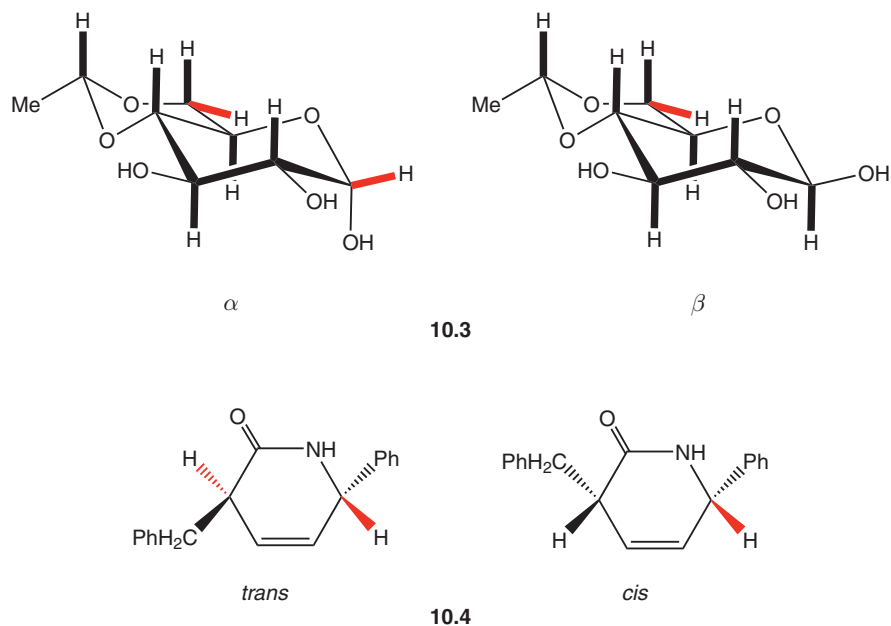


Figure 10.51. The orientational dependence of dipolar couplings. (a) The internuclear vector between spins I and S defines the angle θ with the static magnetic field B_0 . (b) The molecular coordinate system x_m, y_m, z_m rotates with the molecule and defines the time-dependent angles β_x, β_y and β_z with the static magnetic coordinate system.



bond vectors of the stereogenic centres share the same orientation within the molecular frame and hence have similar $^1D_{CH}$ RDC values, whereas in the *cis* isomer, these differ by virtue of the bond vectors pointing in different directions. More generally the assignment of stereochemistry demands the computational approach indicated above. Such investigations have to date focussed mostly on studies of rigid organic molecules [127–129], and the problem of accounting for conformational flexibility within molecules still presents a significant barrier to the wider application of RDCs. The selection of suitable alignment media for small molecules is also an area subject to further refinement. Two types have found most use to date, liquid crystals and polymeric gels. The use of chiral liquid crystals such as poly(γ -benzyl-L-glutamate) (PBLG) is well proven as a method for distinguishing the spectra of enantiomers in solution by virtue of differential ordering of the *R* and *S* isomers of a molecule [130], but liquid crystals have tended to impose too great an ordering for the measurement of RDCs. The alternative use of polymeric gels, referred to as ‘strain-induced alignment in a gel’ (SAG) [131], involves solvation of a gel within the NMR tube such that as it swells and is confined by the tube, it elongates, introducing anisotropic strain that aligns the organic molecule contained within the solution. Cross-linked polymers such as polystyrene and poly(dimethylsiloxane) have proved effective.

The use of RDCs for small-molecule studies is still in its infancy. It appears that optimisation of sample conditions for RDC measurement is still a non-trivial exercise, and the need for a large number of independent RDCs for definition of the alignment tensor can also impose difficulties in the application of the method. It remains to be seen what impact this has on small-molecule NMR studies, but clearly there is potential for this to provide a powerful adjunct to the tools currently in use for stereochemical assignment.

10.8.4. Parallel acquisition NMR spectroscopy

This final section concludes with mention of a very recent addition to high-resolution NMR methodology that has once again found its inspiration in magnetic resonance imaging methods, as with the initial implementation of pulsed field gradients. The concept is simple yet revolutionary – the use of multiple NMR receivers in a spectrometer such that it can collect responses from different nuclei in parallel. The method has been termed Parallel Acquisition NMR Spectroscopy (PANSY) [132] and offers another approach to more rapid data collection from a sample. Although the presence of multiple receiver channels on modern NMR spectrometers is almost unheard of at the time of writing, modern consoles are already built with the capability to accommodate these and new technique developments based on the multiple receiver concept are to be expected and may even become commonplace in due course.

The philosophy can be readily understood by considering the simplest approach to the simultaneous acquisition of ^1H - ^1H and ^1H - ^{13}C 2D correlation spectra (Fig. 10.52). In this, the proton channel operates as for the conventional 2D COSY sequence incorporating the

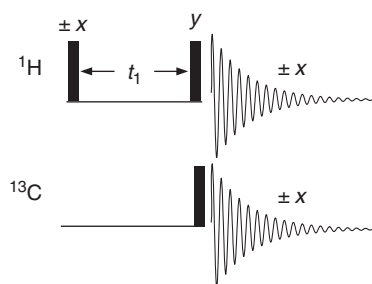


Figure 10.52. A basic scheme for the parallel acquisition of ^1H - ^1H and ^1H - ^{13}C 2D correlation spectra using dual spectrometer receiver channels.

usual ^1H detection. In parallel to this, the carbon pulse applied simultaneously with the second proton pulse initiates ^1H to ^{13}C polarisation transfer which is then followed by ^{13}C detection. This magnetisation has been frequency labelled in t_1 according to the ^1H chemical shifts and thus provides a ^{13}C -detected 2D heteronuclear shift correlation spectrum. The detected ^{13}C signal is acquired in the absence of ^1H decoupling in this case, so although this does not represent an optimum approach it does serve to illustrate the principles behind parallel acquisition spectroscopy. More sophisticated sequences have since been proposed, such as for the simultaneous acquisition of ^1H - ^{13}C and ^{19}F - ^{13}C 2D correlation spectra, for which dual ^1H and ^{19}F receivers and a triple-resonance ^1H - ^{19}F - ^{13}C probe were employed [133]. A yet more sophisticated approach employing dual receivers provides both one-bond and long-range ^1H - ^{13}C correlation spectra, a ^{13}C - ^{13}C INADEQUATE spectrum and a 1D proton-decoupled ^{13}C spectrum in a single experiment, elaborately termed PANACEA: “Parallel Acquisition NMR, an All-in-one Combination of Experimental Applications” [134]. The addition of a third receiver allows for the simultaneous acquisition of a ^{15}N spectrum also! Clearly with these methods there exists enormous future potential, and a whole new area of exploration for those engaged in pulse sequence development.

REFERENCES

- [1] R. Freeman, S.P. Kempell and M.H. Levitt, *J. Magn. Reson.*, 1980, **38**, 453–479.
- [2] M.H. Levitt and R. Freeman, *J. Magn. Reson.*, 1979, **33**, 473–476.
- [3] A.J. Shaka, C. Bauer and R. Freeman, *J. Magn. Reson.*, 1984, **60**, 479–485.
- [4] M.H. Levitt, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1986, **18**, 61–122.
- [5] A.J. Shaka and R. Freeman, *J. Magn. Reson.*, 1983, **55**, 487–493.
- [6] M. Keniry and B.C. Sanctuary, *J. Magn. Reson.*, 1992, **97**, 382–384.
- [7] N.S. Bai, M. Ramakrishna and R. Ramachandran, *J. Magn. Reson. (A)*, 1993, **102**, 235–240.
- [8] R. Ramachandran, *J. Magn. Reson. (A)*, 1993, **105**, 328–329.
- [9] M.H. Levitt and R. Freeman, *J. Magn. Reson.*, 1981, **43**, 65–80.
- [10] R. Tycko, H.M. Cho, E. Schneider and A. Pines, *J. Magn. Reson.*, 1985, **61**, 90–101.
- [11] A.E. Derome, *J. Magn. Reson.*, 1988, **78**, 113–122.
- [12] M. Garwood and L. DelaBarre, *J. Magn. Reson.*, 2001, **153**, 155–177.
- [13] A. Tannús and M. Garwood, *NMR Biomed.*, 1997, **10**, 423–434.
- [14] E. Kupče and R. Freeman, *J. Magn. Reson. (A)*, 1995, **115**, 273–276.
- [15] E. Kupče and R. Freeman, *J. Magn. Reson. (A)*, 1996, **118**, 299–303.
- [16] J.M. Bohnen and G. Bodenhausen, *J. Magn. Reson. (A)*, 1993, **102**, 293–301.
- [17] E. Kupče and R. Freeman, *J. Magn. Reson. (A)*, 1995, **117**, 246–256.
- [18] T.-L. Hwang, P.C.M. van Zijl and M. Garwood, *J. Magn. Reson.*, 1997, **124**, 250–254.
- [19] R.D. Boyer, R. Johnson and K. Krishnamurthy, *J. Magn. Reson.*, 2003, **165**, 253–259.
- [20] E. Kupče and R. Freeman, *J. Magn. Reson.*, 2007, **187**, 258–265.
- [21] M.A. Smith, H. Hu and A.J. Shaka, *J. Magn. Reson.*, 2001, **151**, 269–283.
- [22] T.E. Skinner, K. Kobzar, B. Luy, M.R. Bendall, W. Bermel, N. Khaneja and S.J. Glaser, *J. Magn. Reson.*, 2006, **179**, 241–249.
- [23] R. Fu and G. Bodenhausen, *J. Magn. Reson. (A)*, 1995, **117**, 324–325.
- [24] R. Fu and G. Bodenhausen, *J. Magn. Reson. (A)*, 1996, **119**, 129–133.
- [25] R. Tycko and A. Pines, *Chem. Phys. Lett.*, 1984, **111**, 462–467.
- [26] E. Kupče, R. Freeman, G. Wider and K. Wuthrich, *J. Magn. Reson. (A)*, 1996, **122**, 81–84.
- [27] E. Kupče, R. Freeman, G. Wider and K. Wuthrich, *J. Magn. Reson. (A)*, 1996, **120**, 264–268.
- [28] E. Tenaillon and S. Akoka, *J. Magn. Reson.*, 2007, **185**, 50–58.
- [29] M.H. Levitt, R. Freeman and T. Frenkiel, *J. Magn. Reson.*, 1982, **47**, 328–330.
- [30] A.J. Shaka, J. Keeler and R. Freeman, *J. Magn. Reson.*, 1983, **53**, 313–340.
- [31] A.J. Shaka, J. Keeler, T. Frenkiel and R. Freeman, *J. Magn. Reson.*, 1983, **52**, 335–338.
- [32] A.J. Shaka, C.J. Lee and A. Pines, *J. Magn. Reson.*, 1988, **77**, 274–293.
- [33] A.J. Shaka, P.B. Barker and R. Freeman, *J. Magn. Reson.*, 1985, **64**, 547–552.
- [34] N.S. Bai, N. Hari and R. Ramachandran, *J. Magn. Reson. (A)*, 1994, **106**, 241–244.

- [35] A. Bax and D.G. Davis, *J. Magn. Reson.*, 1985, **65**, 355–360.
- [36] S.P. Rucker and A.J. Shaka, *Mol. Phys.*, 1989, **68**, 509–517.
- [37] M. Kadkhodaie, O. Rivas, M. Tan, A. Mohebbi and A.J. Shaka, *J. Magn. Reson.*, 1991, **91**, 437–443.
- [38] A.J. Shaka and J. Keeler, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1987, **19**, 47–129.
- [39] H. Kessler, S. Mrona and G. Gemmecker, *Magn. Reson. Chem.*, 1991, **29**, 527–557.
- [40] H. Kessler, H. Oschkinat, C. Griesinger and W. Bermel, *J. Magn. Reson.*, 1986, **70**, 106–133.
- [41] R. Freeman, *Chem. Rev.*, 1991, **91**, 1397–1412.
- [42] S. Berger, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1997, **30**, 137–156.
- [43] R. Freeman, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1998, **32**, 59–106.
- [44] H. Geen, S. Wimperis and R. Freeman, *J. Magn. Reson.*, 1989, **85**, 620–627.
- [45] C. Bauer, R. Freeman, T. Frenkiel, J. Keeler and A.J. Shaka, *J. Magn. Reson.*, 1984, **58**, 442–457.
- [46] J. Friedrich, S. Davies and R. Freeman, *J. Magn. Reson.*, 1987, **75**, 390–395.
- [47] L. Emsley and G. Bodenhausen, *J. Magn. Reson.*, 1989, **82**, 211–221.
- [48] H. Kessler, U. Anders, G. Gemmecker and S. Steuernagel, *J. Magn. Reson.*, 1989, **85**, 1–14.
- [49] H. Geen and R. Freeman, *J. Magn. Reson.*, 1990, **87**, 415–421.
- [50] H. Geen and R. Freeman, *J. Magn. Reson.*, 1991, **93**, 93–141.
- [51] L. Emsley and G. Bodenhausen, *Chem. Phys. Lett.*, 1990, **165**, 469–476.
- [52] L. Emsley and G. Bodenhausen, *J. Magn. Reson.*, 1992, **97**, 135–148.
- [53] P.J. Hajduk, D.A. Horita and L.E. Lerner, *J. Magn. Reson. (A)*, 1993, **103**, 40–52.
- [54] D.A. Horita, P.J. Hajduk and L.E. Lerner, *J. Magn. Reson. (A)*, 1993, **103**, 53–60.
- [55] J.-M. Nuzillard and R. Freeman, *J. Magn. Reson. (A)*, 1994, **107**, 113–118.
- [56] E. Kupče, J. Boyd and I.D. Campbell, *J. Magn. Reson. (B)*, 1995, **106**, 300–303.
- [57] T.L. Hwang and A.J. Shaka, *J. Magn. Reson. (A)*, 1995, **112**, 275–279.
- [58] K. Stott, J. Stonehouse, J. Keeler, T.L. Hwang and A.J. Shaka, *J. Am. Chem. Soc.*, 1995, **117**, 4199–4200.
- [59] G.Z. Xu and J.S. Evans, *J. Magn. Reson. (B)*, 1996, **111**, 183–185.
- [60] K.E. Kövér, D. Uhrin and V.J. Hruby, *J. Magn. Reson.*, 1998, **130**, 162–168.
- [61] K. Stott, J. Keeler, Q.N. Van and A.J. Shaka, *J. Magn. Reson.*, 1997, **125**, 302–324.
- [62] G.A. Morris and R. Freeman, *J. Magn. Reson.*, 1978, **29**, 433–462.
- [63] S.L. Patt, *J. Magn. Reson.*, 1992, **96**, 94–102.
- [64] E. Kupče and R. Freeman, *J. Magn. Reson. (A)*, 1993, **105**, 234–238.
- [65] J.-M. Nuzillard and R. Freeman, *J. Magn. Reson. (A)*, 1994, **110**, 252–256.
- [66] J.-M. Bernassau and J.-M. Nuzillard, *J. Magn. Reson. (B)*, 1994, **103**, 77–81.
- [67] P.J. Hore, *Method. Enzymol.*, 1989, **176**, 64–77.
- [68] M. Guéron, P. Plateau and M. Decors, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1991, **23**, 135–209.
- [69] W.E. Hull, Experimental aspects of two-dimensional NMR In: *Two-dimensional NMR Spectroscopy: Applications for Chemists and Biochemists*, 2nd ed. (Eds.: W.R. Croasmun, R.M.K. Carlson), VCH, New York, 1994.
- [70] D.I. Hoult, *J. Magn. Reson.*, 1976, **21**, 337–347.
- [71] R.W. Dykstra, *J. Magn. Reson.*, 1987, **72**, 162–167.
- [72] D. Neuhaus, I.M. Ismail and C.-W. Chung, *J. Magn. Reson. (A)*, 1996, **118**, 256–263.
- [73] H. Mo and D. Raftery, *J. Magn. Reson.*, 2008, **190**, 1–6.
- [74] P. Plateau and M. Guéron, *J. Am. Chem. Soc.*, 1982, **104**, 7310–7311.
- [75] P.J. Hore, *J. Magn. Reson.*, 1983, **54**, 539–542.
- [76] P.J. Hore, *J. Magn. Reson.*, 1983, **55**, 283–300.
- [77] D. Callihan, J. West, S. Kumar, B.I. Schweitzer and T.M. Logan, *J. Magn. Reson. (B)*, 1996, **112**, 82–85.
- [78] M. Piotto, V. Saudek and V. Slenár, *J. Biomol. NMR*, 1992, **2**, 661–665.
- [79] V. Sklenár, M. Piotto, R. Leppik and V. Saudek, *J. Magn. Reson. (A)*, 1993, **102**, 241–245.
- [80] M. Liu, X. Mao, C. Ye, J.K. Nicholson and J.C. Lindon, *J. Magn. Reson.*, 1998, **132**, 125–129.
- [81] D. Marion, M. Ikura and A. Bax, *J. Magn. Reson.*, 1989, **84**, 425–430.
- [82] T. Parella, P. Adell, F. Sánchez-Ferrando and A. Virgili, *Magn. Reson. Chem.*, 1998, **36**, 245–249.
- [83] C. Dalvit, G. Shapiro, J.-M. Böhlen and T. Parella, *Magn. Reson. Chem.*, 1999, **37**, 7–14.
- [84] A.J. Simpson and S.A. Brown, *J. Magn. Reson.*, 2005, **175**, 340–346.
- [85] R.E. Hurd, *J. Magn. Reson.*, 1990, **87**, 422–428.
- [86] S. Macura, Y. Huang, D. Suter and R.R. Ernst, *J. Magn. Reson.*, 1981, **43**, 259–281.
- [87] S. Macura, K. Wüthrich and R.R. Ernst, *J. Magn. Reson.*, 1982, **46**, 269–282.
- [88] M.J. Thrippleton and J. Keeler, *Angew. Chem. Int. Ed.*, 2003, **42**, 3938–3941.
- [89] M. Rance, G. Bodenhausen, G. Wagner, K. Wüthrich and R.R. Ernst, *J. Magn. Reson.*, 1985, **62**, 497–510.
- [90] K.E. Cano, M.J. Thrippleton, J. Keeler and A.J. Shaka, *J. Magn. Reson.*, 2004, **167**, 291–297.
- [91] A.L. Davis, G. Estcourt, J. Keeler, E.D. Laue and J.J. Titman, *J. Magn. Reson. (A)*, 1993, **105**, 167–183.
- [92] G. Lowe, *Chem. Soc. Rev.*, 1995, **24**, 309–317.
- [93] D. Obrecht and J.M. Villalgordo, *Solid-supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries*, Pergamon, Oxford, 1998.
- [94] E. Giralt, J. Rizo and E. Pedroso, *Tetrahedron*, 1984, **40**, 4141–4152.
- [95] G.C. Look, C.P. Holmes, J.P. Chinn and M.A. Gallop, *J. Org. Chem.*, 1994, **59**, 7588–7590.
- [96] E.R. Andrew, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1971, **8**, 1–39.
- [97] W.P. Power and G.A. Webb, *Ann. Rep. NMR Spectrosc.*, 2003, **51**, 261–295.
- [98] M.J. Shapiro and J.S. Gounarides, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1999, **35**, 153–200.
- [99] M.E. Bollard, A.J. Murray, K. Clarke, J.K. Nicholson and J.L. Griffin, *FEBS Lett.*, 2003, **553**, 73–78.

- [100] W.L. Fitch, G. Detre, C.P. Holmes, J.N. Shoolery and P.A. Keifer, *J. Org. Chem.*, 1994, **59**, 7955–7956.
- [101] P.A. Keifer, L. Baltusis, D.M. Rice, A.A. Tymiak and J.N. Shoolery, *J. Magn. Reson. (A)*, 1996, **119**, 65–75.
- [102] R.C. Anderson, M.A. Jarema, M.J. Shapiro, J.P. Stokes and M. Ziliox, *J. Org. Chem.*, 1995, **60**, 2650–2651.
- [103] R.C. Anderson, J.P. Stokes and M.J. Shapiro, *Tetrahedron Lett.*, 1995, **36**, 5311–5314.
- [104] R. Jelinek, A.P. Valente, K.G. Valentine and S.J. Opella, *J. Magn. Reson.*, 1997, **125**, 185–187.
- [105] C. Dhalluin, C. Boutillon, A. Tartar and G. Lippens, *J. Am. Chem. Soc.*, 1997, **119**, 10494–10500.
- [106] P. Rousselot-Pailley, D. Maux, J.-M. Wieruszeski, J.-L. Aubagnac, J. Martinez and G. Lippens, *Tetrahedron*, 2000, **56**, 5163–5167.
- [107] P.A. Keifer, *J. Org. Chem.*, 1996, **61**, 1558–1559.
- [108] E. Kupče, P.A. Keifer and M. Delepiere, *J. Magn. Reson.*, 2001, **148**, 115–120.
- [109] E. Kupče and W. Hiller, *Magn. Reson. Chem.*, 2001, **39**, 231–235.
- [110] T.R. Ramadhar, F. Amador, M.J.T. Ditty and W.P. Power, *Magn. Reson. Chem.*, 2008, **46**, 30–35.
- [111] R. Freeman and E. Kupče, *J. Biomol. NMR*, 2003, **27**, 101–113.
- [112] L. Frydman, T. Scherf and A. Lupulescu, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 15858–15862.
- [113] L. Frydman, A. Lupulescu and T. Scherf, *J. Am. Chem. Soc.*, 2003, **125**, 9204–9217.
- [114] S.B. Duckett and C.J. Sleight, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1999, **34**, 71–92.
- [115] J.H. Ardenkjaer-Larsen, B. Fridlund, A. Gram, G. Hansson, L. Hansson, M.H. Lerche, R. Servin, M. Thaning and K. Golman, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 10158–10163.
- [116] R.A. Wind, M.J. Duijvestijn, C. van der Lugt, A. Manenschijn and J. Vriend, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1985, **17**, 33–67.
- [117] V.S. Bajaj, C.T. Farrar, M.K. Hornstein, I. Mastovsky, J. Viereg, J. Bryant, B. Eléna, K.E. Kreisler, R.J. Temkin and R.G. Griffin, *J. Magn. Reson.*, 2003, **160**, 85–90.
- [118] J. Wolber, F. Ellner, B. Fridlund, A. Gram, H. Johannesson, G. Hansson, L.H. Hansson, M.H. Lerche, S. Mansson, R. Servin, M. Thaning, K. Golman and J.H. Ardenkjaer-Larsen, *Nuc. Instr. Meth. Phys. Res. A*, 2004, **526**, 173–181.
- [119] K. Golman, J.H. Ardenkjaer-Larsen, J.S. Petersson, S. Mansson and I. Leunbach, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 10435–10439.
- [120] I.J. Day, J.C. Mitchell, M.J. Snowden and A.L. Davis, *Magn. Reson. Chem.*, 2007, **45**, 1018–1021.
- [121] L. Frydman and D. Blazina, *Nat. Phys.*, 2007, **3**, 415–419.
- [122] R.M. Gschwind, *Angew. Chem. Int. Ed.*, 2005, **44**, 4666–4668.
- [123] J. Yan and E.R. Zartler, *Magn. Reson. Chem.*, 2005, **43**, 53–64.
- [124] C.M. Thiele, *Concept. Magn. Reson. A*, 2007, **30**, 65–80.
- [125] J. Yan, A.D. Kline, H. Mo, M.J. Shapiro and E.R. Zartler, *J. Org. Chem.*, 2003, **68**, 1786–1795.
- [126] C. Aroulanda, V. Boucard, F. Guibé, J. Courtieu and D. Merlet, *Chem. Eur. J.*, 2003, **9**, 4536–4539.
- [127] C.M. Thiele, *J. Org. Chem.*, 2004, **69**, 7403–7413.
- [128] J. Yan, F. Delaglio, A. Kaerner, A.D. Kline, H. Mo, M.J. Shapiro, T.A. Smitka, G.A. Stephenson and E.R. Zartler, *J. Am. Chem. Soc.*, 2004, **126**, 5008–5017.
- [129] C.M. Thiele, A. Marx, R. Berger, J. Fischer, M. Biel and A. Giannis, *Angew. Chem. Int. Ed.*, 2006, **45**, 4455–4460.
- [130] M. Surfati, P. Lesot, D. Merlet and J. Courtieu, *Chem. Commun.*, 2000, 2069–2081.
- [131] B. Luy, K. Kobzar, S. Knor, J. Furrer, D. Heckmann and H. Kessler, *J. Am. Chem. Soc.*, 2005, **127**, 6459–6465.
- [132] E. Kupče, R. Freeman and B.K. John, *J. Am. Chem. Soc.*, 2006, **128**, 9606–9607.
- [133] E. Kupče, S. Cheatham and R. Freeman, *Magn. Reson. Chem.*, 2007, **45**, 378–380.
- [134] E. Kupče and R. Freeman, *J. Am. Chem. Soc.*, 2008, **130**, 10788–10792.

This page intentionally left blank

Appendix

Glossary of acronyms

Acronym/term	Translation	Section
ACCORDION	A method of increasing one time period in concert with another	6.4.3
ACOUSTIC	Alternate compound 180s used to suppress transients in the coil (for quadrupolar nuclei)	4.5
ADEQUATE	Adequate sensitivity double-quantum spectroscopy	5.9
APT	Attached proton test (spectrum editing)	4.3.2
BEBOP	Broadband excitation by optimized pulses	10.2.2
BIP	Broadband inversion pulse	10.2.2
BIRD	Bilinear rotation decoupling	6.3.3
BPP	Bipolar pair: gradient pulse pair of opposite sign	9.2.3
BURP	Band-selective, uniform-response, pure-phase (selective) pulse	10.4.1
CALIS	Calibration for indirect spins	3.5.1
CAMELSPIN	Cross-relaxation appropriate for minimolecules emulated by locked spins (aka ROESY)	8.8
CHIRP	Frequency swept pulse (adiabatic pulse).	10.2.1
CIGAR-HMBC	Constant-time inverse-detection gradient accordion rescaled HMBC	6.4.3
COLOC	Correlation through long-range coupling	6.5.2
COSY	Correlation spectroscopy	5.2
CPD	Composite pulse decoupling	10.3
CPMG	Carr-Purcell-Meiboom-Gill (T_2 -dependent spin-echo sequence)	2.4.4
CSA	Chemical shift anisotropy	2.5.3
CYCLOPS	Cyclically-ordered phase-sequence (for suppressing quadrature artefacts)	3.2.5
DANTE	Delays alternating with nutation for tailored (selective) excitation	10.4.3
DEPT	Distortionless enhancement by polarisation transfer (spectrum editing)	4.4.3
DEPTQ	Distortionless enhancement by polarisation transfer retaining quaternary centres	4.4.4
DIPSI	Decoupling in the presence of scalar interactions (isotropic mixing)	10.3.2
DNP	Dynamic nuclear polarisation	10.8.2
DOSY	Diffusion-ordered spectroscopy	9.2.5
DPFGE	Double pulsed field gradient spin-echo (selective excitation)	10.4.2
DQF-COSY	Double-quantum filtered correlation spectroscopy	5.6.2
DSTE	Double stimulated-echo	9.3.1
ERETIC	Electronic reference to access <i>in-vivo</i> concentrations	4.1.3
EXORCYCLE	Phase cycle to suppress “ghost” and “phantom” artefacts in spin-echo sequences	7.2.2
EXSY	Exchange spectroscopy	8.7.4
FID	Free induction decay	2.2.2
FLIPSY	Flip-angle adjustable one-dimensional NOESY (solvent suppression)	10.5.1
FLOPSY	Flip-flop mixing sequence (for total correlation spectroscopy)	10.3.2
GARP	Globally-optimised, alternating phase, rectangular pulses (for broadband decoupling)	10.3
HEHAHA	Heteronuclear Hartmann-Hahn spectroscopy	5.7
HETCOR	Heteronuclear correlation	6.5.1
H2BC	Heteronuclear two-bond correlation	6.4.3
HMBC	Heteronuclear multiple-bond correlation	6.4
HMQC	Heteronuclear multiple-quantum correlation	6.3.1
HOESY	Heteronuclear Overhauser effect spectroscopy	8.9.2
HOHAHA	Homonuclear Hartmann-Hahn spectroscopy (aka TOCSY)	5.7
HSQC	Heteronuclear single-quantum correlation	6.3.2
IMPEACH-MBC	Improved performance accordion heteronuclear multiple bond correlation	6.4.3
INADEQUATE	Incredible natural-abundance double-quantum transfer experiment	5.8
INEPT	Insensitive nuclei enhanced by polarisation transfer	4.4.2
J-MOD	J-modulated spin-echo	4.3.1
LED	Longitudinal eddy-current delay	9.2.3
LP	Linear prediction	3.2.3
MAS	Magic angle spinning	10.7
MLEV	Broadband decoupling cycle (from M. Levitt)	10.3
MQF	Multiple-quantum filter	5.6.2
NOE	Nuclear Overhauser effect	8.2
NOESY	Nuclear Overhauser effect spectroscopy	8.7
PANSY	Parallel acquisition NMR spectroscopy	10.8.4
PENDANT	Polarisation enhancement nurtured during attached nucleus testing (spectrum editing)	4.4.5
PEP	Preservation of equivalent pathways	6.3.3
PFG	Pulsed field gradient	5.5

(Continued)

Acronym/term	Translation	Section
PHIP	<i>Para</i> -hydrogen induced polarisation	10.8.2
PURGE	Presaturation utilizing relaxation gradients and echoes (solvent suppression)	10.5.3
RDC	Residual dipolar coupling	10.8.3
RIDE	Ring-down delay sequence (for quadrupolar nuclei)	4.5
ROESY	Rotating-frame Overhauser effect spectroscopy	8.8
SEFT	Spin-echo Fourier transform (spectrum editing)	4.3.1
SPFGSE	Single pulsed field gradient spin-echo (selective excitation)	10.4.2
SPT	Selective population transfer	4.4.1
STE	Stimulated-echo	9.2.2
STEP	Selective TOCSY edited preparation	8.7.2
SUSAN	Spin decoupling employing ultra-broadband inversion sequences generated <i>via</i> simulated annealing (broadband decoupling sequence)	10.3
TOCSY	Total correlation spectroscopy	5.7
TPPI	Time-proportional phase incrementation	5.3.1
Tr-ROESY	Transverse rotating-frame Overhauser effect spectroscopy	8.8
WALTZ	Wideband, alternating-phase, low-power technique for zero-residual splitting (broadband decoupling sequence)	10.3
WATERGATE	Water suppression through gradient tailored excitation	10.5.3
WATR	Water attenuation by transverse relaxation	2.4.4
WURST	Wideband, uniform rate and smooth truncation (adiabatic pulse).	10.2.1

Index

¹¹B
coupling, 34
2D EXSY, 291
linewidths, 33

¹³C
satellites in ¹³C spectra, 178
satellites in ¹H spectra, 103, 191, 195
satellites, selecting, 195, 206

¹⁴N
coupling, 34
linewidths, 33

¹⁵N
INEPT, 119–120
HSQC, 199

¹⁷O
backward linear prediction, 127
RIDE, 126

180° pulse

¹⁹⁵Pt
field dependence, satellites, 31
¹H-¹³C-¹⁹⁵Pt correlation, 229

¹⁹F
coupling to ¹¹B, 34
quantitative, 43

1D NMR, *see* one-dimensional NMR

¹J_{CC}
measuring, 180–183
typical values, 182

¹J_{CH}
typical values, 113, 193

^{2/3}J_{CH}
measuring, 221–223, 235–237
typical values, 210

²⁹Si
HMBC, 211

2D NMR, *see* two-dimensional NMR

²H
coupling, 33
lock, 75–76

3-(trimethylsilyl)propionate, TSP
shift reference, 61

³¹P
¹³C HMQC, 227
³¹P COSY, 137
⁵⁷Fe HMQC, 228
decoupling, 110

3D NMR, *see* three-dimensional NMR

⁵⁷Fe
³¹P HMQC, 228

⁷⁷Se
CSA relaxation, 31

90° pulse, 15

A

Absolute-value
display, 2D, 138, 141, 239

Absorption mode, 16

Acetylisomontanolid
¹³C INADEQUATE, 179

ACCORDION optimisation
in HMBC, 216, 227
in ADEQUATE, 186

ACCORD-HMBC, 216–219

ACOUSTIC, 126

Acoustic ringing, 126

Acquisition parameters
2D NMR, 145–146

Acquisition time, 42–44
2D NMR, 145–146

Acronyms
glossary, 374–375

Active coupling, 161

Active volume, 68

ADC, *see* analogue-to-digital converter

ADEQUATE, 183–186

Adiabatic
decoupling, 344
pulses, 338–341

Adiabaticity factor, 339, 344

Aliasing, 41, 48
2D NMR, 140
folded signals, 49
wrapped signals, 49

Alignment media, for RDCs, 368, 370

Alkynes, unusual behaviour
DEPT, 123
HMQC/HSQC, 194

Allylic coupling
in COSY, 166

Amplitude modulation, 131, 138

Analogue filters, 42

Analogue-to-digital converter, 40,
51–55
overload, 52
resolution, 51

Andrographolide
DQF-COSY, 164
NOEs, 265
NOESY, 278

Anisotropy
in chemical bonds, 31

Anti-J spectrum, 242

Anti-phase
splittings, in COSY, 140, 161–165
vectors, 17

Apodisation, 44, 55

APT, *see* attached proton test

Artefacts
axial peaks, 142
clipped FID, 52
ghosts, 238
phantoms, 238
quad images, 48, 143
SPT, 116, 273
subtraction, 272
t₁-noise, 143, 199, 209
truncated FID, 43

ASTM
test sample, 95

Attached proton test, 113

Audio frequencies, 40, 47

Axial peaks, 142

B

B₀, static magnetic field, 11, 38
inhomogeneity, 24, 36
optimising, 77–82

B₁, rf magnetic field, transmitter, 15, 39
calibrating, 83–87
inhomogeneity, 335–337

B₂, rf magnetic field, decoupler, 343–344
calibrating, 87–88

Bandpass filter, 40

Band-selective
HMBC, 214

Bandwidth
 decoupling, 344
 excitation, 366
 inversion, 366–368

Bar magnet analogy, 12, 30

B_{eff} , effective rf field, 38–39, 335, 339

Benzene
 test sample, 95

Bicycle dynamo, 114

Bi-level decoupling, 345

Bilinear rotation decoupling, 201
 HMQC, 200–202
 HETCOR, 225
 J-resolved spectroscopy, 236
 in HMBC, 213

Bipolar pulse pairs, 306

BIPs, *see* Broadband inversion pulses

BIRD pulse, *see* bilinear rotation decoupling

Bloch-Siegert shift, 88, 106

Boiling points
 deuterated solvents, 60

Boltzmann distribution, 13, 114, 249

Boronic acids, 33

Broadband decoupling, 105, 343–345
 of proton spectra, 239–240, 242–244

Broadband inversion pulses, 342

Bulk magnetisation vector, 13

BURP pulse, 347, 348–350
 excitation trajectory, 348

Butanol
 ^{13}C INADEQUATE, 179

C

Calibrations
 decoupler pulses, 87
 pulsed field gradients, 89
 pulse widths, 84–88
 radiofrequency fields, 84–88
 shaped pulses, 353
 temperature, 91

CALIS
 pulse calibration, 88

CAMELSPIN, *see* ROESY

Carbopeptides
 COSY, 135–136
 HMBC, 215
 HSQC, ^{15}N , 199
 TOCSY, 168
 Tr-ROESY, 294

Carousel analogy
 rotating frame, 14

Carr-Purcell sequence, 25

Carr-Purcell-Meiboom-Gill sequence, 25–26

Chemical exchange
 EXSY, 290–292
 solvent attenuation, 28

Chemical shift
 anisotropy, 31
 refocusing, 18–19
 visualising in vector model, 17–18

Chemical shift anisotropy relaxation, 31

CHIRP pulses, 340

Chloroform
 test sample, 94

Chromium(III) acetylacetonate, 31, 101, 182

CIGAR-HMBC, 218

Cleaning, NMR tubes, 64

Clipping, FID, 52

Coherence, 148–151
 double-quantum, 149, 159, 178
 multiple-quantum, 149
 order, 148
 phase-, 16, 149
 single-quantum, 149
 transfer pathways, 150–151
 zero-quantum, 149

Coherence transfer, 148–151
 echoes, 2D, 141
 in COSY, 134
 in TOCSY, 169

Coherence transfer pathways, 150–151
 in COSY, 150
 in DQF-COSY, 159
 in HMQC, 193
 in HSQC, 196–198
 in INADEQUATE, 179
 in NOESY, 278

COLOC, 225

Composite pulse decoupling, 108, 343–344

Composite pulses, 274, 335–338
 broadband decoupling, 343–344
 inversion vs refocusing, 338
 properties of, selected, 337
 spin locks, 169, 345

Conformational averaging, of NOE,
 268–269

Constant-time experiments, 225
 in HMBC, 214

Constant-time variable-delay, 218

Contour plot, 133, 142, 148

Convection, in diffusion NMR, 311–316
 compensating, 315
 dealing with, 313–316
 diagnosing, 311–313

Cooley-Tukey algorithm
 fast Fourier transform, 20

Correlation spectroscopy, COSY, 134–138,
 158–168
 gradient selected, 154, 160
 interpreting, 135–138, 161–165
 small couplings, 166–167
 which method? 158

Correlation time, τ_c , 29, 251

COSY, *see* correlation spectroscopy

COSY-45, *see* COSY- β

COSY-60, *see* COSY- β

COSY-90, *see* COSY- β

COSY- β , 165
 geminal vs vicinal couplings, 166

Coupling constants
 from DQF-COSY, 163
 in cyclohexanes, 164

CPMG, *see* Carr-Purcell-Meiboom-Gill

Crosspeak, 134, 137
 disappearance, in COSY, 163
 tilting, in COSY, 165

Cross-polarisation, 169

Cross-relaxation, 250
 rates, 250–267

Cryo-probes, 71–73
 temperature calibration, 92

CSA, *see* chemical shift anisotropy relaxation

Cyclodextrins
 NOESY, 289
 ROESY, 297

Cyclohexanes
 couplings, from COSY, 164

CYCLOPS, 50
 in 2D NMR, 143, 181

D

D_2O exchange, 61

DANTE, 351–352
 excitation sidebands, 352

dB scale, 84

Decimation, 64

Decoupler
 calibrating, 87
 gating, 108
 leakage, 107

Decoupling, 105–110, 343–344
 adiabatic, 344
 bandwidths, 344
 bi-level, 345
 broadband, 105, 108, 343–344
 composite pulse, 108, 343–344

Degassing
 NOE, 256, 300
 techniques for, 64

Delayed-COSY, 166

DEPT, 121–125
 editing with, 122
 optimising sensitivity, 123
 retaining quaternaries, 124–125

DEPT-Q, 124–125

Detection, 40–43, 46–50
 2D NMR, 131
 single channel, 46
 two channel, quadrature, 46–50

Deuterated solvents
 changing, 61
 properties, 60

Developments, in NMR, 1–3

Diagonal peak, 134, 137

Diastereotopic protons
 ^1H - ^{13}C correlations, 192

Difference spectroscopy, 105
 NOE, 271–276

Diffusion
 coefficient, 303–304, 320–321
 losses, 26, 157

Diffusion NMR, 303–333
 applications, 321–329
 convection, 311–316
 optimising, 317–320
 sequences, 304–309, 330–333

Diffusion ordered spectroscopy, *See also*
 diffusion NMR, 310, 322, 328
 3D DOSY, 331–333

Digital filters, 53

Digital resolution, 42
 Digital signal processor, 40, 54
 Digital quadrature detection, 49
 Digitisation, 40, 51
 2D NMR, 145–146
 noise, 53
 Digitiser, *see* analogue-to-digital converter
 Dilute spins
 correlating, 178–186
 Dioxane
 shift reference, 61
 test sample, 95
 Dipolar interactions
 coupling, 30, 247, 250–253, 362
 electrons, 30
 residual coupling, 362, 370
 Dipole-dipole relaxation, 30–31, 250–253
 DIPSI-2, 174, 345
 Directional coupler, 75
 DNP, *see* dynamic nuclear polarisation
 DOSY, *see* diffusion ordered spectroscopy
 Double difference phase-cycle, 195
 Double pulsed field gradient spin-echo, 350
 1D NOESY, 283
 1D TOCSY, 175
 Double-quantum filter
 1D, 160
 2D, 159–165
 INADEQUATE, 178, 181
 Doubly selective
 1D TOCSY NOESY, 284–285
 DPFGE, *see* double pulsed field gradient spin-echo
 DQD, *see* digital quadrature detection
 DQF, *see* double-quantum filter
 DQF-COSY, 159–165
 multiplet structures, 161
 Drying, samples, 60
 DSP, *see* digital signal processor
 Dummy scans, 146
 Duty cycle, 107
 Dwell time, 40
 Dynamic nuclear polarisation, 365–368
 Dynamic range, 51, 354

E

EBURP, *see* BURP pulse
 Echo-antiecho method, 155, 198
 Eddy currents, 157
 Editing
 1D proton spectra, 206
 background suppression, 27
 by molecular size, 27, 304
 diffusion filter, 321
 heteronuclear 2D correlations, 203
 multiplicity, 117–118, 122, 203
 T₂ filter, 27–28
 Electric field gradients, 32
 Emerging methods
 DNP, 365
 PANSY, 370
 RDCs, 368
 single-scan 2D, 364

Enantiomeric excess
 improving accuracy, 43, 103
 in liquid crystals, 370
 Equilibrium
 establishing, 21–22
 Equilibrium NOE, *see* steady-state NOE
 ERETIC
 quantification with, 103–104
 Ernst angle, 100
 Ethanediol
 temperature calibration, 92–93
 Ethanol, first high-resolution ¹H spectrum, 2
 Ethylacetoacetate
 DNP ¹³C spectrum, 367
 Ethylbenzene
 test sample, 95
 Ethylene glycol, *see* ethanediol
 Evolution time
 2D NMR, 130
 Excitation bandwidth, 38, 335, 351
 Excitation profiles, 38, 338–351
 binomial pulses, 356, 358
 inversion, 338–342
 shaped pulses, 347–348
 Excitation sculpting, 350
 solvent suppression, 357
 Excitation trajectories
 binomial pulses, 357
 composite pulses, 336
 DANTE, 351
 imperfect pulses, 336
 shaped pulses, 347, 348
 WURST, 340
 EXORCYCLE, 181, 238, 283, 355
 Experiment selection, guidelines for,
 7–10
 Exponential
 decay, 56, 94
 multiplication, 55
 recovery, 21–22
 EXSY, 279, 290–292
 Extreme narrowing limit, 29, 253, 271

F

f₁, 2D NMR, 7, 131
 f₂, 2D NMR, 7, 131
 Field-gradient pulses, *see* pulsed field
 gradients
 Filling factor, 191
 Filtering
 1D proton spectra, 206
 low-pass, HMBC, 212
 noise, 42
 sample solutions, 64
 FLIPSY, 355
 FLOCK, 226
 FLOPSY-8, 344
 Folding, *see* aliasing
 Fourier transformation
 complex, 47
 introduction, to NMR, 2
 in 2D NMR, 131, 147

 mathematical expression, 19
 real, 47
 Free induction decay, 17
 Freeze-pump-thaw method, 64
 Frequency domain, 20
 Frequency labelling, 131
 Frequency sweep
 adiabatic, 339

G

Gadolinium triethylene-tetraamine-
 hexaacetate, 182
 GARP, 343
 Gated decoupling, 108
 in J-resolved spectroscopy, 234
 inverse, 102, 108, 255
 power, 108
 Gaussian pulse, 346–348, 351
 Gel-phase NMR, 362
 Geminal couplings
 in COSY, 136, 165
 Gradient echo
 gradient calibrations, 89–91, 316–317
 selective excitation, 350
 shimming, 80
 signal selection, 152–155
 Gradient image profiles, 81, 91
 Gradient shimming, 90–82
 Gradient-accelerated spectroscopy,
 see gradient-selected spectroscopy
 Gradient-enhanced spectroscopy,
 see gradient-selected spectroscopy
 Gradient-selected spectroscopy, 151–158
 advantages and limitations, 156
 Gramicidin-S
 TOCSY, 171, 173
 NOESY, 288
 Gyromagnetic ratio, *see* magnetogyric
 ratio

H

H2BC, 220
 Hard pulse, 38, 345
 Hartmann-Hahn match, 169–170
 in ROESY, 294
 Heating, rf induced, 108, 335
 HEHAHA, 170
 Heisenberg Uncertainty Principle
 pulse excitation, 38
 relaxation, 21
 HETCOR, 223–225, 245
 Heterogeneous samples, 362–363
 Heteronuclear multiple-bond correlations,
 see also HMBC
 proton detected, 207–223
 X-detected, 225–226
 Heteronuclear single-bond correlations,
 see also HMQC and HSQC
 proton detected, 195–207
 BIRD selection, 200

breakthrough in HMBC, 207
 gradient selection, 197
 hybrid experiments, 203–207
 X-detected, 223–225

History of NMR, 1–3

HMBC, 207–223
 broadband methods, 216–219
 constant time, 214
 editing, 219
 measuring $^1J_{\text{CH}}$, 221
 phase-sensitive, 209

HMQC, 192–195
 for X–Y correlations, 227

HOESY, 298–300

HOHAHA, *see* TOCSY

Homospoil pulse, 156

HSQC, 195–196
 edited, multiplicity, 203
 TOCSY, 204

Hybrid experiments, 203–205,
 330–333

Hydrodynamic radius, 304, 320

Hyperbolic secant, pulse, 340

Hyperpolarisation
 DNP, 365–368
 PHIP, 366

HYSEL, 204

I

IBURP, *see* BURP pulse

IDOSY, 332–333

Imaginary spectrum, 20, 139

Imaging
 gradient shimming, 80

IMPEACH-MBC, 218, 228

INADEQUATE, 178–183
 1D, measuring J_{CC} , 180
 2D, 178
 proton detected, 183–186

INEPT, 116–121, 183
 editing with, 120–121
 DOSY, 330
 in ^1H –X correlations, 195, 223
 in ^1H –X–Y correlations, 229
 multiplet intensities in, 118
 optimising sensitivity, 119
 refocused, 117–118
 signal averaging, 120

INEPT⁺, 118

INEPT-INADEQUATE, 183

Inhomogeneity
 B_0 field, 24, 35
 B_1 field, 335–337

Ionic liquids, 322

INSIPID, 183

Instrumentation, 35–37, 65–73

Integration, 101–103

Interferogram, 132

Inverse spectroscopy, 190–191

Inversion recovery
 for measuring T_1 , 22–24
 in BIRD-HMQC, 201

Isochromats, 25

Isotropic mixing, *see* spin-lock

I-spin, in NOE, 248

J

J-modulated spin-echo, 111–113
 2D analogue, J-resolved, 233–235
 editing in HSQC, 203

J-resolved spectroscopy, 233–246
 absorption mode, 242–244
 heteronuclear, 233–248
 homonuclear, 238–245
 indirect homonuclear, 245–246

L

Laboratory frame of reference, 13

Larmor
 frequency, 12
 precession, 11

LC-NMR, 2, 130

LED, *see* longitudinal eddy-current delay

Leu-enkephalin
 1D DQF, 161
 DQF-COSY, 163

Ligand exchange, EXSY, 290

Linear prediction, 44–46
 backward, 46, 127
 forward, 45
 in 2D NMR, 45, 147

Line-broadening functions, 55

Lineshape
 2D, 138–142
 absorption mode, 16
 defects, 79
 dispersion mode, 16
 Gaussian, 57
 Lorentzian, 25, 57, 103
 tests, 94

Linewidth
 half-height, 25

Liquid crystals, 370

Lock
 optimising, 76
 parameters, 76
 system, 75–76

Longitudinal eddy-current delay, 307

Longitudinal spin relaxation, 20–24, 251

Long-range COSY, *see* delayed-COSY

Long-range couplings
 ^1H – ^{13}C , 109, 208, 225, 235–237
 in COLOC, 225
 in COSY, 166
 in HMBC, 207, 210
 measuring $^1J_{\text{CH}}$, 221–223, 235–237

Low-pass J-filter, in HMBC, 212

LP, *see* linear prediction

M

Magic angle spinning, 362–363

Magic-cycle, decoupling, 343

Magnetic field, B_0 , 11
 optimising, 77–82

Magnetic flux density, 11

Magnetic moment
 molecular, 32
 nuclear, 11

Magnetic susceptibility
 matched NMR tubes, 63
 probe coils, 67
 sample preparation, 63

Magnetogyric ratio, γ , 11, 114, 190

Magnets, superconducting, 2, 35–36
 progress, 36
 shielded, 3

Magnitude calculation, 141

Manganese(II) chloride, 31

Marine alkaloids, 172, 286

MAS, *see* magic angle spinning

Matching
 probehead, 74

MAXY, 204

Melting points
 deuterated solvents, 60

Menthol
 ^1H -decoupled ^1H , 244
 INEPT-INADEQUATE, 184
 J-resolved, 235

Methanol
 temperature calibration, 192

Microprobes, 69–71

Mixing
 2D NMR, 130

Mixing time, τ_{m}
 in NOESY, 277, 281
 in ROESY, 370, 292
 in TOCSY, 169, 173

MLEV-16, 174, 343–344

MLEV-17, 174, 344

Molecular sieves
 drying samples, 60

Monochromatic radiation
 pulsed excitation with, 38, 335

Mosher's acid derivatives
 ee determinations, 43

Multiple-quantum coherence, 148–150
 DEPT, 121
 HMQC, 193
 simplified picture, 150

Multiple receivers, 370

Multiplets
 visualising in vector model, 17

Multiplicities from
 DEPT, 122–123
 HMQC/HSQC, 203
 INEPT, 117–118
 J-resolved spectroscopy,
 234–235

N

Natural abundance
 of quadrupolar nuclei, selected, 32
 of spin- $\frac{1}{2}$ nuclei, selected, 12

NMR spectrometer, schematic, 35

- NOE difference, 271–276, 297
heteronuclear, 297
optimising, 272–275
quantifying enhancements, 276
SPT artefacts, 273
subtraction artefacts, 272
- NOE, *see* nuclear Overhauser effect
- NOESY, 276–290
1D, gradient, 283
internuclear separations, 281
presat, 355
ZQ suppression, 279–281
- Noise
filtering, 42
digitisation-, 53
quantisation-, 53
- Nomenclature, pulse sequence,
5–7
- N-type
echoes, 141
pathway, 150, 154
- Nuclear Overhauser effect: *See also*:
Steady-state NOE; Transient NOE;
Rotating frame NOE, 30,
247–301
applications, examples, 262–266,
286–290
conformational averaging, 268
cross-relaxation, 250
definition, 248
distance dependence, 252–255,
257
experimental aspects, 300
heteronuclear, 114, 297–300
homonuclear, 248, 253
initial rate approximation, 267
internuclear separations,
268, 281
introduction of, to NMR, 2
kinetics, 267
molecular motion, 253
negative, 250, 255, 258
positive, 250
sensitivity enhancement, 2, 119
- Nuclear spin, 11
- Nutation angle, 15
- Nyquist condition, 40–41
2D NMR, 145
oversampling, 53
- O**
- Off-resonance
effects, 38, 335–336
excitation, 357
- One-dimensional NMR
techniques, 99–126, 161, 175, 180, 206,
271, 282, 294
- One-shot
diffusion sequence, 308
- On-resonance
excitation, 16, 38
- Oversampling, 53
decimation, 54
- Overview
of modern NMR methods, 7–10
of NMR spectrometer, 35–37
- P**
- Palladium phosphine complexes
 $^1\text{H}\{^1\text{H}\}$ decoupled, 241
 $^1\text{H}\{^{31}\text{P}\}$ decoupled, 110
Diffusion, 325
HETCOR, 224
NOESY, 290
ROESY, 297
- PANSY, *see* parallel acquisition NMR
- Parallel Acquisition NMR, 370
- Paramagnetic relaxation, 27, 30
agents for, 31, 101
NOE, quenching, 256, 300
removing O_2 , 64, 300
- Pascal's triangle, 162
- Passive coupling, 161
- PBLG, liquid crystal, 370
- PENDANT, 125
- PEP, *see* preservation of equivalent
pathways
- Phantom
in gradient calibrations, 90
- Phase
correction, 1D, 58
correction, 2D, 148
coherence, 16, 149
real and imaginary data, 20
- Phase cycling, 50
axial peak suppression, 143
CYCLOPS, 50, 143
double-difference, 195
EXORCYCLE, 238
multiple-quantum filtration, 159
- Phase errors
aliased signals, 48
correction, 58, 148
zero and first order, 58
- Phase modulation, 141
in J-resolved spectroscopy, 239
- Phase-sensitive
display, 2D, 138, 142
gradient spectroscopy, 15
- Phase-sensitive detector, 47
- Phase-twist lineshape, 141–142
in J-resolved spectroscopy, 239
- Polarisation transfer, 115–116
in COSY, 134, 137
versus NOE, sensitivity, 119
- Population differences, 13, 15–16
equalising, 15
inverting, 15
- Population inversion, 15,
115–116
- Pre-acquisition delay
phase errors, 58
quadrupolar nuclei, 126
- Precession
gyroscope, 11
nuclear, Larmor, 11–12
- Preparation
2D NMR, 130
- Presaturation, solvent, 355
PURGE, 358
- PRESAT-180, 353
- Preservation of equivalent pathways,
199–200
- Probeheads, 65–73
acoustic ringing, 126
actively shielded, 157
cryogenic, 71
magic angle spinning, 362
Q-modulation, 301
tuning and matching, 74
- Processing parameters
2D NMR, 147
- Propagation, of magnetisation
with spin-lock, 169–170, 173
- Protocol, for structure confirmation, 9
- Pseudo-diagonal, 179
- P-type
anti-echoes, 141
pathway, 150, 154
- Pulse excitation, 15–16, 38–40
bandwidth, 38
tip or flip angle, 15
vector model, 13–16
- Pulse imperfections
 B_1 inhomogeneity, 335–337
compensating for, 335–338
excitation trajectories, 336
in HSQC, 196
off-resonance effects, 38, 335–336
- Pulse sequence nomenclature, 6
- Pulse width
calibration, 83–89
definition, 38
miscalibration, 335
- Pulsed field gradients, 151–158
implementing, 157
introduction of, to NMR, 2
shimming with, 80
symbols used for, 6
- Pulses
adiabaticity factor, 339, 344
gradient, 152–154
hard, rf, 38
shaped/selective, rf, 345–353
soft, rf, 345–353
symbols used for, 6
- Pure-phase pulse, shaped, 348–350
- Q**
- Q3, shaped pulse, 349
- Q5, shaped pulse, 349
- Q-modulation, 300
- Quad-images, 48, 143
compensating for, CYCLOPS,
50, 143
- Quadrature detection, 46–50
aliasing, 48, 140
echo-antiecho method, 2D, 155, 198
images, 48
in 2D NMR, 138–142
States method, 2D, 138
States-TPPI method, 2D, 139
TPPI method, 2D, 139
- Quadrupolar nuclei, 32–34
coupling with spin- $\frac{1}{2}$ nuclei, 33–34
observing, 126
- Quadrupolar relaxation, 32–33
NOE, quenching, 256

Quantitative NMR, 101–103
with ERETIC, 103–105
 ^{19}F , 43

Quinine
diffusion, 312, 314

R

Radiation damping, 75, 354

Radio-frequency pulses, *see also* pulses
symbols for, 6

Rayleigh number, 313

RDC, *see* residual dipolar coupling

Real spectrum, 20, 139

REBURP, *see* BURP pulse

Receiver, 35
gain, 51–52

Receiver imbalance
compensating for, 50

Recovery delay, *see* relaxation delay

Reference compounds, 62

Reference deconvolution, 82–83, 145

Reference distances, NOE, 281

Referencing spectra
internal, 61
external, 61
 Ξ scale, 61

Refocusing
chemical shifts, 18–19

Relative sensitivity
of spin- $\frac{1}{2}$ nuclei, selected, 12
of quadrupolar nuclei, selected, 32

Relaxation, 20–34
free induction decay, 17
longitudinal, 21–24
mechanisms, 28–34
spin-lattice, 21
spin-spin, 25
transverse, 24–28

Relaxation delay, 99–101

Relayed- COSY, 167

Relaying of information
in TOCSY, 169–170
in HSQC-TOCSY, 205

REPAY, 204

Repetition rate
Ernst angle, 100
optimum, in 1D NMR, 99–101
optimum, in 2D NMR, 146
optimum, with 90° pulse, 101

Residual dipolar coupling,
368–370

Resolution
tests, 94

Resolution enhancement, 55
linear prediction, 44

RIDE
quadrupolar nuclei, 126

Robotic sample changer, 35

ROE, *see* rotating frame NOE

ROESY, 292–297
1D, 294
mixing time, 270, 292

spin-locks, 293–294
transverse, Tr-, 294

Rotating frame NOE, 269–271, 292–297
appearance in TOCSY, 173
applications, examples, 295–297
false, 293
mixing time, 270, 292
spin-lock, 270, 293–294

Rotating frame of reference, 13–14

Rotaxanes
NOESY, 288

S

Saccharides
HSQC, edited, 204
HSQC-TOCSY, 205
J-resolved, 240
1D TOCSY-NOESY, 285

SAG, 370

Sample preparation, 59–65, 354

Sample spinning, 36, 300
2D NMR, 144
in diffusion NMR, 314
introduction of, to NMR, 2
shimming, 77
sidebands, 79

Sampling, data, 40–43
sequential, 47, 139
simultaneous, 47, 138

Satellites, 31, 103, 178, 191, 195, 207

Saturation, 15, 101
transfer, 260, 275, 356

Sealing tubes, 65

SEFT, 111

Selective detection, 55

Selective excitation, 38, 345–353
DANTE, 351
gradient echoes, 350
shaped pulses, 346

Selective population inversion, 115

Selective population transfer, 115–116
artefacts, 115, 273

SELTRIP-gs, 230

Sensitivity, 67–68, 114–115, 190–191
enhancement, 55
heteronuclear correlations, 190–191
probe, 67–69
optimising, in 1D NMR, 99–101
oversampling, 54
tests, 94

Sequential assignment, peptides, 287–288

Shaped pulse, 345–350
bandwidth factor, 349
calibrating, 353
DANTE, 352
Gaussian, 346
properties of, selected, 349
pure phase, 348

Shift correlation
chemical exchange, 290–292
dipolar couplings, 276–290, 292–297
heteronuclear couplings, 189–230
homonuclear couplings, 129–186
overview, 8

Shim coils, 36, 77

Shimming, 36, 77–82
defects, 80
gradient, 80–82
on the FID, 79

Signal averaging, 52
NOE difference, 272

Signal-to-noise ratio
measuring, tests, 94
in signal averaging, 52
comparing probes, 68

Single-channel detection, 46, 48
TPPI, 139

Single-scan 2D NMR, 364–365, 368

SNEEZE, shaped pulse, 349, 353

Soft pulse, *see also* shaped pulse, 38,
345–350

Solvent suppression, 354–359
binomial sequences, 357
via chemical exchange, 27
via diffusion, 321
jump-return, 356
presaturation, 355, 358
pulsed field gradients, 357
test sample, sucrose, 96, 358

Solvents, 59, 300, 354
viscosity, 314

Spatially selective excitation, 364

Spectral density, 29, 251

Spectral width, 40

Spectrum editing, 111–126
with spin echoes, 27–28,
111–113

Spin coupling
visualising in vector model, 16–19

Spin quantum number, I, 12, 32

Spin rotation relaxation, 32

Spin-diffusion, 258

Spin-echoes, 18–19
diffusion NMR, 304
experimental observation, 25–27
homonuclear, 18, 169
heteronuclear, 18
in J-resolved spectroscopy, 233
in spin-locks, 169
J-modulated, 111–113
 T_2 measurement, 25–27

Spin-lattice relaxation, *see* longitudinal spin
relaxation

Spin-lock, 343–345
in ROESY, 270, 293–294
in TOCSY, 169, 347

Spinning sidebands, 79

Spin-spin relaxation, *see* transverse spin
relaxation

Spin-spin coupling
early observations of, 2
vector model, 16–17

Spontaneous emission, 22

S-spin, in NOE, 248

Stacked plot, 132, 133, 142

States method
2D quad detection, 139
axial peaks, 142

States-TPPI method
 2D quad detection, 139
 axial peaks, 143

Steady-state
 2D NMR, 146
 magnetisation, 99–101

Steady-state NOE, 248–266
 applications, examples, 262–266
 distance dependence, 254, 257
 indirect, 3-spin, 258
 measuring, 271–276
 multispin system, 255–260
 origin, 249
 spin-diffusion, 258
 summary of, key points, 260–262
 two-spin system, 248–255
versus transient NOE, 276

Steroids
 NOESY, 289
 ROESY, 295

Stimulated echo
 diffusion NMR, 306–309
 double, 315
 double, asymmetrical, 312

Stimulated emission, 22

Stokes-Einstein equation, 304

Strong coupling
 in J-resolved spectroscopy, 238, 241
 spin-locks, TOCSY, 169

Sucrose
 test sample, 96, 358

Superconducting magnets, *see* magnets

Supercritical fluids
 as NMR solvents, 33

Super-cycle, decoupling, 343

SUSAN, 344

Symmetrisation
 in COSY, 144
 in J-resolved spectroscopy, 240

Symmetry
 in complexes, 32
 quadrupolar lineshapes, 32

T

t_1 , 2D NMR, 7, 130
 noise, 143, 199, 210

T_1 , relaxation time constant
 definition, 21
 dependence on tumbling rates, 251–252
 measuring, 22–24

T_2^* , relaxation time constant
 definition, 25

t_2 , 2D NMR, 7, 130

T_2 , relaxation time constant
 definition, 25
 measuring, 25–27

Temperature calibration, 91–93

TentaGel resin, 363

Tests, spectrometer, 93–97
 gradient linearity, 91

Tetramethylsilane
 shift reference, 61
 diffusion reference, 320

Three-dimensional NMR
 DOSY, 331–333
 schematic sequence, 133

Three-spin effects, NOE, 258–260

Tilting
 of J-resolved spectra, 240
 within COSY crosspeaks, 165

Time domain, 19
 extension, linear prediction, 44
 extension, zero-filling, 43

Time profile
 of shaped pulses, 347

Time proportional phase incrementation
 2D quad detection, 139
 axial peaks, 143

TMS, *see* tetramethylsilane

TOCSY, 168–178
 1D, selective, 175
 1D, TOCSY-NOESY, 284–285
 applications, 171–173
 breakthrough in ROESY, 293
 gradient-selected, 174
 HMQC/HSQC, 204
 implementing, 174
 mixing schemes for, 343–345
 spin-locks, 169, 345
 ZQ suppression, 176–178

TOE, *see* truncated driven NOE

Top-hat profile, 346, 348

Total Correlation Spectroscopy, *see* TOCSY

TPPI, *see* time proportional phase incrementation

Transient NOE, 266–269, 276–286
 applications, examples, 286–290
 initial rate approximation, 267
 internuclear separations, 268, 281
 interpreting, comments, 285
 mixing time, 267, 277–279, 281
versus steady-state NOE, 276

Transition probabilities, 250

Transitions
 directly connected, 165
 remotely connected, 165

Transmitter
 attenuation, 84
 radiofrequency, 35

Transverse magnetisation
 observable, 15–16
 loss of, 24–25

Transverse spin relaxation, 24–28

Trim pulses, 174

Triple-quantum filter, 165

Triple-resonance methods, 226–230

Trityl radicals, for DNP, 366

Tr-ROESY, 294

Truncated driven NOE, 267

Truncation artefacts, 43
 sinc wiggles, 44, 147
 suppressing, 44, 55–58

TSP, *see* 3-(trimethylsilyl)propionate

Tubes, 62–64
 cleaning, 64
 micro, 62
 sapphire, 33
 sealing, 65
 susceptibility matched, 63

Tumbling rates
 in solution, 29–34, 250–255
 correlation time, 29, 254

Tuning
 probehead, 73–75

Two-dimensional NMR
 introduction, to NMR, 2
 introduction to, 130–133
 practical aspects, 138–148
 schematic sequence, 130

U

Ultrasonic mixing, 354

Universal pulse, shaped, 349

V

Vector model, 13–19

Vibrations, floor, 37

Vicinal couplings
 in COSY, 136, 165

Volume integrals, 2D, 281

Volumes
 NMR samples, 62–63

W

WALTZ-16, 174, 343–344

Water
 suppression, 354–359
 temperature dependence, 354

WATERGATE, 357

WATR, 28

Window functions, 55–58
 2D NMR, 141–142, 147–148

WURST, 340

X

XCORFE, 226

X-Y heteronuclear correlations, 226–230
 ^1H detected, 228

Z

Zanger-Sterk element, 243

Zero-crossing, NOE, 253–254, 269–270

Zero-filling, 43–44, 147

Zero-quantum dephasing, 360–362
 in NOESY, 280, 284
 in TOCSY, 176
 in TOCSY-NOESY, 285

z-filtration, 174, 359
 NOESY, 279

α -pinene
 ^{13}C spectra, 109
 Proton T_1 s, 23

β -peptides
 ROESY, 295–296

γ , *see* magnetogyric ratio

τ_m , *see* mixing time

τ_{null}
 inversion recovery, 24

This page intentionally left blank