New methods in mixture analysis

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List of abbreviations and symbols

1D: One-dimensional
2D: Two-dimensional
ALS: Alternating Least Squares
$B_0$: static magnetic field
$B_1$: radiofrequency field
BSA: Bovine Serum Albumin
BPPLED: Bipolar Pulse Pair Stimulated Echo with Longitudinal-Eddy-current Delay
BPPSTE: Bipolar Pulse Pair Stimulated Echo
CHOCO: Chromatographically-Ordered Correlation NMR
CORCONDIA: CORe CONsistency DIAgnostic
CORE: COmponent RESolved NMR
COSY: Correlation SpectroscopY
CSA: Chemical Shift Anisotropy
CTP: Coherence Transfer Pathway
CW: Continuous Wave
$D$: apparent Diffusion coefficient
DD: Dipole-Dipole interaction
DECRA: Direct Exponential Curve Resolution Algorithm
DEPT: Distortionless Enhancement by Polarization Transfer
DEPTSE: Distortionless Enhancement by Polarization Transfer Spin Echo
DMSO: Dimethyl sulfoxide
DOSY: Diffusion-Ordered SpectroscopY
DRONE: Decaying Relaxation ONEshot
DRONE45: Decaying Relaxation ONEshot45
EPR: Electron Paramagnetic Resonance
EVD: EigenValue Decomposition
$f$: friction factor  
FID: Free Induction Decay  
FT: Fourier Transformation  
$h$: Planck’s constant divided by $2\pi$  
HETP: Height equivalent to a theoretical plate  
HMQC: Heteronuclear Multiple Quantum Coherence  
HR-DOSY: High Resolution Diffusion-Ordered Spectroscopy  
$I$: spin operator  
$I$: observable of the spin operator  
INEPT: Insensitive Nuclei Enhanced by Polarization Transfer  
INEPTSE: Insensitive Nuclei Enhanced by Polarization Transfer Spin Echo  
IR: InfraRed (spectroscopy)  
IRON: Inversion Recovery ONeshot  
$J$: Coupling constant  
$k_B$: Boltzmann constant  
LC: Liquid Chromatography  
LED: Longitudinal Eddy Delay  
$M$: net magnetization  
MAD: Matrix-Assisted Diffusion-ordered spectroscopy  
MCR: Multivariate Curve Resolution  
NMR: Nuclear Magnetic Resonance  
$N$: number of spins  
NIPALS: Nonlinear Iterative Partial Least Squares  
NOE: Nuclear Overhauser Effect  
$p$: coherence order  
$P$: intrinsic angular momentum  
PARAFAC: PARAllel FACtor analysis  
PARALIND: PARAllel profiles with LINear Dependences
PCA: Principal Component Analysis
PEEK: PolyEther Ether Ketone
PFG: Pulsed Field Gradient
PFGSE: Pulsed Field Gradient Spin Echo
PFGSTE: Pulsed Field Gradient STimulated Echo
PTFE: PolyTetraFluoroEthylene
Q: Quadrupolar
\( r_H \): hydrodynamic radius
RF: RadioFrequency
ROLSY: Relaxation Ordered Liquid Spectroscopy
\( s, S \): signal detected
\( s_{\text{ref}} \): signal of the reference peak
\( s_{\text{ref,ideal}} \): ideal shape of the reference signal
\( s_{\text{ideal}} \): ideal shape of the signal detected
SARON: SAuration Recovery Oneshot
SCORE: Speedy Component Resolved NMR
SNR (S/N): Signal-to-Noise Ratio
SR: Spin Rotation
SVD: Singular Value Decomposition
\( t, \tau \): evolution time
\( T \): Temperature
\( T_1 \): longitudinal relaxation time constant
\( T_2 \): transverse relaxation time constant
TMS: Tetramethylsilane
TOCSY: TOTal Correlation Spectroscopy
TSP: 3-trimethylsilylpropionic acid sodium salt
\( u \): velocity
\( u_s \): superficial velocity
UV-Vis: UltraViolet-Visible (spectroscopy)

VT: Variable Temperature

δ: chemical shift or duration of encoding/decoding pulse field gradient

Δ: diffusion time

Δ': diffusion time corrected for effect of finite gradient pulse duration

ε: porosity

γ: magnetogyratic ratio

η: viscosity

ν: resonance frequency

ν₀: Larmor frequency

ν_{ref}: reference frequency

ω₀: Larmor angular velocity

Ω: offset

μ: magnetic moment or magnetic dipole

φ: phase of the magnetization or of a radiofrequency pulse

φₚ: phase of the receiver

φₚ: phaseshift of a coherence

Φ: angle between the radiofrequency pulse \( B_1 \) and the receiver phase (assumed to be on the x axis)

Ψ: sphericity
Abstract

The quest for a complete understanding of mixtures is a challenge which has stimulated the development of several techniques. One of the most powerful NMR-based techniques is known as Diffusion-Ordered Spectroscopy (DOSY), in which it is possible to distinguish the NMR spectra of chemical species with different hydrodynamic radii, i.e. with different self-diffusion coefficients. It allows the study of intact mixtures, providing information on the interactions within the mixture and saving time and money compared to other techniques. Unfortunately, DOSY is not very effective when signals overlap and/or the diffusion coefficients are very similar. This drawback has led to the development of new methods to overcome this problem. The present investigation is focused on developing some of these.

Most DOSY datasets show multiplet phase distortions caused by J-modulation. These distortions not only hinder the interpretation of spectra, but also increase the overlap between signals. The addition of a 45º purging pulse immediately before the onset of acquisition is proposed as a way to remove the unwanted distortions.

Most DOSY experiments use 1H detection, because of the higher sensitivity which is generally achieved. However, acquiring spectra with other nuclei such as 13C can reduce overlap problems. Two new sequences have been developed to maximize the sensitivity of heteronuclear DOSY experiments.

In order to increase resolving power, it is also possible to incorporate another variable into diffusion experiments as a further dimension. If this results in an approximately trilinear dataset (each dimension varying independently), it is possible to extract physically meaningful information for each component using multivariate statistical methods. This is explored for the cases where the new variable is either the relaxation behaviour or the concentration variation (which can be measured during a reaction or in a set of samples with different concentrations for each component). PARAllel FACtor (PARAFAC) analysis can obtain the spectra, diffusional decay and relaxation evolution or kinetics for each of the components.

In a completely different approach, the separation power of liquid chromatography has been combined in a novel way with the NMR potential for elucidating structures. NMR has been used previously as a precise way to measure average flow velocities, even in porous media. Using this capability to detect the different average velocities of solutes that occur in chromatographic columns ought to provide a new way of analysing mixtures with the same potential as LC-NMR, but faster and more simple. In such a flow system, a chromatographic column is introduced into the NMR probe and a 2D dataset is acquired and Fourier transformed to obtain the velocity distribution for each of the detected NMR signals.
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Chapter 1

Introduction

*Dubium sapientiae initium*

(Doubt is the origin of wisdom)

René Descartes
1. Introduction

Human beings have always been full of doubts and questions: where can I find food? how can I cure this pain? how did life begin? what are the lottery winning numbers? Over time we were able to discover some answers from mere observation and deduction; but with the answers also came more questions. We constructed tools to evaluate hypotheses and obtain a deeper understanding of our surroundings to answer our questions, and each time we developed more and more sophisticated tools. Nowadays we are at a stage in which we are developing instruments to find out the physical properties and chemical structure of both substances that surround us, and substances that we create. Increasing our wisdom in this field can allow us to find new chemical species, understand interactions between molecules, develop new medicines, optimize industrial processes…; and in the end, what all this means, is that this knowledge can be used to improve our quality of life.

Nuclear Magnetic Resonance (NMR) spectroscopy is one of these tools and is, if not the most, one of the most powerful to obtain structural chemical information.\textsuperscript{1} NMR spectroscopy studies radio-frequency-induced transitions between magnetic energy levels of atomic nuclei. In 1945 Bloch\textsuperscript{2} and Purcell\textsuperscript{3} established the basis of this spectroscopy with the discovery that magnetic nuclei could absorb energy in the radio-frequency band (resonate) when exposed to a magnetic field. The potential of NMR to elucidate chemical structures was not revealed until the late 1940s and early 1950s, when the chemical shift was discovered\textsuperscript{4}, i.e., that nuclei would resonate at different frequencies in different chemical environments.
After more than 60 years of progress\footnote{5}, this technique has become one of the most versatile methods for identifying chemical structures, allowing even the determination of the structures of complex proteins. However, it has a serious drawback: the analysis of mixtures by conventional NMR methods is very complex. There are several techniques used for mixture analysis by NMR (such as TOCSY\footnote{6,7}, multiple quantum spectroscopy\footnote{8} or statistical analysis of 1D or 2D spectra when a set of samples is available\footnote{9-16}), but the most powerful are probably Liquid Chromatography-NMR (LC-NMR)\footnote{17,18} and Diffusion-Ordered SpectroscopY (DOSY)\footnote{19-22}:

DOSY relies on the different diffusion properties (random motion) of compounds to resolve mixture spectra, rather than on the coherent motion studied in chromatography. It is performed on a standard NMR spectrometer using standard sample handling, with consequent savings in time, solvents and equipment, as compared to LC-NMR. Hahn showed in 1950\footnote{23} that NMR techniques could be used for the study of self-diffusion coefficients. In 1965 Stejskal and Tanner\footnote{24} applied the newly-developed gradient coil technology to improve the technique. This methodology was used by Stilbs in 1981 to study complex mixtures\footnote{25}, but DOSY itself did not appear until 1992, with the introduction by Morris and Johnson\footnote{26} of a data processing method that created a 2D plot correlating the spectrum of a sample with the diffusion coefficients of the NMR signals. The method is very powerful, but as was realised from the start, DOSY is unfortunately not very effective when signals overlap and/or the diffusion coefficients are very similar.\footnote{27,28} This drawback has led to the development of new methods; the present investigation is focused on further
developing some of these, as well as completely new, methods. The fundamental principles to understand this work are presented in Chapter 2.

Most DOSY datasets show multiplet phase distortions caused by $J$-modulation$^{29}$. These distortions not only hinder the interpretation of 1D spectra, they also increase the overlap between signals and may cause unexpected and misleading apparent diffusion coefficients in DOSY spectra. The methods commonly used to suppress such effects$^{30}$ are not entirely satisfactory. The addition of a 45° purging pulse immediately before the onset of acquisition is proposed as a way to remove the unwanted distortions. Chapter 3 studies the effects of $J$-modulation on DOSY spectra and the effect of this 45° pulse.

Most DOSY experiments use $^1$H detection because of the higher sensitivity which is generally achieved. However, acquiring spectra with other nuclei such as $^{13}$C will normally reduce overlap problems. Chapter 4 presents two new sequences that have been developed to maximize the sensitivity of heteronuclear DOSY experiments, and are compared with previous pulse sequences.

In order to increase resolving power in diffusion measurements by NMR, it is also possible to incorporate another variable as a further dimension to the chemical shift and the diffusion encoding. If each dimension varies independently, it is possible to extract physically meaningful information for each component using multivariate statistical methods. Chapter 5 explores the cases where the new variable is either relaxation behaviour
or concentration variation (measured during a reaction). The resulting spectra can be analysed with PARA llel FACtor analysis (PARAFAC)\textsuperscript{31,32} to obtain the spectra, diffusional decay and relaxation evolution or kinetics for each of the mixture components.

In a completely different approach to the analysis of mixtures, we propose a novel way to combine the separation power of liquid chromatography (LC) with the NMR potential of elucidating structures. In traditional LC-NMR, measurements are made directly on the eluent stream from a chromatography column. The different compounds in the mixture are forced through a stationary phase, which consists of packing material in a column, where they will be retained for a time dependent on the interactions between the mobile phase and the stationary phase. At the end of the column they generally elute one at a time and are analysed in an NMR spectrometer. Carried out for the first time in the late 1970s by Watanabe and Niki,\textsuperscript{33} this technique has been widely adopted for the analysis of complex mixtures due to its flexibility (different stationary and mobile phases, operation modes, combinations with other techniques\textsuperscript{34}) and its power to separate compounds. However, it is very time-consuming and expensive, both in solvent consumption and in instrumentation\textsuperscript{35}. NMR has previously been used as a precise way to measure average flow velocities, even in porous media.\textsuperscript{36,37} Using this capability to detect the different average velocities of solutes that occur in chromatographic columns ought to provide a new way of analysing mixtures with the same potential as LC-NMR, but more simple, because no additional software is required to coordinate LC and NMR,\textsuperscript{38} and faster, because all mixture components are measured at the same time, providing the Fellgett advantage.\textsuperscript{39} Chapter 6 illustrates this system, where a chromatographic column is introduced into the NMR probe.
and a 2D dataset is acquired and Fourier transformed to obtain the velocity distribution for each of the detected NMR signals.

Appendices include the 4 papers already published as a result of this work, and additional content supporting the chapters.
Chapter 2

Theory

Theoretical physicists live in a classical world, looking out into a quantum-mechanical world. The latter we describe only subjectively, in terms of procedures and results in our classical domain.

John Stewart Bell
2. Theory

NMR is a phenomenon that is explained in as many ways as the number of people that try to explain it. It has aspects both from quantum mechanics and from classical mechanics. The basics of NMR are explained by the classical Faraday law of induction: a changing magnetic field can induce an electric current, and this is what is detected in NMR. The problem is that the intensity of this electric current cannot be completely explained with classical mechanics, only with quantum mechanics. In this chapter both perspectives are introduced.

2.1. The NMR phenomenon

Many nuclei have a property called spin, which means that they have a nonzero intrinsic angular momentum. It is represented by the observable $\hat{h}I$ (or $\hat{P}$), where $\hbar$ is the Planck constant divided by $2\pi$. $I$ is the observable of the spin operator $\hat{I}$, for which projection onto a given direction $z$ gives quantized eigenvalues: 0 for a spin-0 particle, $+\frac{1}{2}$ and $-\frac{1}{2}$ for a spin-$\frac{1}{2}$ particle, and so on. Only nuclei with non-zero spin quantum number can be observed by NMR. Nuclei have a magnetic moment or magnetic dipole $\mu$, which is proportional to the spin angular momentum and originates from the positive charge and dynamic properties of its nucleus,

$$\mu = \gamma \hbar I$$  \hspace{1cm} [2.1]

with $\gamma$ being the magnetogyric ratio, specific to a given species of nucleus. However, the behaviour of a single spin is not normally studied by NMR, but rather the behaviour of an
ensemble of spins. The ensemble sum of all magnetic moments of the $N$ spins in the sample is the net magnetization $M$.

$$M = \sum_{i=1}^{N} \mu_i$$  \hspace{1cm} [2.2]

In the absence of an external magnetic field, the net magnetization at equilibrium is zero. Under the effect of a magnetic field $B_0$, aligned along the $z$-axis by convention, a bulk magnetization develops aligned with the field. This magnetization is the equilibrium magnetization of magnitude $M_0$. When the net magnetization $M$ is not collinear with $B_0$, $M$ rotates around the $z$-axis in a motion described, to a first approximation, by the torque equation [2.3]:

$$\frac{dM}{dt} = -\gamma B_0 \times M$$  \hspace{1cm} [2.3]

This may be expressed in terms of the Larmor angular velocity $\omega_0$ as in equation [2.4]:

$$\omega_0 = -\gamma |B_0|$$  \hspace{1cm} [2.4]

or in the form of the Larmor frequency:

$$v_0 = \frac{|\gamma|}{2\pi} |B_0|$$  \hspace{1cm} [2.5]

A radiofrequency (RF) field $B_1$, perpendicular to $B_0$ and with a frequency close to the Larmor frequency, is used to tip the magnetization away from the $z$-axis, as described by the equation

$$\frac{dM}{dt} = -\gamma (B_0 + B_1) \times M$$  \hspace{1cm} [2.6]
The field $B_1$ is several orders of magnitude smaller than $B_0$; in order to understand why it has a strong effect on magnetization it is studied in the rotating frame of reference. This reference system rotates around the $z$-axis at a frequency $\omega_{\text{rot}}$ equal to the frequency of the RF pulse, so the apparent frequency of the Larmor precession, or offset $\Omega$, is equal to $\omega_0 - \omega_{\text{rot}}$. When the offset is zero, the apparent magnetic field along the $z$-axis is also zero. This is why it is possible to tilt the magnetization with a small, close to resonance (i.e., with a frequency similar to the Larmor frequency) field.

After perturbation, the magnetization $M$ will precess around the $z$-axis while it progressively returns to its equilibrium state, collinear with $B_0$. This precession will induce a current in a coil which is the same used to generate the RF field $B_1$ and is located in the probe. This current is amplified, its frequency is “down-converted” (the signal is subtracted by a reference signal so that the frequency is reduced from hundreds of MHz to several kHz), and is then digitized as a Free Induction Decay (FID). It is then possible through a Fourier Transform (FT) of the FID to obtain a spectrum. This spectrum, in the form of peaks at the Larmor frequencies of the spins involved, contains information about the local electronic environment of the spins. The frequency of each spin is usually given in a dimensionless form called the chemical shift $\delta$:

$$\delta = 10^6 \frac{(\nu - \nu_{\text{ref}})}{\nu_{\text{ref}}}$$  \hspace{1cm} [2.7]

where $\nu_{\text{ref}}$ is the frequency of a resonance from a reference compound, e.g. tetramethylsilane (TMS) resonance, a typical reference in $^1\text{H}$ spectra.
An initial approximation for the magnetization behaviour was introduced by Bloch in 1946; it describes the variation of the 3 Cartesian components of the magnetization ($M_x$, $M_y$ and $M_z$) due to the effects of $B_1$, $B_0$ and relaxation:

\[
\frac{dM_x}{dt} = \gamma |B_1| M_z \sin \Phi - \gamma |B_1| M_y \cos \Phi - \frac{(M_z - M_0)}{T_1}
\]
\[
\frac{dM_y}{dt} = -\Omega M_y - \gamma |B_1| M_z \sin \Phi - \frac{M_z}{T_2}
\]
\[
\frac{dM_z}{dt} = \Omega M_z + \gamma |B_1| M_y \cos \Phi - \frac{M_y}{T_2}
\]

where $\Phi$ is the angle between $B_1$ and the $x$ axis (phase of the receiver), $T_1$ and $T_2$ are time constants characterizing longitudinal and transverse relaxation respectively (see section 2.7). This classical description is good enough to describe the behaviour of isolated spin systems, but not to describe spins that are interacting with other spins.

### 2.2. Spin-spin coupling

Interactions between spins are called spin-spin couplings, and can be divided into two categories: direct and indirect dipole-dipole couplings. The direct coupling is generated by the interaction through space of nuclear spin magnetic fields. The magnitude of this coupling depends on the relative orientation of the spins involved and the static field $B_0$. In an isotropic liquid, molecules tumble rapidly and this effect does not produce any splitting in the spectrum. Indirect spin-spin coupling (scalar coupling or $J$-coupling) is the result of interactions mediated by electrons. This coupling is not averaged to zero in liquids, because the electrons change the orientation dependence of the interaction. The signals belonging to each spin will thus be split according to the possible energy eigenstates each neighbouring
spin can adopt, e.g. the signal belonging to a spin-$\frac{1}{2}$ nucleus will, in the simplest case, be split in two signals when coupled to another spin-$\frac{1}{2}$ nucleus; the magnitude of this separation is the coupling constant $J$, which is independent of the magnetic field. The resulting multiplet patterns provide valuable information on the chemical bonds. When $J$ is much smaller than the difference in chemical shift between the coupled spins, the coupling is known as weak coupling; when this is not the case the coupling is strong and the resulting multiplet typically has a more complicated pattern of lines. $J$-coupling is responsible for an effect called $J$-modulation, where the lineshapes of multiplets are distorted when the magnetization is allowed to precess before acquisition; $J$-modulation is further described in the chapter 3.1 of this thesis.

2.3. Product operator formalism

The Bloch equations provide a description of NMR from a classical perspective. Unfortunately, they do not describe what happens when there are spin-spin couplings. Quantum mechanics provides rigorous tools to study NMR, such as density matrix theory. However, these tools do not provide an intuitive understanding of the underlying physics of NMR experiments. Another method called the product operator formalism\textsuperscript{41} has been developed as a simplification of density matrix calculation valid for weakly coupled spin systems. This is an algebraic description of NMR that uses operators with physical meaning, in which the effects of pulses and delays can be understood as geometrical rotations. For a single spin, the operators are the Cartesian components of the spin angular momentum $\hat{L}_x$, $\hat{L}_y$, $\hat{L}_z$ and the unity operator $\hat{I}_x$. For any number of spins, the operators
needed are all possible combinations of the 4 basic operators, i.e., $4^N$ where $N$ is the number of coupled spins.

For the case of a system with two coupled spins $I$ and $S$ the operators would be $\hat{E}_x, \hat{I}_x, \hat{I}_y, \hat{I}_z, \hat{S}_x, \hat{S}_y, \hat{S}_z, \hat{I}_x\hat{S}_x, \hat{I}_y\hat{S}_y, \hat{I}_z\hat{S}_z, \hat{I}_x\hat{S}_y, \hat{I}_y\hat{S}_x, \hat{I}_x\hat{S}_z, \hat{I}_y\hat{S}_z, \hat{I}_z\hat{S}_y, \hat{I}_z\hat{S}_x$. $\hat{I}_x$ and $\hat{S}_x$ represent the $z$-magnetization operators. $\hat{I}_x, \hat{I}_y, \hat{S}_x$ and $\hat{S}_y$ represent in-phase magnetization, where the lines in a multiplet have the same sign and lineshape. $\hat{I}_x\hat{S}_z, \hat{I}_y\hat{S}_z, \hat{I}_z\hat{S}_x$ and $\hat{I}_z\hat{S}_y$ represent anti-phase magnetization, where the lines in a multiplet have opposite phases (see sections 2.4.1 and 3.1). $\hat{I}_x\hat{S}_y, \hat{I}_y\hat{S}_x, \hat{I}_z\hat{S}_x$ and $\hat{I}_z\hat{S}_y$ are mixtures of so-called zero quantum and double quantum coherences, which are not directly detectable. $\hat{I}_x\hat{S}_z$ represents anti-phase $z$-magnetization for both spins. The order of coherence determines the effect of rotation about the $z$-axis on the coherence, so single quantum coherences rotate at the Larmor frequency of a spin, double quantum coherences rotate at the sum of Larmor frequencies of the coupled spins, and zero quantum coherences rotate at the difference between the Larmor frequencies of the coupled spins. Only single quantum coherences are directly detectable by NMR. More information about coherences is given in section 2.6.

Product operators are rotated using the right-hand rule in a diagram like that shown in Figure 2.1. For example, a 90º pulse about the $y$ axis would transform $z$-magnetization into magnetization on the $x$ axis, which using this notation would be represented as:

$$\hat{I}_z \xrightarrow{(\pi/2)\hat{I}_y} \hat{I}_x$$
During a delay $\tau$ product operators are rotated by the offset operator $\Omega \tau \hat{I}_z$. For uncoupled spins, this free precession can be expressed as:

$$\hat{I}_x \xrightarrow{\Omega \tau \hat{I}_z} \hat{I}_x \cos \Omega \tau + \hat{I}_y \sin \Omega \tau$$

For coupled spins, the evolution would be:

$$\hat{I}_x \xrightarrow{\Omega \tau \hat{I}_z, \Omega \tau \hat{S}_z} \hat{I}_x \cos \Omega \tau \cos \pi \tau J_{IS} + 2 \hat{I}_y \hat{S}_x \sin \Omega \tau \sin \pi \tau J_{IS} + \hat{I}_y \sin \Omega \tau \cos \pi \tau J_{IS} - 2 \hat{I}_x \hat{S}_z \sin \Omega \tau \sin \pi \tau J_{IS}$$

This interconversion between in-phase and anti-phase terms is known as $J$-modulation. This is a phenomenon used in several experiments, particularly in 2D FT NMR, but it is often an inconvenience when it results in spectra difficult to interpret due to the presence of peaks with different phases (see next section).

### 2.4. Data processing techniques

Data acquired from NMR spectrometers normally comes in the form of an FID. This is not directly interpretable; it is first necessary to Fourier transform it (and in the case of some manufacturers, deal with digital filtering so that the FID starts at time zero\(^{42,43}\)) in order to obtain a spectrum.
2.4.1. Fourier transform

In NMR, the signal detected \( s(t) \) can be expressed as a result of the \( x \) and \( y \) components of the magnetization that, assuming that there is an initial magnetization \( M_0 \) along the \( x \)-axis after being rotated by an RF pulse, can be represented for a single resonance as:

\[
 s(t) = \alpha \left( M_x(t) + iM_y(t) \right) \tag{2.9}
\]

where \( \alpha \) is a factor of proportionality that depends on the instrument, and \( M_x \) and \( M_y \) are given by

\[
 M_x(t) = M_0 \cos \Omega t \, e^{-\gamma t/T_2} \tag{2.10}
\]

\[
 M_y(t) = M_0 \sin \Omega t \, e^{-\gamma t/T_2}
\]

Using the Euler relationship it may be represented as:

\[
 s(t) = \alpha e^{\alpha t} \, e^{-\gamma t/T_2} \tag{2.11}
\]

This signal is known as the Free Induction Decay (FID). Fourier transformation of this signal gives a complex spectrum, but for practical purposes, only the real part is typically used. Taking into account the phase difference between the magnetization and the receiver, the actual FID would be:

\[
 s(t) = \alpha e^{\alpha t} \, e^{-\gamma t/T_2} \, e^{i\Phi} \tag{2.12}
\]

where \( \Phi \) is the angle between the phase of the magnetization (direction in which the magnetization is initially oriented) and the receiver phase (phase of a reference signal in the spectrometer). When the phase difference is zero, the real part of the spectrum will have peaks with phase-dependent lineshapes, as pictured in Figure 2.2. The imaginary part has a phase difference of 90º in relation to the real part. Multiplying the FID by a phase factor in order to obtain absorption lineshapes in the real part of the spectrum is known as phasing.
Figure 2.2: Lineshape dependence of the real part of the spectrum on difference between receiver phase and receiver magnetization. a) $\Phi=0^\circ$, positive absorption lineshape, b) $\Phi=90^\circ$, dispersive lineshape, c) $\Phi=180^\circ$, negative absorption lineshape, d) $\Phi=270^\circ$, dispersive lineshape

The algorithm normally used for Fourier transformation is the Fast Fourier Transform (FFT), a very efficient algorithm but, as a form of the standard Discrete Fourier Transform (DFT), it has some limitations. DFT requires that the data points in the FID have to be evenly spaced (it needs to fulfil the Nyquist theorem, i.e., sampling frequency must be at least twice as high as the highest frequency expected in the signal). In principle, this is only a small inconvenience for 1D spectra, but in multidimensional Fourier transformed experiments this limits the achievable resolution in a reasonable time. If for example, a 1D spectrum takes 1 min to acquire with a few scans (SNR of the spectrum increases as a function of the square root of the FIDs acquired) and 256 data points sampled in additional dimension would provide sufficient resolution, a 2D spectrum would require more than 4 hours, and a 3D experiment more than 1090 hours, which is already a prohibitive experimental time. Some processing alternatives developed to enhance resolution are multidimensional one-step Discrete Fourier Transform (also known as Multidimensional Fourier Transform), back-projection reconstruction, multiway decomposition,
maximum entropy method,\textsuperscript{44,50,51} filter diagonalization method, covariance spectroscopy\textsuperscript{52} and Hadamard spectroscopy\textsuperscript{53,54}. All of these processing schemes have inherent disadvantages, increased processing time being common to all of them. Some alternatives to reduce acquisition time are exponential sampling,\textsuperscript{55} radial sampling\textsuperscript{56,57} and random sampling\textsuperscript{44,58-60}. None of these sampling alternatives can provide reliable results without high SNR.\textsuperscript{61}

2.4.2. Resolution and sensitivity

Both sensitivity and resolution are major concerns in NMR development. NMR is one of the less sensitive spectroscopic methods; it looks for changes in the orientation of a net magnetization in an ensemble of spins that corresponds to tiny differences in the populations of spin states, of the order of 1 in 10\textsuperscript{6} (see section 2.7).\textsuperscript{62} The SNR is typically defined as the signal height divided by twice the root mean square noise.\textsuperscript{63} It typically depends on a number of parameters, some of them given by equation [2.13]:

\[
    SNR \propto NAT_{i}^{-1}B_{0}^{1/2}\gamma^{5/2}T_{2}^{-1}\frac{1}{2}(NS)^{1/2}
\]

where \(N\) is the number of spins in the observed sample volume, \(A\) is a term that represents the natural abundance of the nuclide, \(T_{i}\) is the temperature of the sample and surrounding RF coil, \(T_{2}\) is the effective transverse relaxation time and \(NS\) is the total number of averaged scans. The SNR is typically proportional to \(\gamma^{5/2}\) due to factors for higher resonant frequency (\(\gamma^{1}\)), larger Boltzmann population difference (\(\gamma^{1}\)), stronger magnetic moment (\(\gamma^{1}\)), and noise increases with the square root of the resonance frequency (\(\gamma^{1/2}\)).\textsuperscript{64} SNR is dependent also on the distance from the coil to the sample, which has led to the use of
microcoils to achieve a better filling factor when there is a limited amount of material.\textsuperscript{65-68}

The electrical resistance of the sample and the RF coil (and also other electrical losses in the probe) contribute to the noise, and in order to reduce this resistance it is possible to use cryoprobes, where the RF coil is kept at a very low temperature (typically around 25 K); this allows for an improvement in SNR of the order of a factor of 5.

Resolution is a spectral quality relating to the width of the spectral lines and the degree of overlap between them. Higher resolution can be typically achieved with higher $B_0$ homogeneity (less variation in the Larmor frequency of the spins), slow relaxation, detecting nuclei with fewer or better disperse resonances (such as $^{13}$C instead of $^1$H), or suppressing multiplet structure (for example, by irradiating coupling partners in what is known as decoupling). Just as sensitivity is fundamental in order to discover resonances in a spectrum, resolution is essential in order to distinguish them chemically. It is possible to improve resolution or SNR by multiplying the FID by time-dependent function.

The resolution of a signal in an NMR spectrum is determined by how fast it decays in the FID, e.g., a rapid decay results in a wide resonance. In the tail of an FID, the noise has a higher relative contribution to the signal than at the beginning. Because of this, it is common to multiply the FID by a time-dependent function (weighting function) in order to change the relative contribution of each time point, altering both the SNR and the resolution of the spectrum. If the FID is multiplied by an exponential decay, both the relative contribution of the noise and the resolution will be decreased. The optimal balance to achieve the best SNR without excessive line broadening can be obtained by multiplying
the FID by an exponential decay that decays in the same way as the FID, i.e., $e^{-t/T_2}$, thus the spectral linewidth will be doubled. This kind of a weighting function is known as a matched filter. To improve resolution, the FID should be multiplied by a function that lowers the contribution of the beginning of the FID, so the signal decay is slower. This is typically done with a combination of a Gaussian function and a rising exponential.

2.4.3. Lineshape correction

Lineshapes obtained by Fourier transform of equation [2.11] are known as Lorentzian and are described by:

$$s(\Omega) = \frac{1}{T_2 \left( \frac{1}{T_2} \right)^2 + (\Omega - \Omega_c)^2} \cdot i \frac{\Omega - \Omega_c}{\left( \frac{1}{T_2} \right)^2 + (\Omega - \Omega_c)^2}$$

[2.14]

This lineshape is theoretical, and there are many reasons why an experimental spectrum may not have this kind of lineshape. The main cause is magnetic field ($B_0$) inhomogeneity. It is possible to reduce field inhomogeneity by shimming;\textsuperscript{69-71} this consists in modifying the electrical currents that pass through some gradient or shim coils so that the field shape can be modified. Even though NMR experiments are normally carried out in very stable superconducting magnets, the field tends to drift over time and so will the Larmor frequency. In order to compensate for this variation, spectrometers include a channel that monitors the frequency of the signal of a second nucleus and compensates for frequency variations. This is called the field-frequency lock and is normally carried out using $^2$H, which is why it is preferable for experiments to use deuterated solvents.
Although spectrometers are designed to minimize sources of spectral variations such as frequency, lineshape or phase changes, not all instabilities can be neutralized. Reference deconvolution\textsuperscript{72-74} is a method that can minimize lineshape and/or frequency variations if all peaks in a given spectrum are affected in the same way (frequency variations due to temperature or pH changes can affect different peaks to different extents, so this method can only partially correct these variations). This is achieved by comparing the ideal shape of a reference peak $s_{\text{ref,ideal}}(\Omega)$ with the experimental one $s_{\text{ref}}(\Omega)$, computing a correction function $g(\Omega)$, and applying it to the full experimental spectrum $s(\Omega)$. Mathematically,

$$s_{\text{ref}}(\Omega) = s_{\text{ref,ideal}}(\Omega) \ast g(\Omega)$$

or in the time domain:

$$s_{\text{ref}}(t) = s_{\text{ref,ideal}}(t) \ast g(t)$$

So the ideal spectrum can be calculated as:

$$s_{\text{ideal}}(\Omega) = \mathcal{F}T \left\{ \frac{s(t) \cdot s_{\text{ref,ideal}}(t)}{s_{\text{ref}}(t)} \right\}$$

It may not be always possible to obtain a well-resolved reference peak, so other methods have appeared to try to improve data quality. One of them is Global Spectral Deconvolution, which tries to fit all peaks to a master lineshape function, e.g., to a Lorentzian lineshape with additional parameters that take into account common lineshape distortions.\textsuperscript{75,76} Other advances have been done on individual peak fitting,\textsuperscript{77} but this is a technique likely to fail with severe overlap.\textsuperscript{78} Other techniques to improve lineshapes are based on peak alignment,\textsuperscript{79,80} and PCA and linear regression for phase and frequency
variations. Obtaining undistorted lineshapes is especially important for multivariate analysis, otherwise the results will suffer from these distortions, but it is also important for obtaining the best results when a spectrum is screened to match the spectra of reference compounds. Further information on these effects is given in chapter 5.

2.5. Pulsed Field Gradients (PFGs)

PFGs are pulses that result in a variation of the magnetic field across the sample and are generated by a gradient coil, a standard component in modern spectrometers. The Larmor angular velocity of spins under the presence of a gradient will be:

\[ \omega_0 = -\gamma |B_0| - \gamma Gz \]  \[2.18\]

Thus, after a gradient pulse of amplitude \( G \) and duration \( \delta \), the phase change of the magnetization will be given by:

\[ \varphi = -p\gamma \delta Gz \]  \[2.19\]

It is proportional to the order of coherence because the phase acquired as a result of a \( z \)-rotation of a coherence of \( p \) order through an angle \( \varphi \) is \( p\varphi \). Gradients dephase coherences in the plane perpendicular to the direction in which the gradient is applied. This dephasing is performed in a reproducible way and, thus, can be refocused by applying an opposite gradient. This is a property used in diffusion measurements (section 2.8.1) and in coherence selection (section 2.6), where it allows cleaner results in one scan. PFGs are generally designed to cause a linear variation of the \( B_0 \) field along the gradient axis (the \( z \)-axis for most experiments), but in practice, gradients are not perfectly linear. This nonlinearity has
to be taken into account to obtain precise results when gradient characterization is required, such as in measurement of diffusion coefficients.84

Another aspect to be taken into account when gradients are implemented in pulse sequences is that they generate eddy currents. These are electrical current in the conducting parts of the probe and the magnet induced by a sudden change in magnetic field. These currents can be greatly reduced by an outer coil (shield) that generates a field outside the inner coil opposite to that generated by the latter. It is not possible to cancel the external field perfectly, so usually after PFGs, pulse sequences use a stabilization delay to let these currents dissipate.

2.6. Coherence selection

It has been explained in section 2.3 that product operators represent different types of coherences. Coherences are an average relationship between the phases of wavefunctions. They can be represented by product operators; in terms of energy diagrams they correspond to transitions and, thus, they are ultimately what define NMR spectra. The order of coherence in an $N$ spin system can be any integer from $-N$ to $+N$. The orders of coherence represented by a product operator are determined taking into account that $\hat{I}_{z}$ (raising operator), $\hat{I}_{+}$ (lowering operator) and $\hat{I}_{z}$ have +1, -1 and 0 coherence orders respectively, and that the following relationships exist:

\[ \hat{I}_{+} = \frac{1}{2}(\hat{I}_{z} + \hat{I}_{-}) \]

\[ \hat{I}_{-} = \frac{1}{2i}(\hat{I}_{z} - \hat{I}_{-}) \]

[2.20]
Therefore, $\hat{I}_x$ and $\hat{I}_y$ have equal mixtures of $+1$ and $-1$ coherence order, known as single-quantum coherence.

For a spin system at thermal equilibrium the order of coherence is zero. Detectable magnetization has an order of coherence of $-1$ by convention (it could have been $+1$, but only one of these coherences can be detected). Each time an RF pulse is applied, different orders of coherences can be generated. The way coherences convert into other coherences in a pulse sequence is called the coherence transfer pathway (CTP), and can be represented in a diagram like the example shown in Figure 2.3. In most sequences, only one or a few CTPs are of interest and the other pathways can result in unwanted responses in the spectrum. For example, if a desired CTP has coherences of $+2$ or $-2$ at some point, the final spectrum should only show resonances from coupled nuclei, otherwise it means unwanted CTPs have survived. It is possible to remove unwanted CTPs either by phase cycling or by selection of pathways with pulsed field gradients.

![Figure 2.3: Example of the coherence transfer pathway diagram for a spin echo pulse sequence. The pulse with phase $\varphi_1$ rotates $z$ magnetization by $90^\circ$, generating $+1$ and $-1$ coherences, but only $+1$ coherences are selected in this case. The pulse with phase $\varphi_2$ rotates magnetization by $180^\circ$, selecting a coherence order change of $-2$. Magnetization is acquired with a receiver phase $\varphi_R$.](image-url)
In phase cycling the phases of the pulses and the receiver are varied in a systematic way to add up signals from the desired pathways and cancel out signals from all other pathways. This requires repeating an experiment a number of times to select the wanted pathway. When a pulse with phase $\varphi_1$ is applied, a pathway with an order of coherence difference $\Delta p$ experiences a phase shift $\varphi_{1p} = -\Delta p \varphi_1$. If this is the only pulse, and the phase of the receiver is kept equal to $\varphi_{1p}$ as the phase of the pulse $\varphi_1$ is varied, the wanted pathways add up.

For the spin echo pictured in Figure 2.3, it is possible to select the wanted coherence with a phase cycling of 4 steps, although 16 steps would allow for instrument non-ideality (to compensate for phase variations between the RF pulses and the receiver). This can be done altering the phase of each of the pulses through each of the 4 different phases (typically phase cycling has been done using 4 phases multiple of 90°, but it is possible to use any phase). In phase cycling, it is common to notate the phases as multiples of 90° (0 = 0°, 1 = 90°, 2 = 180°, 3 = 270°), with subscripts denoting repetition. In the first pulse $\Delta p$ is equal to +1 and in the second pulse $\Delta p$ is equal to -2. The phase of the receiver $\varphi_R$ should be set to the sum of the phase shift of both pulses, i.e, $\varphi_{1p}$ and $\varphi_{2p}$ (and normally expressed as a positive number between 0 and 3). Table 2.1 shows the full phase cycling in the extended form and Table 2.2 in a more commonly used form. Table 2.3 shows the minimum phase cycling.
Table 2.1: Phase cycling for the spin echo pictured in Figure 2.3 showing each individual pulse phase and the effect on the phase of the desired coherence pathway. Phases are notated as multiples of 90° (0 = 0°, 1 = 90°, 2 = 180°, 3 = 270°).

<table>
<thead>
<tr>
<th>Step</th>
<th>$\phi_1$</th>
<th>$\phi_{1p}$</th>
<th>$\phi_2$</th>
<th>$\phi_{2p}$</th>
<th>$\phi_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
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<td>0</td>
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<td>2</td>
</tr>
<tr>
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<td>3</td>
<td>-3</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
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<td>2</td>
</tr>
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<td>2</td>
<td>1</td>
</tr>
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<td>-2</td>
<td>1</td>
<td>2</td>
<td>0</td>
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</tr>
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<td>-3</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
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<td>2</td>
<td>2</td>
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</tr>
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<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>-3</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.2: Full phase cycling for the spin echo pictured in Figure 2.3 in the compact format. Phases are notated as multiples of 90° (0 = 0°, 1 = 90°, 2 = 180°, 3 = 270°), with subscripts denoting repetition.

<table>
<thead>
<tr>
<th>$\phi_1$</th>
<th>0123</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi_2$</td>
<td>$0_41_22_33_4$</td>
</tr>
<tr>
<td>$\phi_R$</td>
<td>$0321+0_42_4$ ($\phi_1-2\phi_2$)</td>
</tr>
</tbody>
</table>

Table 2.3: Minimum phase cycling for the spin echo pictured in Figure 2.3 in the compact format. Phases are notated as multiples of 90° (0 = 0°, 1 = 90°, 2 = 180°, 3 = 270°), with subscripts denoting repetition.

<table>
<thead>
<tr>
<th>$\phi_1$</th>
<th>0123</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi_R$</td>
<td>0321</td>
</tr>
</tbody>
</table>
Pulsed field gradients are, as described in the previous section, short periods during which the applied magnetic field is made dependent on position (dependent on $z$ for all experiments found in this thesis). As a result, coherences dephase as explained in equation [2.19] and are apparently lost, but it is possible to restore them by the application of another gradient pulse. Unlike phase cycling, this method does not require repetition of the experiment. By applying a gradient after an RF pulse, it is possible to refocus the phase distribution caused by a gradient applied before the pulse for specific coherence orders. Thus, using the subscript 2 to refer to the gradient applied after the RF pulse, the condition for selecting a coherence order change from $p_1$ to $p_2$ is:

$$\varphi_1(z) + \varphi_2(z) = 0$$
$$- p_1 \gamma G_1 z \delta_1 - p_2 \gamma G_2 z \delta_2 = 0$$
$$\frac{G_1 \delta_1}{G_2 \delta_2} = - \frac{p_2}{p_1}$$

This means that there will be always two pathways selected. For example, two gradients must be equal to select coherence order change from +1 to -1 (this would typically be the wanted pathway for the 180º pulse of a spin echo), but this will also select the pathway from -1 to +1.

### 2.7. Relaxation

At thermal equilibrium, there are no coherences and populations follow the Boltzmann distribution, where the population of the lower energy state is higher than that of the higher energy state. The population or fractional occupation $\rho_{mm}$ is the probability of finding a spin in the eigenstate $m$ ($-\frac{1}{2}$ or $+\frac{1}{2}$ for spin-$\frac{1}{2}$ nuclei) from all the $j$ possible eigenstates:
where \( k_B \) is the Boltzmann constant and \( T \) is the temperature. The net magnetization in equilibrium is aligned with the static field \( B_0 \) and has a magnitude defined by the Curie’s law:

\[
M_0 = \frac{N\gamma^2\hbar^2 I (I + 1) B_0}{3k_B T}
\]  

where \( N \) is the number of spins. The restitution of populations or \( z \)-magnetization to the equilibrium state \( M_0 \) is called longitudinal relaxation (spin-lattice relaxation), and it involves a loss of energy of the spins transferred to the surroundings in the form of heat. Simultaneously with longitudinal relaxation, the motion of the spins loses phase coherence (coherences decay), which implies that transverse magnetization \( M_{XY} \) returns to its equilibrium state in a process called transverse relaxation (spin-spin relaxation).

### 2.7.1. Longitudinal relaxation and its mechanisms

Longitudinal relaxation represents the movement of spin populations back to their Boltzmann distribution values. It is often assumed to be first order, and is characterized by a relaxation time \( T_1 \):

\[
\frac{dM_Z}{d\tau} = \frac{M_0 - M_Z}{T_1}
\]
where $\tau$ is the time for which the relaxation occurs. Integrating equation [2.24] shows that an exponential recovery is to be expected:

$$M_0 - M_Z(\tau) = [M_0 - M_Z(0)]e^{-\tau/T_1}$$

Equation [2.25] can be expressed in a more practical way for the case of recovery following a single pulse of flip angle $\alpha$, through which the magnetization has been rotated by the $B_1$ field:

$$M_Z(\tau) = M_0[1 - (1 - \cos \alpha)e^{-\tau/T_1}]$$

Longitudinal relaxation is sometimes explained as the coupling between the spins and the lattice (environment) via molecular motion. This (Brownian) motion creates small fluctuating fields that interact with nuclear magnetic dipoles. Several types of fluctuating fields contribute to relaxation:

$$\frac{1}{T_{1,\text{obs}}} = \sum_{\text{mechanism}} \frac{1}{T_{1,\text{mechanism}}} = \frac{1}{T_{1,\text{IDD}}} + \frac{1}{T_{1,\text{CSA}}} + \frac{1}{T_{1,\text{SR}}} + \frac{1}{T_{1,\text{para}}} + \frac{1}{T_{1,\text{Q}}} + \frac{1}{T_{1,\text{J}}} + \frac{1}{T_{1,\text{IDD}}}$$

$1/T_{1,\text{IDD}}$, $1/T_{1,\text{CSA}}$, $1/T_{1,\text{SR}}$, $1/T_{1,\text{para}}$, $1/T_{1,\text{Q}}$, $1/T_{1,\text{J}}$ and $1/T_{1,\text{IDD}}$ are the terms due to intramolecular dipole-dipole interactions, chemical shift anisotropy (CSA), spin rotation, paramagnetic interactions, quadrupolar interactions, scalar coupling and intermolecular dipole-dipole interactions respectively.

Dipole-dipole interactions couple a spin to other spins and to the environment (except in solids, where they do not cause longitudinal relaxation unless the interactions become time-
dependent as in liquids, due to internal motions with similar rates to Larmor frequencies\textsuperscript{87}. They may be direct, or more rarely indirect (through the involvement of electrons, also known as $J$-coupling), and are usually the largest influence on longitudinal relaxation in spin-$\frac{1}{2}$ nuclei. The direct dipole-dipole coupling can be investigated through its effects on relaxation rate, but it cannot normally be observed directly in a liquid sample due to molecular tumbling (which changes the orientation of the internuclear axis on a time scale much faster than that of dipole-dipole coupling interactions thus averaging to zero its direct effects on the spectrum). The interactions between spins in the same molecule depend on the rotational diffusion of the molecule.\textsuperscript{88} The interactions between spins in different molecules depend on the relative translational diffusion coefficient,\textsuperscript{88} but these interactions are small when the solute studied is dissolved in a solvent without significant nuclear magnetic moments.\textsuperscript{89}

CSA (related to the dependence of the chemical shift on the orientation of the molecules) is another effect to be considered. Spin-rotation is significant in small or highly symmetrical molecules as a result of the nuclear spin interactions with the magnetic fields generated by the rotational motion of the molecules. The electron spin or paramagnetic contribution dominates the relaxation in paramagnetic substances (those with unpaired electrons). For nuclei with spin $I$ higher than $\frac{1}{2}$, i.e. quadrupolar nuclei, relaxation is generally dominated by the quadrupolar mechanism, which originates in the interaction between the quadrupolar moment and the electric field gradient at a nucleus. Such gradients, formed by the non-symmetrical distribution of the surrounding electrons, fluctuate with the molecular motion.
The scalar coupling mechanism affects relaxation when a spin is coupled with another spin undergoing rapid quadrupolar relaxation or chemical exchange.

2.7.2. Longitudinal relaxation measurements

There are a number of different ways of measuring longitudinal relaxation. The most common experiment is inversion recovery. This experiment consists of inverting the $z$-magnetization with a 180° pulse, and then, after a recovery delay $\tau$, flipping the magnetization into the $xy$ plane by applying a 90° pulse before acquisition. This is repeated for a set of $\tau$ values so that each peak height can be measured as a function of the recovery delay, and the resulting relaxation curve can be fitted to the theoretical expression to estimate $T_1$.

Another experiment is saturation recovery. In this, a saturation sequence is applied to leave the spins in a state of saturation, i.e. with no net magnetization, and after a delay $\tau$ the ($z$-)magnetization recovered is measured with a 90° pulse. An array of experiments with different $\tau$ values would allow the extraction of the $T_1$. However, true saturation is hard to achieve: the saturation sequence typically consists of a series of 90° pulses or a 90° pulse and a gradient pulse, but these techniques result only in an apparent saturation, and the application of a combination of pulses may partially restore the initial magnetization. To achieve true saturation any hidden coherence must be destroyed; this may be achieved with the help of diffusion in an inhomogeneous field. Other sequences to determine $T_1$ are described in the book of Martin et al.
2.7.3. Transverse relaxation

Random motions of molecules lead to fluctuations of the local magnetic field perceived by spins, which result in random variations in the instantaneous precession of different spins. As a result, any initial phase coherence of the spins is lost. This decoherence is characterized by a relaxation time $T_2$. If the field $B_0$ were perfectly homogeneous, this relaxation time could be extracted from the linewidths of the peaks, but instead, the width at half-height is commonly approximated to $1/\pi T_2^*$, where $T_2^*$ is a time constant dependent on the homogeneity of the field. The decoherence due to magnetic field inhomogeneity is not random, but dependent on the location of the spins in the magnet. By performing a spin echo experiment is possible to refocus the magnetization, compensating for these inhomogeneities. By using spin echoes, or a train of spin echoes, generally in the form of Carr-Purcell-Meiboom-Gill (CPMG) sequence\textsuperscript{94}, it is possible to determine $T_2$s.

$T_{1\rho}$ describes the relaxation of the longitudinal magnetisation while the system is irradiated at its resonance frequency, i.e., while it is spin locked. $T_{1\rho}$ is called the spin-lattice relaxation in the rotating frame, but it would be more accurate to define it as a decay time constant for transverse magnetization in the presence of an RF field.\textsuperscript{64}

Transverse relaxation is caused by the same mechanisms that affect longitudinal relaxation. All these mechanisms share in common that they cause magnetic field oscillations of the order of the Larmor frequency. But transverse relaxation is also affected by additional mechanisms that cause slower field fluctuations. As a result, fast tumbling molecules
typically have similar $T_1$ and $T_2$, but slow tumbling molecules have a noticeably smaller $T_2$. Other effects like $J$-coupling and chemical exchange contribute to transverse relaxation.

2.7.4. Multi-spin effects

There are additional effects to take into account when studying coupled nuclei. Cross-correlated relaxation occurs through the interference of several relaxation mechanisms, like the interference between two dipole-dipole interactions or between the dipole-dipole interaction of a spin pair and the CSA. These interferences influence both longitudinal and transverse relaxation. The major consequence is the multiplet or differential effect which causes differences in relaxation between the inner and the outer lines of a multiplet. This effect can be suppressed by using a nonselective 90º measuring pulse, which converts multi-spin longitudinal order into undetectable multiple quantum coherences and thus suppresses the multiplet effect.$^{87,95,96}$ Cross-correlated relaxation may also alter the apparent $J$-coupling, through interference with passive longitudinal relaxation.$^{97}$

Nonselective 90º pulses acting on strongly coupled or equivalent spins cause a mixing of the populations and, therefore, multiexponential behaviour with lines in the multiplet decaying at different rates.$^{98,99}$ This effect can be observed in AX$_2$ spin systems, for example, in some bimetallic trihydride complexes there is a 40% difference in relaxation rates between the central and outer lines of the triplet belonging to the $^1$H next to the metal.$^{100}$
Cross-relaxation is a multi-spin contribution to relaxation; it is always present in multi-spin systems, as described by the Solomon equations\textsuperscript{101} (except for certain combinations of Larmor frequencies and correlation times which result in cancellation of the cross-relaxation terms), although it is possible to suppress its effects.\textsuperscript{102} In a two-spin system it leads to biexponential relaxation, which will be simple exponential if any of the following applies:\textsuperscript{95}

- The two spins are magnetically equivalent.
- One of the spins is characterized by a much faster relaxation mechanism (e.g., an electron spin in a paramagnetic system)
- One of the spins is saturated by a radiofrequency field.

Cross-relaxation causes spin diffusion, which involves transfer of magnetization to neighbouring spins. This should not be confused with the more common uses of the word diffusion to mean molecular motion.

2.8. Diffusion

Molecules in a fluid system are constantly experiencing both rotational and translational Brownian motion. The translational motion experienced by a molecule is characterized by a self-diffusion coefficient $D$. Einstein explained that this motion is caused by particle collisions with surrounding molecules, and showed that its magnitude is correlated with the temperature, as described by the Debye-Einstein theory:
\[ D = \frac{k_B T}{f} \]  

where \( f \) is the friction factor. For a spherical particle of hydrodynamic radius \( r_H \) in a continuous solvent of viscosity \( \eta \), the friction factor is given by given by the Stokes equation:

\[ f = 6\pi \eta r_H \]  

In these conditions the equation [2.29] can be expressed as the Stokes-Einstein relation:

\[ D = \frac{k_B T}{6\pi \eta r_H} \]  

The behaviour for other geometries has been explained by Cussler\textsuperscript{103}, and Hansen and McDonald\textsuperscript{104}. The average displacement of a molecule in any direction is zero; however, the mean square displacement is given by the Einstein relation:\textsuperscript{105}

\[ \left\langle \Delta r^2 \right\rangle = 6DA \]  

where \( A \) is the diffusion time.

2.8.1. Diffusion measurement by NMR

Diffusion measurements by NMR can be explained with the Pulsed Field Gradient Spin Echo (PFGSE) pulse sequence. This sequence (shown in Figure 2.5) contains a 90° pulse to rotate the magnetization (Figure 2.4a) to the transverse plane, a delay with a 180° pulse in the middle to refocus the magnetization at the end of the delay, and two gradient pulses responsible for encoding and decoding the magnetization.
The first gradient pulse dephases the magnetization as a function of position, as explained by equation [2.19], resulting in a helical distribution of the magnetization along the z-axis pictured in Figure 2.4b. The second gradient has the same duration and amplitude as the first; allowing the 180° to refocus the magnetization if spins have not moved. But spins experience diffusion at all times, so they will have moved and they will not necessarily have experienced the same field as they would if they had not moved, thus resulting in dephasing of the magnetization as pictured in Figure 2.4 c and d. Higher diffusion coefficient will result in stronger dephasing. Hence, the attenuation of the signal is dependent on the diffusion coefficient (or mean square displacement of the particles, or the particle size) as described initially by Stejskal and Tanner\textsuperscript{24}:

\[ S = S_0 e^{-D\gamma^2\delta^2G^2\Delta'} \]  

[2.32]

where \( S \) is the signal amplitude, \( S_0 \) is the amplitude that would have resulted without diffusion, \( D \) is the diffusion coefficient, \( \delta \) is the gradient pulse width, \( \gamma \) is the magnetogyrat ratio, \( G \) is the gradient amplitude, and \( \Delta' \) is the diffusion time corrected for the effects of finite gradient pulse width (which in the case of a simple Pulsed Field Gradient Spin Echo is equal to \( \Delta - \delta^2 \)). A more advanced description of the attenuation can be made by taking into account gradient non-uniformity.\textsuperscript{84}
Figure 2.4: Representation of the evolution of magnetization with z-position in a PFGSE. a) Magnetization after the first 90° pulse. B) Magnetization after the first gradient has been dephased as a function of z-position. c) Magnetization before the second gradient is affected by self-diffusion. d) Magnetization after the spin echo has been refocused, but the refocusing is not complete due to spins diffusing.
2.8.2. Pulse sequences for diffusion measurement by NMR

One of the simplest pulse sequences for diffusion measurements is the PFGSE, shown in Figure 2.5, which is a modification of the original spin echo. It consists of a single 90° pulse, one 180° pulse, and two equal PFGs of length $\delta$ separated by a time delay $\Delta$, and it works as described in section 2.8.1.

![Figure 2.5: Pulsed Field Gradient Spin Echo (PFGSE) sequence](image)

One of the problems with this sequence is that the magnetization is in the transverse plane throughout the diffusion period, where it is subject to transverse relaxation, potentially causing a rapid signal loss, especially in macromolecules. Another serious problem is $J$-modulation: the signals for coupled spins in the $xy$-plane are modulated because the components of the multiplet do not all precess at the same rate. This may cause signals with negative amplitudes and/or different phase components in the spectrum. $J$-modulation and its effects are explained further in Chapter 3.

The Pulsed Field Gradient STimulated Echo (PFGSTE) sequence, shown in Figure 2.6, replaces the 180° pulse of the PFGSE by two 90° pulses. The first of these two pulses flips
half of the in-phase magnetization in the transverse plane (either the $y$ or the $x$ magnetization) to the $z$-axis, the rest of the magnetization is converted into unobservable terms or is dephased during $\Delta$. The second pulse returns the stored magnetization to the transverse plane so that it can be measured. Although half of the signal is lost, this disadvantage is usually compensated by reduced relaxation losses and $J$-modulation.

Figure 2.6: Pulsed Field Gradient STimulated Echo (PFGSTE) sequence

The field homogeneity perturbations caused by PFGs disturb the deuterium lock signal and can compromise the quality of the acquired spectrum. The Bipolar Pulse Pair STimulated Echo (BPPSTE) sequence\textsuperscript{106,109} (Figure 2.7) decreases these effects by combining bipolar PFGs with 180° pulses. The 180° pulse will invert the magnetization, thus the encoding produced during the first PFG will develop further in the second PFG, however the deuterium signal does not experience the 180° pulse, so the PFG effects cancel each other for deuterium spins.\textsuperscript{106} In addition, the bipolar pulses reduce the detrimental effects of eddy currents\textsuperscript{110} as well as minimizing spin diffusion in macromolecules\textsuperscript{106}. Effects of eddy currents can be further minimized by using a Longitudinal Eddy current Delay. This delays is a combination of two 90° pulses and a delay with a gradient pulse between them.
Further improvements on the BPPSTE sequence are achieved in the Oneshot sequence\textsuperscript{111}, represented in Figure 2.8. In this sequence the bipolar PFGs are unbalanced, so each pair is in the ratio 1+$\alpha$:1-$\alpha$ (with $\alpha$ being usually 0.1-0.2). This dephases any magnetization not refocused by the 180° pulses, thus minimizing the phase cycling required. Balancing pulses with strength $\alpha G$ are added at the beginning and at the end of the diffusion delay $\Delta$ to refocus the deuterium lock signal. Another PFG is added at the beginning of the diffusion delay to dephase coherences of non-zero order, and a balancing pulse is placed just before the initial 90° pulse. Optionally, a bipolar pulse pair of variable strength dummy pulses (decreasing in amplitude as the diffusion-encoding pulses increase) may be added at the beginning of the sequence to keep the energy supplied to the gradient coil constant. The corrected diffusion time $\Delta'$ for this sequence is $\Delta+\delta(\alpha^2+3\alpha-2)/6+\tau_d(\alpha^2+2\alpha-1)/2$, where $\tau_d$ is the delay between the two PFGs of the bipolar pulse pair. This sequence allows the acquisition of high resolution spectra with only one scan, and provides good lineshapes, although quality can be improved with more scans.
2.8.3. Applications of diffusion measurements by NMR

The fact that in diffusion experiments signals belonging to small molecules decay faster than signals from larger molecules is used to edit out small molecule signals in some experiments. A typical application would consist in removing the large solvent signal, but it can also be used in the same way to eliminate from the spectrum the signals from the fastest diffusing species, thus simplifying the spectrum and allowing a clearer spectral interpretation of the slowest, larger species. This can also be combined with other experiments, for example, with NOE experiments of macromolecules to detect ligands; in these experiments polarization will be transferred from the irradiated macromolecules to bound molecules through spin diffusion, so if small molecules have been previously edited out, only those that are bound to macromolecules will be observed.

The determination of diffusion coefficients of spectral signals allows the study of physical properties of systems. Studies of self-aggregation, complexation, micellization or even the
degree of binding are feasible with this technique. In the form of Diffusion-Ordered Spectroscopy (DOSY) it can distinguish the spectra of different species according to their diffusion coefficients, as explained in section 2.8.4.

Studies of restricted diffusion can give details of the structure in which a fluid is embedded. In MRI, diffusion-weighted techniques are extensively used to detect microstructural changes in tissues, such as those caused by tumours or injuries. This is normally done studying water, but it can be done with other species like noble gases.\textsuperscript{114,115}

The same experiments to measure diffusion by NMR can also be used to measure coherent motions, i.e., flow.\textsuperscript{116} The difference is that flow will cause spins to be displaced coherently and, therefore, spin phases will be refocused in another position, rather than being attenuated. By correlating phase changes with gradient changes it is possible to determine flow rates. In chapter 6, this concept will be developed further to study differences in the flow rate of analytes in a chromatographic column.

2.8.4. Diffusion-Ordered Spectroscopy (DOSY)

DOSY allows the separation of the NMR spectra of different compounds in a sample by exploiting their self-diffusion properties (NMR diffusion data are often, loosely, referred to as DOSY data). A two-dimensional dataset is acquired, with one dimension comprising the NMR signal and the other showing attenuation of the signals caused by diffusion. This dataset is acquired by measuring a series of spectra using one of the sequences in section 2.8.3 or a similar with increasing gradient strengths. The diffusion coefficient of each component can be obtained by fitting the peak heights as a function of the squared gradient.
pulse area $(\gamma\delta G)^2$. With the frequency offset $\Omega$ in the first dimension, $N$ peaks $j$ are extended into a second dimension with Gaussian shapes centred on the diffusion coefficients and widths determined by the standard errors $\sigma$, as described in Equation [2.33]. Figure 2.9 shows an example of a DOSY plot. When it is assumed that each peak belongs to a single species this technique is termed High Resolution DOSY (HR-DOSY). In low resolution DOSY, overlap limits the extraction of diffusion coefficients and other alternatives may provide further information as discussed in the subsection following.

$$S(D,\Omega) = \sum_{j=1}^{N} \frac{S_j(\Omega)}{\sqrt{2\pi \sigma_j^2}} e^{-\frac{(D-D_j)^2}{2\sigma_j^2}} [2.33]$$

Figure 2.9: $^1$H DOSY plot of a mixture of 1-propanol and 3-methyl-3-pentanol. Intermediate apparent diffusion coefficients are observed in the regions where signals overlap, as discussed in section 2.8.5
2.8.5. Limitations of diffusion measurement by NMR

Diffusion measurements are limited by experimental conditions, hardware constraints and mathematical analysis. It is not possible to have a perfect control of the conditions of the experiment, the most relevant being the temperature. Small temperature variations affect the chemical shift of the signal and the diffusion coefficient of the species. In addition, temperature gradients may cause convection under certain conditions (convection will start at the critical Raleigh number, a value which depends on the geometry of volume occupied by the liquid). Convection causes coherent motion in the form of Bénard cells, i.e., small circular flow currents throughout the sample. Since the motion is not coherent throughout all the volume measured, this will lead to a reduction of the signal and also, because some coherent motion is still detected, to phase changes. Typical measures to reduce the possibility of convection include reducing the space occupied by the liquid, improving temperature control, and reducing the oscillation in temperature over time of the air surrounding the sample. When it is not possible to avoid convection, convection compensated sequences should be used.117,118

The currents generated in the RF and gradient coils will dissipate heat that not only will further destabilize the temperature of the sample, but can endanger the physical integrity of the coils and their surroundings. Molecules that diffuse slowly will require stronger diffusion encoding, but the strength of the gradients is limited to avoid coil damage, so the typical approach is to increase the diffusion encoding delay and the gradient duration. A longer delay will incur further relaxation losses and longer gradient widths will increase the heat and the time spent by the magnetization in the transverse plane, so compromised
parameters are necessary. In addition, lock is disturbed when gradients are applied and the rapid pulsing of gradients results in eddy currents that will degrade the lineshape of the peaks.

The exponential nature (in the diffusion dimension) of diffusion-weighted data poses a significant limitation to the accuracy of the determination of diffusion coefficients. When the NMR signal is well-resolved the problem is relatively small and a simple non-linear fit to a single exponential curve yields diffusion coefficients with good accuracy; differences as small as 1% or less can be detected\(^{106}\). However, signal overlap is the norm in all but the simplest mixtures, resulting in superimposed exponential decays. The inversion of such decays is, for experimental (noisy) data a well known ill-posed mathematical problem\(^{119,120}\). If the analytical expression was known, the solution could be calculated through the inverse Laplace transform; in experimental data multiple solutions are valid, thus an approximation is required.

A number of methods have been developed for resolving the superimposed exponential decays of DOSY data. These methods can usefully be divided into univariate and multivariate methods. In univariate methods, each NMR signal (peak) is analysed individually (as in HR-DOSY) where different methods such as biexponential fitting\(^{28}\), CONTIN\(^{20,121}\), the Bayesian transform\(^{122}\), Hopfield neural network analysis\(^{123}\) or entropy maximization\(^{124}\) have been proposed. Multivariate methods take into account the fact that the whole spectrum from a given molecular species showed identical diffusion attenuation; this covariance aids the resolution of individual components spectra. Multivariate methods
include COMponent REsolved NMR (CORE),\textsuperscript{125} Multivariate Curve Resolution (MCR),\textsuperscript{126} Direct Exponential Curve Resolution Algorithm (DECRA),\textsuperscript{127} and Speedy COMponent REsolution (SCORE).\textsuperscript{128} Although these methods can deal with overlaps they may also show spurious cross-talk between components, especially when the diffusion coefficients of the components are close. In practice, the best resolution for superimposed decays is about 20\% difference in diffusion coefficient\textsuperscript{108}.

Another approach is to increase spectral resolution. This may be achieved in experiments where the multiplet structure is collapsed, as in experiments like Pure Shift DOSY\textsuperscript{129,130} or in a $J$-resolved DOSY\textsuperscript{131}. Heteronucleus spectra such as $^{13}$C typically have better resolution than $^1$H\textsuperscript{25,132}. Another alternative is to add another spectral dimension, where a series of two-dimensional spectra are measured with increasing diffusion weighting. In such 3D DOSY experiments, the objective is to reduce greatly the possibility of spectral overlap to allow the use of a simple monoexponential fit. Among these methods are COSY-DOSY,\textsuperscript{133,134} HMQC-DOSY,\textsuperscript{135,136} and 2DJ-DOSY\textsuperscript{137,138}. These experiments usually require long data acquisition times,\textsuperscript{20} and the analysis of 3D DOSY data is also much more tedious and time-consuming.

An emerging field\textsuperscript{139} relies on the application of multi-way analysis to NMR data that depend on more variables than the chemical shift in a 3D or higher dimensionality dataset. As an example, Bro \emph{et al.}\textsuperscript{140} used PARAFAC to untangle the spectra of the components in a set of diffusion-encoded samples with different concentrations. Further information is given in Chapter 5.
2.9. Further reading

This is a small selection of books and reviews that are useful to gain a better grasp of the underlying theory in this thesis. Three of them require special mention. The book written by Tim Claridge\textsuperscript{141} is excellent for starters, explaining basic theory and practicalities of several experiments. Understanding NMR Spectroscopy\textsuperscript{29} by James Keeler provides an insight into the mathematics and physics of NMR. And Spin Dynamics\textsuperscript{64} by Malcolm Levitt goes further into the theory with a rigorous treatment of the NMR theory using density matrix analysis\textsuperscript{142} without forgetting about the physical interpretation. Other books with a practical insight were written by Derome (now superseded by Claridge’s book),\textsuperscript{143} Fukushima and Roeder\textsuperscript{144}, Sanders and Hunter\textsuperscript{145} and Martin et al.\textsuperscript{99} A more conceptual approach is given by Freeman\textsuperscript{63,146} and Günther\textsuperscript{147}. A deeper insight into the mathematical physics of this phenomenon can be found in the books written by Abragam\textsuperscript{148}, Goldman,\textsuperscript{149} Ernst et al.\textsuperscript{150} and Slichter\textsuperscript{151}.

For a deeper insight into relaxation the book of Bakhmutov,\textsuperscript{99} Kowalewski and Mäler\textsuperscript{95} and the paper of Vold and Vold\textsuperscript{152} provide good reviews of the subject. Longitudinal relaxation theory has been analysed by Goldman.\textsuperscript{153} Cross-correlations have been reviewed by Kumar et al.\textsuperscript{154}

Diffusion experiments have been extensively reviewed in an book written by Price.\textsuperscript{155} DOSY has been reviewed by Morris, Johnson and Price.\textsuperscript{19-22} Stallmach et al.\textsuperscript{156} have carried out an extensive study of spin echo NMR diffusion. Antalek has explained how to optimize
DOSY experiments\textsuperscript{108} and how to analyse a sample quantitatively using diffusion measurements.\textsuperscript{157}

For chapter 5, the book Multi-way analysis,\textsuperscript{158} Bro’s thesis\textsuperscript{159} and Dyrby’s thesis\textsuperscript{160} provide an excellent coverage of the basics and applications of PARAFAC. Khajeh has carried out most of the experimental work related to timecourse DOSY, and her thesis \textsuperscript{161} is complementary to this chapter. For chapter 6, Callaghan has written a book that is useful to develop a good understanding of the study of motion with NMR.\textsuperscript{162}
Il semble que la perfection soit atteinte non quand il n'y a plus rien à ajouter,
mais quand il n'y a plus rien à retrancher.

(It would seem that perfection is attained not when no more can be added,
but when no more can be removed.)

Antoine de Saint Exupéry
3. Oneshot45

DOSY has been extensively applied to small molecules, where there is little overlap; but this does not mean that it is not possible to obtain useful results when analysing mixtures of large molecules. The application of DOSY to samples containing large, slowly diffusing species such as micelles\textsuperscript{163,164}, hydrocarbon mixtures\textsuperscript{165,166}, proteins\textsuperscript{167,168} and DNA\textsuperscript{169} is increasing demand for strong diffusion-encoding, often requiring longer diffusion encoding times leading to more pronounced $J$-modulation. This causes signal broadening and increased spectral overlap, and unwanted signal cancellation/distortion due to intruding dispersive signals. When positive signals overlap, the resultant apparent diffusion coefficients in HR-DOSY are a compromise between the values for the species involved. However, in this chapter it is shown that overlap between positive and negative signals results in the opposite effect. A new method is presented to simplify spectra by purging dispersive signals (thus minimizing this problem), using a single 45º pulse at the end of the DOSY pulse sequence.

3.1. $J$-modulation

$J$-coupling is an important phenomenon to take into account in the design and application of pulse sequences. In order to explain $J$-coupling, a spin echo with two coupled spins $\hat{I}_1$ and $\hat{I}_2$ is analysed here. The application of a pulse about the $y$ axis will rotate the initial $z$-magnetization $\hat{I}_{1z} + \hat{I}_{2z}$ into in-phase magnetization on the $x$ axis $\hat{I}_{1x} + \hat{I}_{2x}$ (Eqs. 3.1 and 3.2). The effect of chemical shift is determined by the effect of the offset operators for the spins, $\Omega t \hat{I}_{1z}$ and $\Omega t \hat{I}_{2z}$, and the $J$-coupling evolution by the effect of the operator $2\pi J_{12}\hat{I}_{1z}\hat{I}_{2z}$. The
offset evolution is refocused at the end of the spin echo by the effect of a 180° pulse about the x axis, so the resulting terms are described by Eqs. [3.3] and [3.4].

\[
\hat{I}_{1z} \xrightarrow{\pi/2(I_{y1} + I_{y2})} \hat{I}_{1x} \tag{3.1}
\]

\[
\hat{I}_{2z} \xrightarrow{\pi/2(I_{y1} + I_{y2})} \hat{I}_{2x} \tag{3.2}
\]

\[
\hat{I}_{1x} \xrightarrow{2\pi I_{1z}I_{1z}} \cos(\pi I_{1z}t)\hat{I}_{1x} + \sin(\pi I_{1z}t)2\hat{I}_{1y}\hat{I}_{2z} \tag{3.3}
\]

\[
\hat{I}_{2x} \xrightarrow{2\pi I_{1z}I_{1z}} \cos(\pi I_{1z}t)\hat{I}_{2x} + \sin(\pi I_{1z}t)2\hat{I}_{2y}\hat{I}_{1z} \tag{3.4}
\]

\(\hat{I}_{1z}\hat{I}_{2z}\) and \(\hat{I}_{2y}\hat{I}_{1z}\) are anti-phase terms. When the acquisition is started, all the terms evolve under the effect of the J-coupling and the offset. The in-phase terms give rise to modulated absorption peaks, and the anti-phase terms produce peaks with dispersive lineshape. The combination of both terms produces J-modulated spectra, as simulated in Figure 3.1.

![Echo time: 0 1/5J 1/2J 1/J](image)

**Figure 3.1:** Simulated spin echo spectra of a spin-1/2 weakly coupled to another for different echo times. Arbitrary amplitudes are represented vs. arbitrary frequency. At 0 and 1/J echo times there are pure absorption lineshapes, at 1/(2J) dispersion lineshapes and at any other echo time there is a mixture of absorption and dispersion lineshapes.
3.2. $J$-modulation effects in DOSY experiments

For a well-resolved NMR spectrum, $J$-modulation does not cause trouble in the calculation of apparent diffusion coefficient. However, NMR spectra of most mixtures will contain overlap to different degrees. In DOSY experiments, the diffusional attenuation of a signal resulting from the overlap of two different peaks can ideally be described as:

$$ S = S_1 e^{-D_1\gamma^2\delta^2 G^2 A'} + S_2 e^{-D_2\gamma^2\delta^2 G^2 A'} $$

[3.5]

where $S_1$ and $S_2$ are the amplitudes of the overlapping signals in the absence of diffusion and $D_1$ and $D_2$ are the diffusion coefficients. Ideally the signal should be fitted to a biexponential function; however, it is not simple to distinguish which signals combine contributions from more than one species. They can sometimes be distinguished by the error of the fit, but only when the diffusion coefficients of the species are substantially different. In the HR-DOSY approach, all signals are fitted to a monoexponential function, and in the interpretation of the resulting DOSY spectrum it is taken into account that some apparent diffusion coefficients may be compromises. A monoexponential fit of two positive overlapping signals will result in a calculated diffusion coefficient $D$ that is intermediate between $D_1$ and $D_2$, as shown in Figure 3.2a. When $D_1$ and $D_2$ have a similar order of magnitude, the extra error of the fit is very small, preventing recognition of the decay as non-monoexponential. The wider peaks of dispersive lineshapes present with $J$-modulation enhance the likelihood of this problem.

Another complication arises from the overlap of positive and negative signals. In this case the estimated $D$ does not lie between $D_1$ and $D_2$. Assuming that $|S_1| > |S_2|$ and that $S_2 < 0$,
there are 3 possible cases, (if $S_2 > 0$, the same cases apply, but with the opposite sign of $S_0$), as mapped in Figure 3.3:

**case 1:** $D_1 > D_2$ and $S_0 > 0$: the fitted $D$ will be greater than both $D_1$ and $D_2$, as illustrated in Figure 3.2b. The error for the estimated $D$ can be noticeably larger than for the rest of the peaks.

**case 2:** $D_1 > D_2$ and $S_0 < 0$: the fitted $D$ will be lower than both $D_1$ and $D_2$, as illustrated in Figure 3.2c. The relative error for $D$ is very large, which may cause a peak to be ignored (as not statistically significant) by programs for synthesising DOSY spectra.

**case 3:** $D_1 < D_2$: the fitted $D$ will be lower than both $D_1$ and $D_2$, as illustrated in Figure 3.2d. The error of the estimated $D$ is not noticeably higher than in the case of 2 positive overlapping signals.
Figure 3.2: Simulations of monoexponential fitting of biexponential functions resulting from the addition of two exponential functions $A = S_0 e^{-Dx}$ with different amplitudes $S_1$ and $S_2$ and diffusion coefficients $D_1$ and $D_2$; on the right side is shown the simulated DOSY plot of two doublets from separate species where one of the peaks (that in the centre) is completely overlapped with a peak from the other species, with the projected 1D spectrum obtained in the absence of diffusion. In each case the calculated $D$ depends on the exact choice of points to sample. Green dashed and red dot-dashed lines show the evolution of the amplitude $A$ with $x$ for two exponentials with diffusion coefficients $D_1$ (green) and $D_2$ (red). Brown dots and continuous brown line show sampled points from the sum of the two exponentials and the monoexponential fit to the brown points. a) Monoexponential fit of the sum of two positive exponential decays results in a calculated $D$ intermediate between $D_1$ and $D_2$. b) Monoexponential fit of the sum of exponentials decays resulting in a calculated $D$ greater than either $D_1$ or $D_2$ (case 1). c) Monoexponential fit of the sum of exponentials decays resulting in a calculated $D$ lower than either $D_1$ or $D_2$ (case 2), but with negative estimated $S_0$ (case 2). d) Monoexponential fit of the sum of exponentials decays resulting in a calculated $D$ lower than either $D_1$ or $D_2$ (case 3).
Figure 3.3: Contour plots of calculated a) relative diffusion constant $D/D_1$ and b) relative amplitude $S_2/S_1$ from a monoexponential fit of a biexponential decay function, as a function of the ratios $S_2/S_1$ and $D_2/D_1$. 
The examples pictured in Figure 3.2 show what happens when a negative signal overlaps with a positive signal of twice its amplitude. Even in these extreme cases, the reported errors from some of these fits are not large enough to be distinguishable from those for the fits of true monoexponential decays. Thus in practice it can be hard to find out whether a peak is not aligned in apparent diffusion coefficient with others because it belongs to a different species, or because of overlap. Some species may not be detected at all if the peaks disappear, as can happen in case 2. The presence of negative signal also prevents an effective use of non-negativity constraints, typically used in multivariate analysis 125-128.

3.3. Suppression of J-modulation effects

Various different methods have been developed that avoid these spectral distortions. One approach consists of acquiring homonuclear decoupled spectra170-173, thus collapsing the multiplet structure. Some such methods have already been incorporated into DOSY experiments129,130, but their low sensitivity prevents them from being widely used. Another approach consists of refocusing the J-evolution; this can be readily done for heteronuclear couplings, but a straight forward sequence to achieve this for all homonuclear couplings in a spectrum has not yet been developed. Takegoshi et al.174 and van Zijl et al.175 discovered that the combination of pulses 90º-180º-90º-180º, with equal delays between the pulses, refocuses the signal for a two spin system, but this refocusing is incomplete for higher order spin systems. This approach has been recently implemented for PFG spin echo experiments176. The oscillating-gradient spin echo method177 has been used to obtain pure phase spectra for specific Js at the cost of broadening peaks and the difficulty of finding the
optimal conditions. Homonuclear decoupling during spin evolution avoids the appearance of anti-phase terms, but this cannot effectively decouple all spins at once.

A different approach consists of removing the anti-phase terms responsible for the dispersive lineshapes of peaks. The main pulse sequence elements previously developed for this purpose have been evaluated by Torres et al. in PFG spin echo and in a stimulated echo sequence (without bipolar pulses). These elements are a spin lock, a Longitudinal Eddy Delay (LED), and a chirp-based z-filter. A spin lock (a long pulse to keep spin phases aligned) or a long pulse before the acquisition dephases anti-phase magnetization without affecting in-phase magnetization; however, the lineshape may deteriorate due to lock disturbances (the sample cannot be locked while a pulse is applied) and the amount of power deposited is considerable, which causes troublesome sample heating. The LED element, which is a combination of two 90º pulses and a delay with a gradient pulse between them, was originally designed to let eddy currents dissipate, but it also removes anti-phase magnetization to some extent. The first 90º pulse converts in-phase magnetization into z-magnetization, and anti-phase magnetization into multiple and zero quantum coherences. Multiple quantum coherences ought to be dephased by the gradient, but zero quantum coherences are unaffected by the gradient. The second 90º pulse returns the longitudinal magnetization into in-phase magnetization, and zero quantum coherences are converted back into anti-phase magnetization. Extensive phase cycling is required for the LED element. The last alternative evaluated is the chirp-based z-filter, which is based on the LED, but with a chirp pulse synchronous with the gradient. This allows the removal of zero quantum coherences, thus leaving only in-phase magnetization. All these
three elements lose sensitivity due to relaxation, limiting the range of molecules that can be studied, and the LED-based elements require a minimum phase cycling of 4 steps. Here, an alternative is proposed which consists of the addition of a 45° pulse orthogonal to the preceding 90° pulse.

### 3.4. Oneshot45

Periods involving $J$-evolution in typical DOSY experiments involve spin echo elements. The $J$-evolution for a spin echo lasting a time $t$ for two coupled spins $\hat{I}_1$ and $\hat{I}_2$ is analysed here. In the 2 spin system analysed (Eqs. [3.3] and [3.4]), in-phase terms remain unaffected by the 45° pulse (Eqs. [3.5] and [3.7]), and the anti-phase terms cancel (Eqs. [3.6] and [3.8]):

$$\cos(\pi J_{12} t)\hat{I}_{1x} \xrightarrow{\frac{\pi}{4} (\hat{I}_{1x} + \hat{I}_{2x})} \cos(\pi J_{12} t)\hat{I}_{1x}$$  \[3.5\]

$$\sin(\pi J_{12} t)2\hat{I}_{1y} \hat{I}_{2x} \xrightarrow{\frac{\pi}{4} (\hat{I}_{1x} + \hat{I}_{2x})} \cos(\frac{\pi}{4})\sin(\pi J_{12} t)2\hat{I}_{1y} \hat{I}_{2x} - \sin(\frac{\pi}{4})\sin(\pi J_{12} t)2\hat{I}_{1z} \hat{I}_{2y}$$  \[3.6\]

$$\cos(\pi J_{12} t)\hat{I}_{2x} \xrightarrow{\frac{\pi}{4} (\hat{I}_{1x} + \hat{I}_{2x})} \cos(\pi J_{12} t)\hat{I}_{2x}$$  \[3.7\]

$$\sin(\pi J_{12} t)2\hat{I}_{2y} \hat{I}_{1x} \xrightarrow{\frac{\pi}{4} (\hat{I}_{1x} + \hat{I}_{2x})} \cos(\frac{\pi}{4})\sin(\pi J_{12} t)2\hat{I}_{2y} \hat{I}_{1x} - \sin(\frac{\pi}{4})\sin(\pi J_{12} t)2\hat{I}_{2z} \hat{I}_{1y}$$  \[3.8\]

As a result the remaining magnetization is in-phase, as described by Eq. [3.9]:

$$\cos(\pi J_{12} t)\hat{I}_{1x} + \cos(\pi J_{12} t)\hat{I}_{2x}$$  \[3.9\]

This 45° purging pulse allows the removal of anti-phase terms for small echo times with negligible increase of heating and relaxation. The minimum theoretical phase cycling for this pulse element is 2 transients, which makes it ideal where rapid measurements are required, such as in the analysis of mixtures or reactions. Oneshot, as a pulse sequence routinely used in DOSY with a phase cycling of one transient, is a suitable
sequence with which to evaluate the performance of this purging element. This combination, called Oneshot45, is depicted in Figure 3.4, with the phase cycle shown in Table 3.1.

The analysis for higher order spins is analogous, and is shown in Appendices A and B. All bilinear terms (those of the form $2\hat{I}_i\hat{I}_j$ with $i$ and $j$ being the coupled spins) are removed, and half of the magnetization from higher order terms is transformed into undetectable multiple quantum coherences.

Figure 3.4: Oneshot45 pulse sequence, which consists of Oneshot plus a 45° pulse orthogonal to the preceding 90° pulse.
Table 3.1: Phase cycling for Oneshot45. Phases are notated as multiples of 90° (0 = 0°, 1 = 90°, 2 = 180°, 3 = 270°), with subscripts denoting repetition.

<table>
<thead>
<tr>
<th>( \phi )</th>
<th>0_{4/4}^{+02}</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \phi_2 )</td>
<td>0_{128/2}^{128}</td>
</tr>
<tr>
<td>( \phi_3 )</td>
<td>0_{32/32}</td>
</tr>
<tr>
<td>( \phi_4 )</td>
<td>0_{2/2}^{2/2}+0_{18/18}^{18/18}</td>
</tr>
<tr>
<td>( \phi_5 )</td>
<td>0_{64/64}^{64/64}+0_{16/16}^{16/16}</td>
</tr>
<tr>
<td>( \phi_6 )</td>
<td>( \phi_1-2\phi_2+\phi_3-\phi_4+2\phi_3+1+0_{4/4}^{+02} )</td>
</tr>
<tr>
<td>( \phi_R )</td>
<td>( \phi_1-2\phi_2+\phi_3-\phi_4+2\phi_5 )</td>
</tr>
</tbody>
</table>

The product operator analysis of Oneshot45 for a two-spin system is as follows. After the first spin echo the terms are:

\[
\cos(\pi J_{12} t) \hat{I}_{1x} + \sin(\pi J_{12} t) 2\hat{I}_{1y} \hat{I}_{2z} \\
\cos(\pi J_{12} t) \hat{I}_{2x} + \sin(\pi J_{12} t) 2\hat{I}_{2y} \hat{I}_{1z}
\]

After the second 90°, in-phase magnetization is converted into longitudinal magnetization, and anti-phase into the terms \( 2\hat{I}_{1x} \hat{I}_{2x} \) and \( 2\hat{I}_{1y} \hat{I}_{2y} \), which can be expressed as a sum of zero quantum coherences:

\[
2\hat{I}_{1x} \hat{I}_{2y} - 2\hat{I}_{1y} \hat{I}_{2x}
\]

and multiple quantum coherences:

\[
2\hat{I}_{1x} \hat{I}_{2x} + 2\hat{I}_{1y} \hat{I}_{2y}
\]

Throughout the delay \( \Delta \), multiple quantum terms dephase due to the gradients present in the delay, and zero quantum coherences will evolve as:

---

80
\[-\cos(\pi J_{12} t) \hat{I}_{1x} + \sin(\pi J_{12} t)(-\hat{I}_{1y} \hat{I}_{2x} + \hat{I}_{1x} \hat{I}_{2y}) \cos((\omega_{I_{2x}} - \omega_{I_{1y}})\Delta) - (\hat{I}_{1x} \hat{I}_{2x} + \hat{I}_{1y} \hat{I}_{2y}) \sin((\omega_{I_{2y}} - \omega_{I_{1x}})\Delta)\]
\[-\cos(\pi J_{12} t) \hat{I}_{2x} + \sin(\pi J_{12} t)(-\hat{I}_{2y} \hat{I}_{1x} + \hat{I}_{2x} \hat{I}_{1y}) \cos((\omega_{I_{1x}} - \omega_{I_{2y}})\Delta) - (\hat{I}_{2x} \hat{I}_{1x} + \hat{I}_{2y} \hat{I}_{1y}) \sin((\omega_{I_{1y}} - \omega_{I_{2x}})\Delta)\]

After the third 90°, zero quantum coherences will convert into antiphase magnetization and zero and multiple quantum coherences:

\[-\cos(\pi J_{12} t) \hat{I}_{1x} + \sin(\pi J_{12} t)(\hat{I}_{1y} \hat{I}_{2x} - \hat{I}_{1x} \hat{I}_{2y}) \cos((\omega_{I_{2x}} - \omega_{I_{1y}})\Delta) - (\hat{I}_{1x} \hat{I}_{2x} + \hat{I}_{1y} \hat{I}_{2y}) \sin((\omega_{I_{2y}} - \omega_{I_{1x}})\Delta)\]
\[-\cos(\pi J_{12} t) \hat{I}_{2x} + \sin(\pi J_{12} t)(\hat{I}_{2y} \hat{I}_{1x} - \hat{I}_{2x} \hat{I}_{1y}) \cos((\omega_{I_{1x}} - \omega_{I_{2y}})\Delta) - (\hat{I}_{2x} \hat{I}_{1x} + \hat{I}_{2y} \hat{I}_{1y}) \sin((\omega_{I_{1y}} - \omega_{I_{2x}})\Delta)\]

Antiphase terms cancel out:

\[-\cos(\pi J_{12} t) \hat{I}_{1x} - \cos(\pi J_{12} t) \hat{I}_{2x} - \sin(\pi J_{12} t) \sin((\omega_{I_{1x}} - \omega_{I_{1y}})\Delta)(2\hat{I}_{1x} \hat{I}_{2x} + 2\hat{I}_{1y} \hat{I}_{2y})\]

After the second spin echo:

\[-\cos^2(\pi J_{12} t) \hat{I}_{1x} - \cos^2(\pi J_{12} t) \hat{I}_{2x} - \cos(\pi J_{12} t) \sin(\pi J_{12} t) \hat{I}_{1y} \hat{I}_{2z} - \cos(\pi J_{12} t) \sin(\pi J_{12} t) \hat{I}_{2y} \hat{I}_{1z} - \sin(\pi J_{12} t) \sin((\omega_{I_{1x}} - \omega_{I_{1y}})\Delta)(2\hat{I}_{1x} \hat{I}_{2x} + 2\hat{I}_{1y} \hat{I}_{2y})\]

After the orthogonal 45° pulse:

\[-\cos^2(\pi J_{12} t) \hat{I}_{1x} - \cos^2(\pi J_{12} t) \hat{I}_{2x} - \cos(\pi J_{12} t) \sin(\pi J_{12} t) \hat{I}_{1y} \hat{I}_{2z} + \cos(\pi J_{12} t) \sin(\pi J_{12} t) \hat{I}_{2y} \hat{I}_{1z} + \sin(\pi J_{12} t) \sin(\pi J_{12} t) \hat{I}_{1x} \hat{I}_{2z} + \sin(\pi J_{12} t) \sin(\pi J_{12} t) \hat{I}_{2x} \hat{I}_{1z}\]
\[-\cos(\pi J_{12} t) \sin((\omega_{I_{2x}} - \omega_{I_{1y}})\Delta)(2\hat{I}_{1x} \hat{I}_{2x} + 2\hat{I}_{1y} \hat{I}_{2y})\]
\[-\sin(\pi J_{12} t) \sin((\omega_{I_{1x}} - \omega_{I_{1y}})\Delta)(2\hat{I}_{1x} \hat{I}_{2x} + 2\hat{I}_{1y} \hat{I}_{2y})\]

The cosine and sine of π/4 are equal, so antiphase terms cancel out, leaving only in-phase magnetization and invisible coherences:

\[-\cos^2(\pi J_{12} t) \hat{I}_{1x} - \cos^2(\pi J_{12} t) \hat{I}_{2x} - \sqrt{2} \sin(\pi J_{12} t) \sin((\omega_{I_{1x}} - \omega_{I_{1y}})\Delta)(2\hat{I}_{1x} \hat{I}_{2x} + 2\hat{I}_{1y} \hat{I}_{2y})\]
3.5. Methods

Two mixtures were prepared, one of 1-propanol (250 mM) and 2-pentanol (75 mM) in deuterated dimethyl sulfoxide (DMSO-d$_6$) with 27 mM TSP (sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d$_4$) as a reference, and the other containing camphene (20 mM), geraniol (23 mM) and quinine (19 mM), dissolved in methanol-d$_4$ with TMS (tetramethylsilane) as a reference. Measurements were carried out non-spinning on a Varian VNMRS 500 spectrometer in an air-conditioned room at approximately 20 °C, with spectrometer temperature regulation set at 25 °C and with an active air preconditioning system used to minimize temperature variations.

Using an echo time of 5.6 ms, with a gradient duration of 3.6 ms and a strength of 5 G cm$^{-1}$, the differences between the spectrum obtained using 1 transient and that obtained using 4 transients, as depicted in Figure 3.5, are small. In quantitative terms, a least squares fit of the spectral region depicted in 3.5 obtained with Oneshot with 1 transient to the obtained with Oneshot45 with 4 transients provides a regression coefficient $R^2$ equal to 0.928, while $R^2$ is 0.993 for the fit between Oneshot45 with 4 transients and Oneshot45 with 1 transient. This shows that although it would be necessary to cycle the 45° pulse with at least 2 scans to remove undesired signals, a one scan experiment with Oneshot45 can still be an improvement over Oneshot.
Figure 3.5: Comparison using the mixture with quinine, camphene and geraniol between a) Oneshot with 1 transient, b) Oneshot45 with 1 transient and c) Oneshot45 with 4 transients.

A series of experiments was carried out with Oneshot and Oneshot45 to evaluate the effects of $J$-modulation on DOSY spectra and their suppression with Oneshot45. Data for the mixture of 1-propanol and 2-pentanol were acquired in 5 min with 10 gradient amplitudes ranging from 10 to 30 G cm$^{-1}$ in equal steps of gradient squared using 4 transients, 8192 complex data points, a total diffusion-encoding gradient duration of 1.8 ms and a diffusion time of 0.2 s. The spin-echo time was varied from 2.8 to 13.8 ms by incrementing the delay after the gradient pulses to observe $J$-modulation effects for a range of echo times. Data for
the mixture of quinine, camphene and geraniol were acquired in 7 min with 8 gradient amplitudes ranging from 10 to 30 G cm\(^{-1}\) in equal steps of gradient squared using 4 transients, 16384 complex data points, a total diffusion-encoding gradient duration of 3.6 ms and a diffusion time of 0.1 s. The gradient stabilization delay was varied from 1 to 8 ms to observe \(J\)-modulation effects for a range of echo times.

### 3.6. Results

The Oneshot DOSY spectrum for the mixture of 1-propanol and 2-pentanol using an echo time of 13.8 ms shows peaks with 3 apparent diffusion coefficients (Figure 3.6), if the solvent multiplet at 2.5 ppm is disregarded. The rightmost peak belonging to a triplet of the 2-pentanol, at 0.90 ppm, appears with an apparent diffusion coefficient lower than the apparent diffusion coefficient of either species. This region of the 1D spectrum is pictured in Figure 3.7, and compared with the results obtained using Oneshot45, where the anti-phase magnetization has been effectively suppressed. Figure 3.8 shows the cleaner DOSY spectrum obtained with Oneshot45 sequence. The variation with echo time of the apparent diffusion coefficient of the highlighted signal in Figure 3.7, and the apparent diffusion coefficient obtained for resolved peaks of the 2 species, are shown in Figure 3.9. The results obtained with Oneshot45 are largely unaffected by \(J\)-modulation for this range of echo times, but with Oneshot these effects are already noticeable for low echo times. Oneshot45 thus effectively increases the range of samples that can be analysed, allowing larger molecules and samples with a higher dynamic range of concentrations to be studied.
Figure 3.6: Oneshot DOSY spectrum with an echo time of 13.8 ms of a mixture of 1-propanol (blue) and 2-pentanol (red) in DMSO (black). The highlighted signal belongs to 2-pentanol, but the overlap with 1-propanol signals has notably altered its different apparent diffusion coefficient.

Figure 3.7: Detail of the spectrum obtained with Oneshot (left) with an echo time of 13.8 ms compared with the spectrum obtained with Oneshot45 (right) for the mixture of 1-propanol and 2-pentanol. The signal highlighted is severely distorted by the dispersive negative tail of a 1-propanol peak, causing misleading results in the apparent diffusion coefficient (Figure 3.6).
Figure 3.8: Oneshot45 DOSY spectrum with an echo time of 13.8 ms of a mixture of 1-propanol (blue) and 2-pentanol (red) in DMSO (black).

Figure 3.9: Evolution of the apparent diffusion coefficient with their error bars for a range of echo times of the highlighted signal in Figure 3.6 from 2-pentanol (red), a resolved peak at 1.1 ppm from 2-pentanol and a resolved peak at 0.89 ppm from 1-propanol.
The mixture of quinine, camphene and geraniol was used to assess the sequences on a more complex example. Although the overlap of small signals between 1 and 2 ppm makes it difficult to resolve the signals with HR-DOSY, the rest of the spectrum appears to be resolved using Oneshot (Figure 3.10). However, it is misleading. A doublet at 2.6 ppm appears to belong to geraniol, but the DOSY plot obtained with Oneshot45 (Figure 3.11) indicates that the doublet actually belongs to the camphene. A positive dispersive tail from a quinine multiplet overlaps with this doublet, altering its apparent diffusion coefficient. This effect is enhanced by the relatively lower intensity of the doublet and by the large echo time used (about 4 times the value normally used).

Figure 3.10: Oneshot DOSY spectrum with an echo time of 19.6 ms of a mixture of quinine (red), geraniol (green) and camphene (blue). The highlighted doublet shows the same apparent diffusion coefficient as the geraniol, but actually belongs to camphene, as shown in Figure 3.11.
Figure 3.11: Oneshot45 DOSY with an echo time of 19.6 ms of a mixture of quinine (red), geraniol (green) and camphene (blue). The apparent diffusion coefficient of the signal highlighted is much closer to the correct value than in figure 3.10

3.7. Conclusions

In DOSY experiments, $J$-modulation can cause signal overlap, leading to misleading apparent diffusion coefficients. When one peak overlaps with the positive dispersive tail of another peak, the apparent diffusion coefficient calculated in HR-DOSY is intermediate between those of the two species; however, when the dispersive tail is negative, the effect is the opposite: the apparent diffusion coefficient lies outside the range of species involved. The anti-phase dispersion mode terms responsible can be suppressed for moderate echo times by adding a 45° purging pulse immediately before acquisition. The Oneshot45 pulse
sequence improves the reliability of Oneshot DOSY data at no significant cost in sensitivity and only a doubling of the very short minimum experiment time, making it ideal wherever rapid and reliable measurements are required, such as for the analysis of unstable mixtures \(^\text{184}\) or reactions \(^\text{185}\). This sequence is likely to prove most useful for analyses involving species with large \(J\)-coupling constants and/or slowly diffusing species.
Chapter 4

$^{13}\text{C DOSY}$

*We've got no money, so we've got to think.*

Ernest Rutherford
4. $^{13}$C DOSY

Two major problems affecting NMR are lack of sensitivity and lack of resolution, and the endeavour to reduce these problems has been the main driving force to increase the field strength of NMR magnets. This increase in magnetic field comes at a very high cost, and therefore any technique that can increase the sensitivity or resolution is highly valuable. Merely doubling the signal-to-noise ratio (SNR) represents the difference between being capable of running an experiment overnight and requiring two complete days of instrument time. In this chapter, a new approach that allows doubling of the SNR in a $^{13}$C DOSY experiment is presented and compared against the current alternative.

4.1. Introduction

Standard DOSY experiments generally use $^1$H nuclei for the majority of compounds because they provide the highest SNR of all nuclei; however, $^1$H spectra of complex molecules are crowded with signals. Crowded spectra are not only harder to interpret, but the performance of DOSY is severely hindered by spectral overlap between different species; where two signals overlap the calculated diffusion coefficient is normally a compromise between those of the species involved. This has led to the development of experiments using nuclei which present a less crowded spectrum, such as $^{13}$C. Although the first published mixture analysis by NMR using this nucleus was carried out as early as in 1981, the low sensitivity made it impractical for general use. Since then, the development of more powerful and stable magnets and the improvements in sensitivity achieved with cryoprobes and microcoils, or other methods have contributed to the improvement
of SNR. In addition to hardware factors, SNR is dependent on several factors such as abundance of the nuclei, multiplicity, and magnetogyric ratio $\gamma$. The abundance of the nuclei is determined not only by the number of atoms of the chemical element (e.g. C) studied, but by the natural abundance of the relevant isotope (e.g. $^{13}$C). Couplings between nuclei will cause signals to split into several peaks, reducing the SNR for each individual peak. The SNR is typically proportional to $\gamma^{5/2}$ (see section 2.4.2). For these reasons, the NMR spectra observed for $^1$H are generally those with the highest SNR (as a comparison, the natural abundance of $^1$H is almost 100% while that of $^{13}$C is 1.1%, and $\gamma_{^1H} \approx 4 \times \gamma_{^{13}C}$).

A low SNR makes it difficult to distinguish signals from noise, increases fitting errors in the construction of DOSY spectra and, as a consequence, makes DOSY spectra harder to interpret. Increasing the number of scans allows the enhancement of SNR, but only in proportion to the square root of the number of scans. The time needed for the acquisition of data is potentially a problem for NMR experiments on lower $\gamma$ nuclei like $^{13}$C, but, the information obtained can be useful, or even essential, to determine the structural characteristics of the compounds studied. In DOSY, increased resolution decreases overlap problems, thus DOSY experiments using other nuclei than $^1$H (commonly referred as heteronuclear DOSY), can be valuable tools for analysing complex mixtures. In order to reduce the time needed to acquire such data (i.e. to increase the SNR), there are different options, such as heteronuclear decoupling to collapse multiplets, signal enhancement by nuclear Overhauser effect (NOE), or polarization transfer.
NOE transfers magnetization from a nucleus I to a nearby nucleus S by perturbing the equilibrium population of I. It provides a maximum signal enhancement (given positive $\gamma_I$ and $\gamma_S$) of $\gamma_I/2\gamma_S$ if the dipole-dipole interactions between the low $\gamma$ nuclei S and the nuclei I are the dominant effects in the longitudinal relaxation of the S nuclei. The effects of other competing relaxation mechanisms reduce the signal enhancement.

Polarization transfer techniques transfer nuclear spin polarization from spins I with large Boltzmann population differences (and hence large $\gamma$ due to the increased energy difference $h\gamma B_0/2\pi$ between spin states) to spins S with lower $\gamma$. This allows an improvement in SNR of up to $\gamma_I/\gamma_S$ (independent of $\gamma$ sign). Unlike the NOE, it is independent of relaxation mechanism and it needs a delay time between experiments dependent on the relaxation time of the source, not the destination nucleus. In a basic experiment, magnetization is transferred from spin I to spin S through anti-phase magnetization: spin I magnetization is first converted into transverse magnetization by an RF pulse (Eq. [4.1]).

$$\hat{I}_z \rightarrow \hat{I}_x^{\pi/2 \gamma_I}$$

[4.1]

Then it is allowed to evolve for a period $\tau$; the scalar coupling with S will convert part of this transverse in-phase magnetization into anti-phase magnetization (Eq. [4.2]).

$$\hat{I}_x^{2\pi J_{IS}\tau} \rightarrow \cos(\pi J_{IS}\tau)\hat{I}_x + \sin(\pi J_{IS}\tau)2\hat{I}_y \hat{S}_z$$

[4.2]

A 90º pulse applied to both nuclei (with an orthogonal phase to the previous pulse applied) will convert the anti-phase magnetization transverse in spin I into antiphase magnetization transverse in spin S (Eq. [4.3]).
\[ \sin(\pi J_{12} \tau) 2\hat{I}_y \hat{S}_z \xrightarrow{\frac{\pi J_{12}}{2}(I_z, S_z)} -\sin(\pi J_{12} \tau) 2\hat{I}_z \hat{S}_y \]  

[4.3]

The amount of anti-phase magnetization obtained depends on the sine of \( \pi J/\tau \) for two coupled spins. In the case of an SI\(_2\) system it depends on \(2\sin(\pi J/\tau)\cos(\pi J/\tau)\) and for an SI\(_3\) system it is dependent on \(3\sin(\pi J/\tau)\cos^2(\pi J/\tau)\).

Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) is based on this concept\(^{189}\). Refocused INEPT (Figure 4.1) incorporates 180° pulses to refocus the magnetization, obtaining in-phase multiplets. \(^{13}\)C signals with different coupling constants will be enhanced to different extent, and if not decoupled, the relative intensities of the multiplets do not follow the binomial relationship unless a 90° purging pulse is added\(^{190}\).

\[ \text{Figure 4.1: Refocused INEPT pulse sequence. I and S are normally } ^1\text{H and } ^{13}\text{C respectively} \]

Distortionless Enhancement by Polarization Transfer (DEPT)\(^{191}\) is a technique where the binomial form of the multiplets is undistorted (Figure 4.2). The anti-phase magnetization transverse for spin I is transferred via multiple quantum coherence. The signal amplitude
obtained for each peak depends on the flip angle $\theta$ of the last proton pulse rather than $\pi/J\tau$, as summarized in Table 4.1

Figure 4.2: DEPT pulse sequence. I and S are normally $^1$H and $^{13}$C respectively

The signal intensity obtained using polarization transfer sequences is dependent on the carbon multiplicity, as indicated in Table 4.1 and illustrated in Figure 4.3. This allows spectral editing for structural elucidation; in DOSY, to identify the apparent diffusion coefficient for each signal it would normally be convenient to maximize the signal obtained. A flip angle of 45° has been chosen as a compromise value, but other values may be chosen depending on the signals of interest.
Table 4.1: Signal amplitude dependence of the angle $\theta$ in DEPT experiments (expressions are equivalent to INEPT experiments using $\theta = \pi J\tau$).

<table>
<thead>
<tr>
<th>Spin System</th>
<th>Signal amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI</td>
<td>$\sin(\theta)$</td>
</tr>
<tr>
<td>SI$_2$</td>
<td>$2\sin(\theta)\cos(\theta)$</td>
</tr>
<tr>
<td>SI$_3$</td>
<td>$3\sin(\theta)\cos^2(\theta)$</td>
</tr>
</tbody>
</table>

Figure 4.3: Signal amplitude dependence of the angle $\theta$ in DEPT experiments (expressions are equivalent for INEPT experiments using $\theta = \pi J\tau$).

The INEPT pulse sequence is more sensitive to miscalibrations due to the larger number of pulses and to being dependent on delays for spectral editing (which are dependent on the $J$-
coupling as well).\textsuperscript{191,192} It also has lower overall duration than DEPT (4*1/8J against 3*1/2J, when using an angle $\theta$ of 45º), thus it is possible to achieve slightly higher SNR due to the lower relaxation losses. When $T_2$ times are large enough, DEPT may be a better option due to the lower sensitivity to deviations from the ideal parameters, not only because it is less sensitive to a wide range of coupling constants, but also because it uses fewer pulses.

Standard sequences for DOSY experiments can be combined with these techniques to enhance their sensitivity. A compromise flip angle $\theta$ in the case of DEPT (Figure 4.2), or delay $\tau$ in the case of INEPT (Figure 4.1), has to be chosen in order to achieve a reasonably good SNR for all multiplicities. An angle of 43º would provide the maximum total intensity if all three types of spin systems (SI, SI$_2$ and SI$_3$), had the same abundance, which is rarely the case; but usually the optimum angle will be close to this value or slightly above (SI$_3$ are usually the least common; without these systems, the optimum value is 54º).

4.2. $^{13}$C DOSY experiments

Stilbs proposed\textsuperscript{25} a Pulsed Field Gradient Spin Echo (PFGSE) experiment for $^{13}$C to study mixtures, where, as a contrast with proton experiments, $J$-modulation is not problematic in natural abundance samples, there is usually no solvent signal, $T_2$ relaxation times are longer, and the smaller magnetogyric ratio of the nucleus makes it less sensitive to magnetic field inhomogeneities. However, the low sensitivity of the method (and the strong gradients required for $^{13}$C diffusion encoding) prevented the popularization of this
technique. Although in the experiment performed by Stilbs no $^1$H decoupling was done during acquisition, decoupling is nowadays a standard procedure. The spectrum is less crowded, and therefore easier to analyse, not only for the identification of the peaks but for the DOSY processing as there will be less overlap; and multiplets are collapsed, so the signal-to-noise ratio is increased. When heteronuclear coupling information is required, it is more time-efficient to obtain it in a separate experiment, either in a $^{13}$C spectrum with NOE enhancement or in an edited polarization transfer experiment. In PFGSE sequences, spins experience transverse relaxation throughout the whole sequence, rendering these sequences inappropriate for the study of macromolecules due to the fast transverse relaxation. For larger molecules the use of sequences derived from the stimulated echo is preferred. One example of this type of sequence is the Bipolar-gradient Pulse Pair Longitudinal-Eddy-current Delay (BPPLED) sequence, which has been successfully used for the analysis of hydrocarbon mixtures$^{165,193}$, or Oneshot, used for the analysis of siloxanes$^{136}$. This kind of sequence is employed when it is necessary to obtain diffusion coefficients for quaternary carbons. When this is not essential, polarization transfer sequences will provide increased SNR.

Wu et al. developed the Stimulated Echo-Insensitive Nucleus Enhancement by Polarization Transfer (STE-INEPT), LED-INEPT and LED-Distortionless Enhancement by Polarization Transfer (LED-DEPT) sequences$^{132}$, but their use in publications appears to be scarce, with BPPLED-INEPT the main sequence employed$^{194,195}$. These sequences perform the diffusion encoding with a STE in $^1$H, followed by magnetization transfer to $^{13}$C for detection. Performing diffusion encoding for the $^{13}$C magnetization could be beneficial
when the $T_2$ relaxation times of $^{13}$C are longer than, or at least of the same order as, $T_1$ relaxation times for $^1$H (protons tend to have rapid relaxation rates because the larger $\gamma$ causes stronger dipole-dipole interactions\(^9^5\), which are generally the largest influence on spin-$\frac{1}{2}$ nucleus longitudinal relaxation). Diffusion encoding in $^{13}$C would allow using spin echoes instead of stimulated echoes, doubling the SNR. The simplicity of the spin echo also makes it less vulnerable to pulse miscalibration. These considerations have led here to the development of sequences combining DEPT or INEPT with a gradient spin echo (DEPTSE and INEPTSE respectively, represented in Figure 4.4 and Figure 4.5 and the phase cycle shown in Table 4.2). There are two main factors that limit their use for the analysis of larger molecules. In large molecules the relaxation times are shorter, limiting the length of the diffusion time, and longer gradient pulses are required to achieve an acceptable attenuation to discriminate between signals with different diffusion coefficients. This is caused by the fact that the lower $\gamma$ of the $^{13}$C makes it less sensitive to gradients (about 4 times stronger gradients are required for diffusion encoding in $^{13}$C) as described by the Stejskal-Tanner equation (Eq. [2.32]).
Figure 4.4: DEPTSE pulse sequence. I and S are normally $^1\text{H}$ and $^{13}\text{C}$ respectively.

Figure 4.5: INEPTSE pulse sequence. I and S normally are normally $^1\text{H}$ and $^{13}\text{C}$ respectively.
Table 4.2: Phase cycle used in DEPTSE and INEPTSE pulse sequences

<table>
<thead>
<tr>
<th>Phase\Pulse sequence</th>
<th>DEPTSE</th>
<th>INEPTSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>φ₁</td>
<td>02</td>
<td>(02)₄(20)₄</td>
</tr>
<tr>
<td>φ₂</td>
<td>0₈1₂₃₈</td>
<td>(02)₃(20)₂ (13)₂ (31)₂</td>
</tr>
<tr>
<td>φ₃</td>
<td>1₂2₃₀₂</td>
<td>0₃21</td>
</tr>
<tr>
<td>φ₄</td>
<td></td>
<td>(01)₂(12)₂</td>
</tr>
<tr>
<td>φᵣ</td>
<td>(02)₄(13)₄(20)₄(31)₄</td>
<td>0₁2₃</td>
</tr>
</tbody>
</table>

Although these sequences are presented with hard (high power) pulses, substituting them by adiabatic pulses can be convenient: they excite large bandwidths, are insensitive to RF field inhomogeneity and partially compensate for \(^1J_{CH}\) variations.\(^{196}\) Further improvements could be achieved by introducing a \(J\)-compensated excitation element.\(^{197,198}\) It has to be taken into account that the duration of the adiabatic pulses causes spins with different chemical shifts to be inverted at different times, thus causing phase variations. This effect can be compensated for by using pairs of adiabatic inversion pulses,\(^{199}\) meaning that the last pair of 180° pulses for \(^{13}C\) can be readily substituted by adiabatic inversion pulses.

4.3. Methods

Data were acquired using a mixture with 1-propanol, 1-butanol, 2-pentanol and 3-methyl-3-pentanol with a concentration of 14 mg mL\(^{-1}\) each in deuterated water with 4 mg mL\(^{-1}\) TSP (sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d₄) as a reference. Measurements were carried out non-spinning on a Varian VNMRS 500 spectrometer in an air-conditioned room at approximately 18 °C, with spectrometer temperature regulation set at 23 °C and with an active air preconditioning system used to minimize temperature variations. A reference
sample with 1-propanol with a concentration of 32 mg mL$^{-1}$ and 2-butanol with a concentration of 46 mg mL$^{-1}$ in deuterated water with 4 mg mL$^{-1}$ TSP (sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d$_4$) as a reference was used for signal-to-noise ratio comparisons.

Oneshot measurements on the mixture sample were carried out in 21 min with 12 gradient amplitudes ranging from 6 to 27 G cm$^{-1}$ in equal steps of gradient squared using 16 transients, 16384 complex data points, a total diffusion-encoding gradient duration of 1.4 ms and a diffusion time of 0.1 s. DEPTSE and INEPTSE measurements on the mixture sample were carried out using 10 gradient amplitudes ranging from 3 to 27 G cm$^{-1}$ in equal steps of gradient squared, 1024 transients, 65536 complex data points, pulse flip angle $\theta$ (Figure 5.2) or $\pi/\tau$ for INEPT of 45º, a total diffusion-encoding gradient duration of 6.0 ms and a diffusion time of 0.05 s. Oneshot-DEPT, BPPSTE-DEPT, Oneshot-INEPT and BPPSTE-INEPT measurements on the mixture sample were carried out using 10 gradient amplitudes ranging from 3 to 27 G cm$^{-1}$ in equal steps of gradient squared, 1024 transients, 65536 complex data points, pulse flip angle $\theta$ (Figure 5.2) for DEPT or $\pi/\tau$ for INEPT of 45º, a total diffusion-encoding gradient duration of 1.4 ms and a diffusion time of 0.05 s.

All spectra were manually phase corrected, and either Fourier transformed with a line broadening of 1 Hz Lorentzian in order to minimize the effect of variations in lineshape between spectra with minimal increase in the overlap for $^{13}$C DOSY or reference deconvoluted$^{200}$ to a line width of 1 Hz for $^1$H DOSY. All processing was done using the DOSY Toolbox$^{201}$ in the MATLAB environment.
4.4. Results and discussion

An array of experiments using 64 transients using the reference sample (1-propanol and 2-butanol) was carried out to compare the SNR achievable with the results summarized in Table 4.3. As expected, SE-based pulse sequences showed approximately double SNR over STE-based pulse sequences. INEPT-based experiments offered a marginal gain over DEPT-based experiments.

Table 4.3: SNR achieved in the same conditions with different $^{13}$C DOSY sequences with polarization transfer.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>SNR</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPPSTE-DEPT</td>
<td>207</td>
</tr>
<tr>
<td>BPPSTE-INEPT</td>
<td>225</td>
</tr>
<tr>
<td>Oneshot-DEPT</td>
<td>212</td>
</tr>
<tr>
<td>Oneshot-INEPT</td>
<td>227</td>
</tr>
<tr>
<td>DEPTSE</td>
<td>452</td>
</tr>
<tr>
<td>INEPTSE</td>
<td>460</td>
</tr>
</tbody>
</table>
The $^1$H DOSY spectrum of the mixture with 1-propanol, 1-butanol, 2-pentanol and 3-methyl-3-pentanol is not straightforward to interpret (Figure 4.6). The severe overlap in the region between 0.8 ppm and 1.6 ppm and the very similar diffusion coefficients make it difficult to disentangle the spectra of the individual components. The spread of signals along the diffusion dimension prevents the identification of the number of species in the mixture. Increasing the number of scans would not improve the results; as shown in the plot, the fitting errors in the diffusion dimension are relatively small and results can only be improved by using other methods or increasing the spectral resolution. This mixture was analysed with the proposed $^{13}$C pulse sequences with the results shown in Figures 4.7 and 4.8.
Figure 4.7: \(^{13}\text{C}\) DOSY spectrum of the mixture of 1-propanol, 1-butanol, 2-pentanol and 3-methyl-3-pentanol acquired with DEPTSE (left) and INEPTSE (right)

Figure 4.8: \(^{13}\text{C}\) DOSY spectrum of the mixture of 1-propanol, 1-butanol, 2-pentanol and 3-methyl-3-pentanol acquired with Oneshot-DEPT (left) and Oneshot-INEPT (right)

The best results were obtained with DEPTSE, which showed a signal-to-noise ratio similar to INEPTSE (it might be slightly higher than INEPTSE because of imperfect pulse calibration). Peaks belonging to each of the alcohols are lined up with the corresponding diffusion coefficient, allowing the identification of the molecules (except for quaternary...
carbon signals, which cannot be observed using one-bond polarisation transfer techniques). The SNR for the first increment in Oneshot-DEPT / Oneshot-INEPT experiments is about 200, which is around the threshold of what is acceptable for a good DOSY experiment. Oneshot-DEPT / Oneshot-INEPT DOSY spectra show larger uncertainties in the apparent diffusion coefficients than DEPTSE / INEPTSE results for the same experimental time.

### 4.4. Conclusions

The use of polarization transfer techniques is a successful technique for reducing spectral overlap. In DOSY diffusion encoding through SE sequences has a sensitivity advantage over STE sequences where transverse relaxation in the heteronucleus is of the same order or slower than longitudinal relaxation in $^1$H. It must be noted that to achieve the same attenuation for the case of $^{13}$C, gradients must be 4 times longer if the diffusion delay is kept constant, making diffusion encoding in $^{13}$C only suitable for small molecules if a standard gradient coil unit is used. Nevertheless, doubling the SNR is a considerable step forward for experiments that may take several hours. This technique would excel in the analysis of mixtures of small molecules, where overlap is substantial, such as liquid foods.\textsuperscript{202} Although its use has been illustrated with $^{13}$C as heteronucleus, under the right conditions it should be feasible to use it for other nuclei such as $^{29}$Si.\textsuperscript{203}
Chapter 5
Multiway DOSY

Above all, it's creative thinking that lies at the basis of discoveries. You must dare to think differently, see things from different sides, in order to come across fortuitous new ideas frequently. You should develop even the most stupid ideas and when you do this systematically, there will always come something useful out of it.

Simon van der Meer
5. Multiway DOSY

The previous two chapters have dealt with improving resolution, sensitivity and data quality in order to obtain resolved component spectra from DOSY data. Sometimes signal overlap is unavoidable and the differences in diffusion coefficient of the components are small. It is then necessary to gather additional information to increase the resolving power. If this new information is added as another dimension in the dataset, a multiway dataset is created that, given certain conditions, can be analysed with a multiway method to extract the component spectra. If the data are trilinear, i.e., each of the three dimensions varies independently, it is possible to analyse the dataset with the PARAllel FACtor (PARAFAC) method to create a model of data variations in each dimension. Each independent variation constitutes a component, as in standard multivariate analysis, but with the characteristic that under suitable conditions each component represents one of the detectable species in the mixture. PARAFAC is ‘model-free’, in the sense that no prior knowledge is needed about the variations in each dimension. If DOSY experiments are run on a set of samples that have different concentrations for each species, the spectra will form a trilinear dataset. A PARAFAC analysis on this dataset can extract the spectrum, diffusion decay and concentration profile for each of the components.\(^{139,140,204,205}\) Here, two other alternatives which do not require a set of different samples as an additional dimension are evaluated.

In the first of these alternatives longitudinal relaxation provides the added contrast. Relaxation and diffusion have already been used to study sets of samples without spectral resolution,\(^{206}\) so relaxation should be a feasible candidate to a third dimension for a DOSY dataset. However, relaxation behaviour is different for different chemical sites, i.e., for
different multiplets, thus making the dataset only locally trilinear. It is necessary to divide the dataset in such a way that each segment contains a maximum of one multiplet per component. PARAFAC is applied in each region, and then the complete spectra can be recovered by concatenating the spectra of those components that present the same diffusion behaviour.

The other alternative uses the evolution of the concentrations of components for a reacting sample as a third dimension, so that spectra, diffusion behaviour and kinetics can be extracted. Traditionally, reactions have been monitored using other spectroscopies such as UV-VIS, IR or Raman, but it is possible to extract additional chemical information by monitoring reactions by NMR, and in some instances it may be easier to extract quantitative information.\textsuperscript{207,208} This has led to the use of NMR to study reactions in different conditions and for different purposes,\textsuperscript{209-218} but there are still at least 4 limiting factors for widespread use: sensitivity, resolution, cost, and timescale that can be monitored. NMR spectrometers are notably more expensive to buy and operate than other spectrometers, but can also provide information that other methods cannot. Some reactions occur too fast to be monitored by NMR. Sensitivity prevents NMR from detecting low concentrations, and also limits the usage of NMR for studying fast reactions; resolution is an obstacle to distinguishing resonances from different environments, and the better resolution and sensitivity that a spectrometer can provide, the more expensive it becomes. The method presented here requires sensitivity and resolution orders of magnitude lower than conventional experiments, allowing more reactions to be studied (even if they have highly overlapped spectra) and reducing the cost.
N.B. In this chapter, arrays of 2 or more dimensions will be underlined to distinguish them from vectors. Experimental work with sucrose reactions was carried out by Maryam Khajeh, and additional analyses are presented in her thesis. 78

5.1. Multivariate analysis

Multivariate analysis\(^{219}\) is a statistical tool used to study how a system is affected by several variables at a time. In standard multivariate analysis, data are arranged in a two-way structure. However, some data would benefit from a structure in three ways or more. A two-way structure typically describes two characteristics at a time (e.g. the sample number and its spectrum, or the spectrum and its diffusion decay), while a general multi-way can describe the evolution of several characteristics at once (e.g. the variation of the spectrum with diffusion decay and with relaxation).

Several methods are available for performing multi-way analysis and are discussed below. Principal Component Analysis (PCA)\(^{220-224}\) is one of the most widely used for standard two-way analysis. A generalisation of PCA to more than two dimensions (multi-way) can be done with Tucker models,\(^{225,226}\) but here the PARAllel FACtor analysis (PARAFAC) will be described, which can be used to analyse data efficiently where the evolution in each dimension is independent. More information about these and other models for multi-way analysis was detailed by Bro\(^{32,159,227}\).
5.1.1. Principal Component Analysis (PCA)

Principal Component Analysis is a two-way analysis in which the two dimensional data ($X$) are decomposed into a set of score ($S$) and loading ($L$) vectors, as shown in equation [5.1]:

$$X = \sum_{p=1}^{P} S_p \otimes L_p + R$$  \[5.1\]

where $R$ is the residuals matrix and $p$ is one component of a total of $P$ components. This decomposition can be carried out using algorithms like Singular Value Decomposition (SVD), Nonlinear Iterative Partial Least Squares (NIPALS), power method or EigenValue Decomposition (EVD).\textsuperscript{228,229} Figure 5.1 is a pictorial representation of a two-component analysis.

![Figure 5.1: Graphical representation of PCA analysis with 2 components](image)

In all bilinear models, including PCA, there is a problem of rotational freedom. This means that there is an infinite range of solutions, which can be interconverted by non-trivial (i.e., not limited to combinations of permutation and scaling matrices) linear transformations, and still fit the data equally well. Thus, it is only possible to obtain physically meaningful solutions of the system by applying constraints on the bilinear model that use prior knowledge about the underlying phenomena. Understanding bilinear models is not always straightforward, and this has led to common misunderstandings on data interpretation.\textsuperscript{230} A
method that could provide models with physical meaning would, therefore, be less liable to misinterpretation.

5.1.2. PARallel FACtor analysis (PARAFAC)

Catell introduced the principle of parallel proportional profiles in 1944;\(^{231}\) this states that the same set of loading vectors describing the variation in more than two dimensions, only in different proportions, will lead to a model that is not subject to rotational freedom. He argued that this is the fundamental property for obtaining meaningful decompositions. This was the basis followed by Harshman\(^{31}\) to develop PARAFAC (PARallel FACtor analysis), which was formulated independently by Carroll and Chang as CANDECOMP (CANonical DECOMPosition).\(^{232}\) This model is also known as trilinear decomposition,\(^{233}\) but can easily be extended to higher dimensions.

A three-way PARAFAC analysis requires a trilinear dataset to provide a unique parameterization of the model; this means that the elements \(x_{ijk}\) of the three-dimensional dataset \(X\) can be decomposed into a product of 3 independent factors, i.e.:

\[
x_{ijk} = \sum_{p=1}^{P} a_{ip} b_{jp} c_{kp} + r_{ijk}
\]

[5.2]

where \(r\) is the corresponding residual and \(a\), \(b\) and \(c\) are the elements of the loading matrices \(A\), \(B\) and \(C\) respectively, representing the parameters of the system. If the data are, at least approximately, trilinear, parameters with physical meaning can be found given acceptable signal-to-noise ratio.\(^{234,235}\) The rank would be the number of components to fit \(X\)
exactly, i.e. fitting even the noise; this is not very practical for chemometrics applications. The minimum number of components \( P \) that describes \( X \) approximately is the pseudo-rank of \( X \). A further definition is the k-rank of the loadings, which is related to the uniqueness of the solution. The k-rank of a matrix is given by the rank in which all subsets of the matrix have linearly independent columns. A first approach is that unique solutions can be expected if “the loading vectors are linearly independent in two of the modes, and in the third mode no two loading vectors are linearly dependent”.\(^{234,236}\) Kruskal has given a less restricted condition for uniqueness: if the sum of the k-ranks of the loadings \( k_1+k_2+k_3 \) is equal to or bigger than \( 2P+2 \), then the PARAFAC solution is unique;\(^{237,238}\) e.g. if only 2 datapoints have been acquired in each dimension, the maximum number of components that can be found by PARAFAC without rotational ambiguity is 2. However, new studies keep appearing in relation to uniqueness conditions,\(^{239}\) showing that there are still many aspects of PARAFAC that remain unknown, and it is not simple to evaluate where the limits are within which PARAFAC analysis will be successful.

A PARAFAC model of a three-way array can be described by the equation:

\[
X = \sum_{p=1}^{P} A_p \otimes B_p \otimes C_p + R
\]  

[5.3]

where \( A_p, B_p, \) and \( C_p \) are the \( p \)th columns, corresponding to the component \( p \), of the matrices \( A, B \) and \( C \). Figure 5.2 represents this equation:
The solution to the PARAFAC model can be found by alternating least squares (ALS)\textsuperscript{240} by successively assuming the loadings in some modes known, and then estimating the unknown set of parameters of the last mode until no change is observed in the parameter values or in the fit of the model to the data. Other algorithms can also be used, but there is no one option clearly better than the others, and ALS represents a good trade-off between computational expense and quality of solution.\textsuperscript{241,242}

The number of components is the basic input required to obtain a PARAFAC model of the data. If this is unknown, different PARAFAC models with different numbers of components must be evaluated, and ideally, the best model will be the one with the highest number of components giving a unique solution. There are methods available for estimating the right number of components for the PARAFAC model, like CORe CONsistency DIAgnostic (CORCONDIA),\textsuperscript{243} based on evaluating the magnitude of the variation described by the components, evaluation of the magnitude of the residuals with the number of components,\textsuperscript{244} half-split analysis,\textsuperscript{32} cross-validation\textsuperscript{245} and comparison with prior knowledge. Given that none of these methods gives a particularly good estimation, there is still plenty of ongoing research to provide better methods, and new general algorithms developed for bilinear models may be valuable as well.\textsuperscript{246}
5.2. $T_1$-DOSY

Relaxation is one of the few intrinsic properties of spins that can be analysed without additional equipment. It is a good candidate to provide more information about a sample, as an addition to the spectral and diffusion data provided by DOSY analysis. Although relaxation and diffusion properties have been used together extensively for the characterization of different materials through measurements on water,$^{206,247-252}$ and relaxation properties have been used to study the structures of complex molecules,$^{253,254}$ and even to analyse paramagnetic mixtures in EPR spectroscopy$^{255,256}$, to the best of our knowledge there are no publications showing DOSY data with high resolution in chemical shift using relaxation as an added contrast.

It is possible to study either transverse relaxation or longitudinal relaxation, or even both. However, the analysis of spectra with transverse relaxation weighting is more complex, with problems like the $J$-modulation, described in chapter 3, that would break the trilinearity of the model. Taking this into account, $T_1$-DOSY is proposed, where a three-way NMR dataset dependent on chemical shift, diffusion and longitudinal relaxation is acquired and analysed with a multi-way method (e.g. PARAFAC). This model-free method can extract the true underlying phenomena, i.e. the spectra, the diffusional decays and the relaxation evolutions.

Longitudinal relaxation is not normally the same for all the multiplets in a spectrum, or even for all lines within a given multiplet; this limits the method to being applied only to
one multiplet per component at a time. However, the resolved spectra from the multiplets with the same diffusion coefficient (obtained from the fit of the diffusion decay loadings) may be combined to recover the spectra of the components of the mixture. Figure 5.3 represents a typical dataset, where two triplets of different species are overlapped, and its decomposition with PARAFAC. Three different pulse sequence schemes, based on the Oneshot sequence, are proposed in the next three subsections for acquiring the three-way dataset.

Figure 5.3: Representation of a $T_1$-DOSY dataset with two overlapped triplets belonging to two different species and its decomposition with PARAFAC
5.2.1. Inversion Recovery ONeshot (IRON)

A $T_1$-DOSY pulse sequence was developed concatenating the inversion recovery\textsuperscript{90} and Oneshot sequences, by adding a 180° pulse and a recovery delay $\tau$ before the Oneshot sequence, as shown in Figure 5.4. This sequence is analogous to the $T_1$-weighted spin echo that has been used for the determination of water diffusion coefficients.\textsuperscript{257} A range of $\tau$ values is used to obtain the relaxation encoding, and for each $\tau$, different gradient strengths encode the diffusion.

![Figure 5.4: Inversion Recovery ONeshot (IRON) sequence. Relaxation is encoded by varying the recovery delay $\tau$. Diffusion is encoded by varying the gradient strength $G$. Phase cycling of the first 180° pulse is as the first 90° pulse.](image)

This sequence requires a long initial delay to remove any magnetization remaining from previous experiments, and it is not possible to use reference deconvolution\textsuperscript{72,74,200} when the reference signal is close to a null. However, the method gives a high dynamic range in the relaxation dimension.
5.2.2. SAturation Recovery ONeshot (SARON)

An alternative experiment to IRON is the combination of a saturation recovery experiment and Oneshot, introducing a saturation pulse and a recovery delay at the beginning of the latter sequence as shown in Figure 5.5. A range of $\tau$ values is used to obtain the relaxation encoding, and again, for each $\tau$ value, different gradient strengths encode the diffusion.

![Diagram of SARON sequence](image)

Figure 5.5: SAturation Recovery ONeshot (SARON) sequence. Relaxation is encoded by varying the recovery delay $\tau$. Diffusion is encoded by varying the gradient strength $G$. Phase cycling of 90°, pulse is 0213, and 1320 for 90°.

This sequence allows data to be acquired in less time than IRON. However, it requires a good saturation sequence to obtain good data.
5.2.3. Decaying Relaxation ONEshot (DRONE)

This reuses the Oneshot sequence, but adapted to acquire data for $T_1$-DOSY experiments. The delay $\tau$, now located between the second and third 90° pulses, is included in the diffusion time. The diffusion is encoded by varying the applied gradient strength. The relaxation is encoded by increasing the diffusion time $\Delta$, while keeping the product $G^2\Delta'$ constant for a given level of diffusion encoding, to maintain the same diffusional attenuation for any $\tau$, according to equation [2.32].

![Diagram of Decaying Relaxation ONEshot (DRONE) sequence]

Figure 5.6: Decaying Relaxation ONEshot (DRONE) sequence. Relaxation is encoded by varying the recovery delay $\tau$. Diffusion is encoded by varying the gradient strength $G$.

By reusing a routine DOSY sequence, no coding of a new sequence is required, and the phase cycling is reduced compared with the other $T_1$-DOSY sequences, as no pulse is added.
5.2.4. $T_1$-DOSY of two alcohols

Medium-chained alcohols are suitable candidates for a test mixture to evaluate the $T_1$-DOSY experiment as they have a range of diffusion coefficients and relaxation times as well as the desired spectral overlap; notably the methyl protons have very similar chemical shifts (~0.9 ppm). The spin-lattice relaxation times and diffusion coefficients for two alcohols (1-propanol and 3-methyl-3-pentanol) were investigated. Three samples were prepared (one with 1-propanol, one with 3-methyl-3-pentanol and a third with both) by adding one drop of alcohol (approximately 15 mg) to 1 ml 0.4% (w/v) aqueous TSP (sodium 3-(trimethylsilyl)-propionate-2,2,3,3-$d_4$) and transferring into a 5 mm NMR tube.

Measurements were carried out without temperature regulation and non-spinning on a Varian Inova 400 MHz spectrometer in an air-conditioned room at approximately 20 ºC, without spectrometer temperature regulation and with a passive probe air preconditioning system used to minimize temperature instabilities. $T_1$ relaxation times were determined by standard inversion recovery experiment using 12 different recovery delays as shown in Table 5.1. Diffusion experiments were carried out using the Oneshot sequence with an imbalance factor ($\alpha$) of 0.2 for the diffusion-encoding gradient pulses, a diffusion delay ($\Delta$) of 0.2 s, a diffusion-encoding pulse width ($\delta$) of 2 ms, and 10 gradient strengths ranging from 3.0 to 27.0 G cm$^{-1}$ (0.03 to 0.27 T m$^{-1}$) in equal steps of gradient squared.
Table 5.1: Longitudinal relaxation times $T_1$ in 1-propanol and 3-methyl-3-pentanol reference samples

<table>
<thead>
<tr>
<th>Freq. / ppm</th>
<th>$T_1 / s$</th>
<th>Freq. / ppm</th>
<th>$T_1 / s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.58</td>
<td>4.89 ± 0.05</td>
<td>1.53</td>
<td>1.12 ± 0.01</td>
</tr>
<tr>
<td>3.56</td>
<td>5.09 ± 0.07</td>
<td>1.51</td>
<td>1.28 ± 0.02</td>
</tr>
<tr>
<td>3.55</td>
<td>4.93 ± 0.06</td>
<td>1.49</td>
<td>1.29 ± 0.03</td>
</tr>
<tr>
<td>1.6</td>
<td>4.46 ± 0.04</td>
<td>1.47</td>
<td>1.14 ± 0.02</td>
</tr>
<tr>
<td>1.58</td>
<td>4.74 ± 0.04</td>
<td>1.14</td>
<td>1.12 ± 0.01</td>
</tr>
<tr>
<td>1.56</td>
<td>4.92 ± 0.04</td>
<td>0.88</td>
<td>1.62 ± 0.03</td>
</tr>
<tr>
<td>1.54</td>
<td>4.92 ± 0.04</td>
<td>0.86</td>
<td>1.71 ± 0.04</td>
</tr>
<tr>
<td>1.53</td>
<td>4.77 ± 0.05</td>
<td>0.84</td>
<td>1.64 ± 0.04</td>
</tr>
<tr>
<td>1.51</td>
<td>4.50 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.92</td>
<td>4.42 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>4.69 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.88</td>
<td>4.42 ± 0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The measured $T_1$ values in the alcohols vary within the multiplets, with a tendency for the outer lines to have a shorter $T_1$, as explained in section 2.7.4. If these effects were due to cross-correlated relaxation between CSA and dipole-dipole interactions, they would be suppressed by a nonselective 90° read-pulse\textsuperscript{87}, but this was not achieved. As an attempt to investigate this, inversion-recovery experiments were repeated using a better composite 90° pulse, but with similar results. The use of a smaller flip angle pulse did not provide statistically significant different results.

For $T_1$-DOSY experiments all three sequences were used. IRON experiments were carried out with 12 gradient strengths $G$ (ranging from 3.0 to 27.0 G cm$^{-1}$ in 12 equal steps of gradient squared) and 8 relaxation delays ($\tau$ ranging from 0.25 to 32 seconds) making a total of 96 spectra, acquired with 16384 complex data points and 16 transients, with an
initial delay $d_1$ of 36 s between experiments. The total acquisition time was 20 h 13 min. SARON was run with 12 gradient strengths ($G$ ranging from 3.0 to 27.0 G cm$^{-1}$ in 6 equal steps of gradient squared) and 10 recovery delays ($\tau$ ranging from 0.1 to 51.2 s) making a total of 36 spectra, with an initial delay $d_1$ of 0.5 s and acquired with 16384 complex data points and 16 transients. The total acquisition time was 6 h. A combination of 2 long pulses for saturation of approximately 750 µs each gave the best results. A DRONE experiment was acquired in only 2 min using 2 recovery delays (0.2 and 3.2 s, selected in order to achieve about 80% attenuation) and 2 gradient amplitudes (3 and 27 G cm$^{-1}$ for the first level of relaxation encoding) using 4 transients, 16384 complex data points, and a delay $d_1$ of 5 s.

All spectra were manually phased, reference deconvoluted with a target line shape of a 2 Hz Lorentzian (except for the IRON data set, where a line broadening of 2 Hz was applied without reference deconvolution) and baseline corrected in VnmrJ 2.2C before exported to a text file. This file was imported into Matlab$^{259}$ and processed with a series of macros and functions created to process such data using the PARAFAC function from the N-way Toolbox.$^{260}$

The DOSY spectrum of this mixture is pictured in Figure 5.7. Apparent diffusion coefficients of well-resolved peaks are not distorted, but the overlap at 0.9 and 1.5 ppm causes the apparent diffusion coefficients for overlapped peaks to adopt compromise values in between those of the overlapping multiplets of the two alcohols. A multivariate method like SCORE can resolve overlapped regions, so it was tried to check how it deals with this problem and the result is plotted in
Figure 5.8 along with reference samples for comparison. Species can be identified, but there is some crosstalk between components even for non-overlapped regions.

Figure 5.7: $^1$H DOSY spectrum of the mixture of 3-methyl-3-pentanol and 1-propanol in D$_2$O.

Figure 5.8: (b) Reference and (d) extracted SCORE spectra of 3-methyl-3-pentanol and (c) reference and (e) extracted SCORE spectra of 1-propanol compared with the spectrum (a) of the mixture of the two alcohols. Reference spectra were measured using samples of the individual alcohols, and extracted spectra were obtained by SCORE processing of the data used to produce Figure 5.7.
IRON experiments (Figure 5.9A) provide the largest dynamic range but, in principle, it is not possible to perform reference deconvolution on them. Although it would be possible to add the last spectrum in a set of relaxation experiments to all previous experiments so all data would be positive, small differences in chemical shift variations between different resonances result in distortions that cannot be corrected by reference deconvolution. Lineshape variations due to stronger gradients and frequency drifts caused by small temperature fluctuations are some of the problems that result in data deviations from strict trilinearity, resulting in significant cross-talk between components. SARON results (Figure 5.9B) are cleaner thanks to the use of reference deconvolution, but achieving clean saturation is not always a simple task. Here, saturation was achieved using an orthogonal pair of long pulses (about 700 µs each).

The DRONE experiment (Figure 5.9C) does not require altering a sequence and can, in principle, provide the cleanest data (except where a longer diffusion delay has effects other than those of diffusion and relaxation, as in the case of chemical exchange). Only two points in each of the diffusion and relaxation dimensions are necessary to distinguish the signals from the two alcohols (as described by the Kruskal condition mentioned in section 5.1.3). The problem of having only two points is that no error estimates can be extracted from the fit, so the error used to produce the DOSY-like plot was calculated from equation [5.4]

$$\sigma_B = D\sigma_n \frac{1}{\sqrt{\frac{1}{I_1} + \frac{1}{I_2}}} \ln\left(\frac{I_1}{I_2}\right)$$

[5.4]
where $\sigma_n$ is the average noise level of the experimental data and $I_1$ and $I_2$ are the amplitudes of the two data points. The calculated error for the apparent diffusion coefficient $\sigma_D$ in the DRONE experiment was set to 5 times the calculated error, in order to compensate for factors other than SNR.

5.2.5. $T_1$-DOSY of a mixture with quinine, camphene and geraniol

A more complex mixture was also evaluated using natural products. These were camphene (20 mM), geraniol (23 mM), and quinine (19 mM), dissolved in methanol-$d_4$ with TMS as a chemical shift reference. A DRONE45 (DRONE using Oneshot45$^{186}$ pulse sequence) experiment was run with 5 gradient strengths ($G$ ranging from 10.0 to 27.0 G cm$^{-1}$ in 3...
equal steps of gradient squared) and 5 relaxation delays (τ ranging from 0.2 to 3.2 s) making a total of 25 spectra, with an initial delay d1 of 5s were acquired with 16384 complex data points and 16 transients. The total acquisition time was 1 h 7 min. Similar results were achieved with a shorter experiment of 23 min using 3 gradient strengths and 3 relaxation delays and also using the sequence Oneshot instead of Oneshot45.

This is a mixture that was previously resolved using DOSY-HMQC\textsuperscript{135} and SCORE\textsuperscript{128}, both with experiments of about 17 h. The T\textsubscript{1}-DOSY approach allows distinguishing the majority of spectral features for each component in the mixture with a notably shorter acquisition time. The results are displayed as a DOSY plot in Figure 5.10 and as 1D spectra in Figure 5.11. Most of the distinguishing features of the mixture components were identified; and, unlike with SCORE, the HOD and methanol signals can be identified as different components. Around 1 ppm the extraction of components is slightly less good, possibly due to a combination of factors such as severe overlap of signals with low SNR.
Figure 5.10: DOSY representation of the $T_1$-DOSY results for a mixture of quinine, camphene and geraniol.

Figure 5.11: Components extracted from a mixture of quinine, camphene and geraniol using $T_1$-DOSY, showing the segmentation and number of components analysed in each region. This representation obtained with the $T_1$-DOSY tools (see section 5.4) was only edited to label the components.
5.3. Timecourse DOSY

Understanding the kinetics of reactions is fundamental in order to optimize them, facilitating cheaper production processes, scale-up and reaction monitoring. One approach for extraction of kinetics consists of using a spectroscopic method to monitor the evolution of a spectral region to extract the kinetics. Information from other possible side-reactions may go unnoticed or may be difficult to interpret with this method. A different way to achieve this information relies on taking samples at different points of the reaction, quenching it, and performing a quantitative analysis, but this is time consuming and not suitable for on-line monitoring. NMR allows a non-invasive and non-destructive analysis of the reaction while providing detailed information on the chemical structures involved once they are resolved. This is straightforward for the simplest reactions where there is minimal overlap between signals, allowing peaks to be integrated and, therefore, providing quantitative information. Unfortunately spectral overlap is present in most reactions, making it necessary to use some method to distinguish between different signals. Multivariate approaches allow monitoring the reaction but may be difficult to physically interpret due the rotational ambiguity. Here, a multiway approach is presented that relies on time and diffusion resolution of NMR signals.

NMR spectra are recorded as a function of time and gradient amplitude, as illustrated in Figure 5.12, to form a trilinear dataset that can be analysed with PARAFAC, allowing the extraction of the spectrum, time evolution and diffusion behaviour for each component. Here, the practicalities of this combination of methods are explored, and its remarkable robustness when analysing data with limited signal-to-noise ratio (S/N) is illustrated.
Four samples with maltotriose and sucrose in different concentrations were prepared to test time-course experiments, all with D$_2$O as solvent: maltotriose 18 mM, pivalic acid 25 mM and sulphuric acid 90 mM; sucrose 540 mM, tert-butanol 135 mM and sulphuric acid 224 mM; sucrose 50 mM, tert-butanol 105 mM and sulphuric acid 464 mM; and sucrose 1 mM, tert-butanol 82 mM, and sulphuric acid 592 mM. All hydrolyses were carried out in thick-walled 5 mm NMR tubes to discourage convection. A 400 MHz Varian Inova spectrometer was used for all measurements. Maltotriose data were acquired at 50 °C and sucrose datasets were acquired without temperature control in an air-conditioned laboratory at about 20 °C, always using a passive probe air preconditioning system to minimize temperature instabilities$^{258}$. One shot experiments were run using the One shot sequence with
6 gradient levels with equal steps in gradient squared, ranging from 3.0 to 27.3 G cm\(^{-1}\). 130 DOSY experiments with a diffusion encoding gradient pulse duration \(\delta\) of 2 ms, a diffusion time \(\Delta\) of 0.2 s, using 32 transients in a total time of 52 h were used to analyse the maltotriose reaction. 46 DOSY experiments with a gradient pulse duration \(\delta\) of 3 ms, a diffusion time \(\Delta\) of 0.1 s, using 16 transients in a total time of 6 h were used to analyse the 540 mM sucrose reaction. 110 DOSY experiments with a gradient pulse duration \(\delta\) of 3 ms, a diffusion time \(\Delta\) of 0.1 s, using 16 transients in a total time of 16 h were used to analyse the 50 mM sucrose reaction. 350 DOSY experiments with a gradient pulse duration \(\delta\) of 3 ms, a diffusion time \(\Delta\) of 0.1 s, using 4 transients in a total time of 15 h were used to analyse the sucrose hydrolysis for the 1 mM sample.

All spectra were Fourier transformed, reference deconvoluted\(^7\) using 2.2 Hz line broadening, and baseline corrected in the spectrometer software Vnmr 6.1C. Data were then exported to Matlab\(^{15}\) as a text file for PARAFAC analysis in the MATLAB environment using the Timecourse DOSY tools, based on the N-way toolbox \(^{256,263}\), described in section 5.4.

### 5.3.1. Timecourse DOSY of sucrose hydrolysis

Sucrose hydrolyses into glucose and fructose. Since glucose and fructose have very similar diffusion coefficients (same molecular weight and similar structure) and their concentrations profiles are the same, they cannot be distinguished with this method and will be detected as one unique component. The spectra from PARAFAC components obtained
with the hydrolysis of sucrose at 540 mM showed some cross-talk with peaks of different apparent phase (Figure 5.13). The nature of these peaks suggests a frequency shift of the resonances. Temperature stability often causes frequency drifts and could be an issue in very exothermic reactions, but it is mostly corrected with reference deconvolution, and the temperature variation in this experiment was smaller than in other experiments where no significant cross-talk was found. This suggested a different origin for the frequency drift. Reducing the concentration decreased the cross-talk of the component spectra. It can be inferred that the cause of this cross-talk was concentration-dependent changes in chemical shifts during the reaction.

Figure 5.13: Spectra obtained with PARAFAC analysis for the hydrolysis of sucrose a) 540 mM and b) 50 mM compared with c) reference spectra of sucrose and an equimolar mixture of glucose and fructose. The cross-talk present at 540 mM hydrolysis have been reduced when the reaction was carried out at lower concentration.
The hydrolysis of 1 mM sucrose was investigated to evaluate the performance of PARAFAC decomposition in noisy datasets. An excerpt from the experimental data is shown in Figure 5.14, where the low SNR is evident (and below the nominal detection level). In this situation most methods would fail, but the effective signal-to-noise ratio of the PARAFAC outputs benefits from the averaging of all datapoints used. The spectral features of the resultant components (Figure 5.15 and Figure 5.16) can be clearly distinguished with the superior SNR (around $\sqrt{350}$ improvement over the average of the array of spectra in any DOSY subset of the experiment). A resulting SNR of about 50 was enough for PARAFAC to extract successfully both components in this mixture. Noisier data resulted in the extraction of only one component (which was a mixture of sucrose, fructose and glucose), although the SNR requirements could be reduced even further by applying constraints. In this case non-negativity constraints in all modes were enough to reduce even further the SNR requirements for a successful decomposition. Thus, this method is a good choice for analysis where the concentrations of the reagents are low but that can still provide an acceptable SNR with a reasonable number of scans. This is typical where there is a limited amount of reagents or their solubility is low, but it would also be useful for decreasing spectral variation such as that caused by reaction-dependent pH changes, temperature variations or intermolecular interactions.
Figure 5.14: A subset of the raw experimental data for the acid hydrolysis of 1mM sucrose, showing attenuation of the spectrum over five gradient amplitudes at three times during the timecourse.

Figure 5.15: Modes obtained with the Timecourse DOSY Toolbox for the acid hydrolysis of 1 mM sucrose. Diffusion coefficient and scaling factor are shown for each component.
5.3.2. Timecourse DOSY of maltotriose hydrolysis

The analysis of the hydrolysis of maltotriose is challenging because of the severe spectra overlap between the three species involved and their similar diffusion coefficients (the ability to measure a complex mixture like this one and being able to resolve the signals from a single analyte has been referred as the cocktail party effect\textsuperscript{205}). Maltotriose ($g_3$) decomposes into glucose ($g$) and maltose ($g_2$), which in turn decomposes to glucose. The kinetics of the reaction can be modelled by:

\[
\begin{align*}
\frac{d[g_3]}{dt} &= -k_1[g_3] \\
\frac{d[g_2]}{dt} &= k_1[g_3] - k_2[g_2] \\
\frac{d[g]}{dt} &= k_1[g_3] + 2k_2[g_2]
\end{align*}
\]

[4.5]
where $k_1$ and $k_2$ are the first order rate constants representing the kinetics for this system. This set of differential equations was solved in Matlab [ \texttt{dsolve('Dg3=-k1*g3','Dg2=k1*g3-k2*g2','Dg=k1*g3+2*k2*g2','g3(0)=g30','g2(0)=g20','g(0)=g0')} ] and the simplified solutions were used to fit the concentration evolutions obtained with PARAFAC analysis to extract the rate constants. This experiment was repeated twice. In the first dataset the component spectra were as expected, but it was necessary to use non-negativity constraints in order to get positive concentrations at all timepoints, and the apparent diffusion coefficients of the components were not correct (maltotriose was apparently diffusing faster than maltose). The reason for these problems was found to be a 2°C drift in temperature during the experiment; this altered the diffusion coefficients of the substances while they were reacting, thus breaking the trilinearity. It was possible to obtain a successful decomposition by compensating for this variation as explained in section 5.4. The experiment was repeated with a more stable temperature (a variation of 0.5°C). PARAFAC analysis revealed correct component spectra and apparent diffusion coefficients, but the concentration profiles revealed a negative component as in the previous experiment, as pictured in Figure 5.17. Non-negativity constraints applied to all modes revealed Figure 5.18, with improved and physically meaningful kinetics statistics. This might be explained by a minimum in the evaluating function of the PARAFAC model introduced by the temperature anomaly. PARAFAC analysis will present a physically meaningful minimum when there are no temperature variations, but with these temperature variations another minimum appeared and the new minimum became the absolute minimum. Non-negativity constraints altered the evaluating function causing the minima to change, thus finding the
physically meaningful minimum in this case. If the absolute minimum had remained the same with non-negativity constraints, the negative datapoints would have been zeroed.

**Figure 5.17:** First order kinetic analysis obtained with the Timecourse DOSY Toolbox without using non-negativity constraints. All data were fitted simultaneously to obtain unique rate constants. Temperature instability causes some deviations from the fit in the concentration evolution of all components, but it is especially noticeable in the maltose concentration profile.

**Figure 5.18:** First order kinetic analysis obtained with the Timecourse DOSY Toolbox using non-negativity constraints. All data were fitted simultaneously to obtain unique rate constants.
Figure 5.19: Spectra of the components obtained for the acid hydrolysis of maltotriose using PARAFAC with the Timecourse DOSY toolbox and non-negativity constraints.

5.4. $T_1$ and Timecourse DOSY tools

A series of MATLAB functions and macros was developed for processing the datasets generated in this chapter and creating publication quality plots. It uses the Matlab implementation of PARAFAC included in the N-way Toolbox. These $T_1$ and Timecourse DOSY tools allow the importing of processed data from VnmrJ and from the DOSY Toolbox.

For $T_1$-DOSY experiments it is necessary to specify the regions to be analysed and the number of components in each of them. It may also be necessary to modify the options for
the DOSY-like plot. With this information the package can automatically extract the PARAFAC components in each region, determine the experiment type (IRON, SARON or DRONE), fit the diffusion decay and relaxation evolution to obtain the apparent diffusion coefficients and $T_1$s, and scale the modes (so the diffusion mode starts at unity for zero gradient strength and the relaxation mode starts at unity (DRONE experiments) or ends at unity (IRON and SARON experiments). It is then possible to plot a DOSY-like graphic with these data, or identify the full spectrum for each global component. This was initially done by sorting all the components obtained according to their diffusion coefficients and assuming that those that have a difference in diffusion coefficient lower than 5% (or any other chosen factor) belong to the same global component. Figure 5.10 and Figure 5.11 are examples of the graphical outputs generated with these tools.

For Timecourse-DOSY experiments other options are taken into account; most of them can be modified in the interface shown in Figure 5.20. Appendices C and D show the rest of the code used in this toolbox for a simple analysis. The inputs required are the number of components present and the spectral range to be used in the analysis. Limiting the spectral range to the region of interest reduces the already low calculation time (of the order of seconds, or minutes if non-negativity constraints are used), especially if other known components are present, such as solvent signals. It is usually convenient to leave such signals out of the analysis because they are more sensitive to external variations that can violate data trilinearity, e.g. frequency shifts due to temperature changes. For kinetic analysis it is necessary to specify the functions that the estimated concentration profiles should be fitted to.
There is a series of additional parameters useful to obtain optimal results. In addition to selecting the spectral range, the timepoints and gradients increments used can be chosen, for example to remove from the analysis points affected by systematic deviations, or to run cross-validated analyses. A reference region can be chosen for normalization purposes. Small variations in receiver sensitivity over the course of the experiment can be corrected for by normalising the integral of the spectrum for each gradient level dividing by the area of the reference peak extrapolated to zero gradient strength, or by the integral of the whole spectrum at the lowest gradient strength acquired. Another approach multiplies each spectrum by the integral of the reference averaged at each gradient strength for all timepoints, and divides it by the integral of the reference; this compensates for variations in
the attenuation of the signals caused by external factors like temperature changes. Auxiliary plots can be generated to evaluate the variation of the integral and apparent diffusion coefficient of the reference.

Publication quality plots of the PARAFAC output are automatically generated, taking into account additional information such as the gradient strengths given in the data and the time used for each experiment specified in the macro. The diffusion mode is fitted, obtaining a diffusion coefficient for each component, and scaled such that the fitted curve extrapolates to unity at zero gradient strength. The spectral mode is scaled to unit integral; this choice can be changed once components have been identified, to reflect the relative numbers of nuclei. The timecourse mode is then rescaled to satisfy the PARAFAC model, providing kinetic data ready for analysis, for example by fitting to a given order of reaction. Examples of the outputs generated with this toolbox are shown in Figure 5.15 to Figure 5.19.

The number of components can be estimated from the PARAFAC results, the correct number of components is given by the maximum number of components that provides physically meaningful results, i.e., positive components are obtained (except for the relaxation evolution in IRON experiments), the diffusion decay follows a Gaussian function (except for restricted diffusion), the relaxation evolution follows an exponential function, and the spectral component resembles an NMR spectrum. These requirements may not be met if the systematic deviations are sufficient to distort the trilinearity of the data. Additional analyses like cross-validation or CORCONDIA might be helpful.
5.5. Systematic deviations

Cross-talk between components in the PARAFAC decomposition of $T_1$-DOSY or Timecourse DOSY data can be caused by unwanted systematic changes; lineshape variations and frequency changes are the most common problems. These can, by a large extent, be corrected by reference deconvolution, but temperature or pH changes can cause differential frequency shifts, breaking the trilinearity of the model.

5.5.1. Frequency drift analysis

It is not unusual to find, from a two-component PARAFAC decomposition of a multiplet originating from a single compound, a second component that appears to be a phase shifted copy (Figure 5.21), while ideally it should be only random noise. The first component is the expected absorption mode triplet, while the second has a distorted lineshape, resembling a dispersion mode lineshape. However, a dispersion mode signal could be phase rotated into absorption mode, but when the second component is phase rotated it shows a lineshape diagnostic of a frequency drift. The latter is typically caused by temperature instability, although possible causes are intermolecular interactions and pH changes.
5.5.2. Phase shift analysis

Another possible source of unwanted systematic deviation is an apparent change in zero order phase shift with gradient strength. This is a well-known problem in DOSY experiments, typically caused by eddy currents.\textsuperscript{108} This effect is difficult to isolate, so the effect of PARAFAC decomposition is best demonstrated by simulated data (Figure 5.22), in which a triplet with phase distortions of 600 °/(T m\textsuperscript{-1}) in a simulated SARON experiment.
The first PARAFAC component obtained is a triplet with phase distortion, related to the average of the different phased spectra, while the second component is related to the average of the phase shift. This reveals a potential problem in PARAFAC analysis if such a gradient dependent phase shift is not corrected. Optimally, a more careful setup of the experiment can be devised to minimize this problem. Another option is to manually or (preferably) automatically correct the phase of each spectrum in the experiment before analysis.

5.5.3. Complex relaxation analysis

Different relaxation behaviour for different multiplet components is a violation of the trilinearity assumed in the PARAFAC decomposition. Such behaviour has been reported in
the literature as well as seen experimentally in the present investigation. In order to understand the consequences for the $T_1$-DOSY analysis, simulations were carried out. A set of spectra was simulated with a triplet for one component and a sextet for the other component (resembling the behaviour of a mixture of 1-propanol and 2-pentanol in a SARON experiment) with 10% difference in diffusion coefficients and 20% difference in $T_1$, and 4% and 8% variation of $T_1$ between the inner lines and the outer lines of the triplet and sextet respectively. The results, in Figure 5.23, show that the proportions of the multiplets are distorted, and a second component appears reflecting the different relaxation rates of the inner and outer lines.

![Simulated Spectrum](image.png)

**Figure 5.23**: Two-component analysis of the simulated region at 3.7 ppm with a 1-propanol triplet and a 2-pentanol sextet, with 4% $T_1$ variation within the triplet.

In experimental data, this may result in a plot such as that shown in Figure 5.24. It is similar to the simulations, but other systematic deviations are also present. However, it is more common to find that for one component the inner peak is larger than expected, and smaller for the other component (Figure 5.25). This may be caused by a mixture of different effects, like frequency drift and variation in peak width.
Figure 5.24: Two-component PARAFAC analysis of the 1-propanol triplet at 3.6 ppm in a DRONE experiment

Figure 5.25: Two-component PARAFAC analysis of the region at 3.7 ppm with a 1-propanol triplet and a 2-pentanol sextet in a DRONE experiment
5.6. Conclusions

The addition of extra information to diffusion experiments, combined with a PARAFAC analysis of the data, can be used to discriminate between the components of a mixture even under severe spectral overlap and very low SNR. This can potentially be useful for the analysis of mixtures or reactions containing species of low availability or solubility, or where high concentrations cause changes in the shape and/or position of the peaks.

The addition of relaxation information to DOSY experiments allows the extraction of spectra, diffusion coefficients and relaxation times for the individual components in very little experimental time. Using the timecourse effects on DOSY experiments to study a reacting mixture will provide information allowing evaluation of the kinetics of the reaction. All these data can be easily analysed using the MATLAB based $T_1$ and Timecourse DOSY tools.

If deviations from trilinearity are kept to a minimum, PARAFAC analysis is a powerful tool to extract information from multiway datasets. Reference deconvolution is a fundamental tool to minimize unwanted variations, but when this is not enough it can be combined with further processing such as compensation of diffusion variations and use of constraints, such as non-negativity, in the PARAFAC analysis. This analysis can pick up very small systematic variations in the data as additional components or distortions incorporated in the expected components; this can be useful for diagnosing either instrumental or environmental problems.
Chapter 6

Chromatographically-Ordered Correlation NMR (CHOCO)

Some years ago it was considered something of an art-form to demolish a grand piano with a sledgehammer. In the vocabulary of NMR this would be known as the impulse response, while the more usual note-by-note excitation would be the equivalent of slow-passage (digitized) frequency sweep.

Ray Freeman
6. Chromatographically-Ordered Correlation NMR (CHOCO)

A fundamental limitation of DOSY techniques is that they cannot distinguish between molecules with the same diffusion coefficients. Some research has focused on adding a substance to the solution that interacts to different extents with the solutes, in order to alter the diffusion coefficients of the species. This approach is called Matrix-Assisted DOSY (MAD). Some of the substances used are surfactants, typically used in the form of micelles, polymers, silica gel and complexation agents such as calix[4]arenes and cyclodextrins. Most of these substances give rise to additional NMR signals, and some of those that do not show signals for the studied nuclei (such as perdeuterated sodium dodecyl sulphate) are very expensive. The use of a solid phase such as silica has the advantage of no extra signal but typically increases the linewidths of NMR signals because of the difference in magnetic susceptibility and, thus, complicates DOSY analysis. This has been partially remedied either by using a solid state spectrometer with Magic Angle Spinning, where the rotation of the samples averages out the anisotropy of the field, thus reducing the linewidths or by using a solvent with the same susceptibility as that of the solid (see section 6.3). But signal overlap is still a major issue for the data processing.

In DOSY techniques, the resolution of the second dimension is typically achieved through some approximation of the Laplace inversion of a sum of exponential decays. This is a numerically unstable problem, as multiple solutions are equally valid for a noisy dataset. This is fundamentally different from the Fourier transform of FIDs, where it is possible to extract the contribution of each oscillatory function to the signal regardless of overlap. The
same applies to the FT in other dimensions in standard multidimensional NMR experiments like COSY, where chemical shift overlap is not a major impediment to the resolution of the other dimensions. If it were possible to use FT processing to generate the second dimension in a DOSY plot, the resolution of overlapped signals would be much simpler. If PFG experiments are performed on a sample under flow conditions, the phases of the signals will be dependent on the gradient strength. This phase encoding allows Fourier transformation of the data with respect to the gradient strength to obtain the velocity distribution for each chemical shift.

In a chromatographic column, different species flow, on average, at different velocities, because they interact to different extents with the stationary phase. It should therefore be possible to encode the spin velocities of each species as a second dimension in a PFG experiment to obtain a 2D dataset that can be processed by 2D FT, obtaining the chemical shift in one dimension and the velocity distribution in the other. This is the principle of Chromatographically-Ordered Correlation NMR (CHOCO). In contrast to conventional LC-NMR, where each species is generally measured independently, in CHOCO the signals of all species are measured simultaneously. The SNR in CHOCO is proportional to the square root of the total experimental time, while in LC-NMR is proportional to the square root of the total experimental time divided by the number of species separately measured. Thus CHOCO provides an increase in SNR of the order of the square root of the number of independent species measured. This is known as Fellgett or multiplex advantage and it is the same principle that allows Fourier Transform NMR to acquire spectra more
efficiently than Continuous Wave NMR, where each resonance is excited at a time. The working principles of CHOCO are developed and presented in this chapter.

6.1. Introduction to chromatography

Chromatography is an analytical technique that allows the separation of mixtures by altering the temporal distribution of analytes in a mobile phase during sequential elution as a result of their different affinities for interaction with a stationary phase. One of the most common types of chromatography is column chromatography, in which the stationary phase or adsorbent is a solid packed in a column. The different analytes are carried by the mobile phase or eluent stream through the stationary phase, where those solutes that interact strongly with it will be retained in the column to a greater extent than those that interact more strongly with the mobile phase. As a result, the solutes will elute at different times, while a detector indicates this by plotting a chromatogram with different peaks or bands representing the different elution times. These peaks should be kept as narrow and as far apart as possible.

The relative separation between two solutes in chromatography depends on the nature and the magnitude of the interactions between each solute and the two phases. There are three main types of attractive interactions: ionic, polar and dispersive forces. Ionic forces depend on the electrical charges present on the molecules. Polar interactions are the result of permanent or induced dipoles in molecules such as alcohols or ketones. Dispersive interactions arise from rapid charge fluctuations. Only dispersive interactions are found for molecules such as hydrocarbons. To separate molecules solely by dispersive forces the
stationary phase must not contain polar or ionic substances and the dominant interactions in the mobile phase must be polar, as is the case for reversed-phase chromatography.

By analogy with distillation columns, the plate theory was developed to describe the efficiency of the column. The column is divided in theoretical plates, each one representing a stage in equilibrium of the solute between the stationary and the mobile phase. This equilibrium is never actually achieved, because there is a continuous exchange of solute between the two phases, so each plate is allotted a finite length in which the solute is assumed to have reached the equilibrium with the two phases; the smaller the plate, the faster the exchange of solute. An ideal column would have infinite plates, so larger number of plates in a real column means that the equilibration rate of the column is closer to the ideal. For this reason, the number of plates is associated with the column efficiency. The concentration of solute $X_m$ in the mobile phase leaving each plate $p$ can be expressed as:

$$X_{m(p)} = \frac{X_0 e^{-V} V^p}{p!}$$

where $V$ is an adimensional way of measuring the volume flow of mobile phase as a function of plate volumes (where plate volume is defined as the volume of mobile phase that contains all the solute that is in the plate at the assumed equilibrium concentration of the mobile phase), and $X_0$ is the initial concentration of solute in the mobile phase. This equation follows a Poisson distribution, which, for a large number, approximates to a Gaussian function. It is represented in Figure 6.1.
Increasing the number of plates makes the peaks broader and more symmetrical, but the peaks of different species (with different interactions) get more spaced out, i.e. the resolution increases. The separation between peaks is proportional to the number of plates and the width of the peaks increases with the square root of the number of plates. As a result, the higher the number of plates, the higher the efficiency. Given a restriction in the height of the column, the maximum efficiency is achieved with the minimum height equivalent to a theoretical plate (HETP). However, the plate theory does not explain the mechanisms that influence the HETP. These parameters are explained by the rate theory. This theory is an approach that takes into account the time taken for the solute to equilibrate between the stationary and mobile phase, and the different paths across the stationary phase.
that the solute can follow. These parameters were initially described by the Van Deemter equation\textsuperscript{293} as a function of the linear velocity $u$.

$$HETP = A + \frac{B}{u} + C \cdot u$$  \hspace{1cm} [6.2]

where $A$ is the eddy diffusion term (or multipath effect), determined by the different paths that the solute may take, and is dependent on the homogeneity of the packing and the particle size. $B$ is the term that describes the longitudinal diffusion effect, related to the difference in concentration of the solute between the centre and the edges of the peak; it is dependent on the homogeneity of the packing and the diffusivities of the solute in the mobile and stationary phases. $C$ is the term related to the resistance to the mass transfer of the solute in the mobile phase (which increases quadratically with the diameter of the particles and decreases with the diffusivity of the solute in the mobile phase) and in the stationary phase (which increases quadratically with the film thickness and decreases with the diffusivity of the solute in the stationary phase).

There is an optimum velocity for which the HETP is minimum. When the effective film thickness (of the liquid phase which is in immediate contact with the stationary phase, small when the liquid film is continuous and large if it fills smaller pores leaving the wider ones unwetted) is much smaller than the diameter of the particles in the stationary phase (which in practice is always true), for well-retained peaks and well-packed columns, this velocity is approximately:

$$u_{opt} \approx 1.62 \frac{D_m}{d_p}$$  \hspace{1cm} [6.3]
where \( D_m \) is the diffusivity of the solute in the mobile phase and \( d_p \) is the diameter of the particles of the stationary phase. The minimum HETP is then approximated by \(^{291}\):

\[
HETP_{\text{min}} \approx 2.48d_p
\]  

[6.4]

These approximations use the Van Deemter equation, but it may not be the best model to describe chromatographic processes. These processes are not perfectly understood, this has led to different interpretations of the terms of this equation \(^{294,295}\) and to other equations \(^{296}\), which generally have a similar degree of agreement with experimental data \(^{297}\), to optimize and find the limits of chromatographic separations \(^{298-300}\). In contrast to these macroscopic models, numerous models have been proposed to try to describe the stochastic nature of the molecular dynamics behind the band spreading, \(^{301}\) an approach introduced by Giddings and Eyring \(^{302}\). One of the latest models introduced a term of heat friction \(^{303,304}\), but there is still plenty of research trying to characterize chromatographic processes better \(^{305-314}\).

### 6.2. Liquid Chromatography – NMR (LC-NMR)

Liquid chromatography has the potential to separate the components of any kind of mixture, given the right stationary and mobile phase. In order to identify the components it is typically necessary to couple it with a detector. The standard option is a UV-VIS or refractive index (RI) detector that will show the bands created by the distribution of the solutes. In order to identify which solute bands belong to which bands it is advantageous to use more powerful detectors which supply more chemical information. Mass spectroscopy is a candidate widely used for the identification of compounds. However, NMR is probably
one of the most powerful methods for the extraction of information on molecular structure and dynamics (which are harder, or impossible, to obtain with MS) and as such, LC-NMR has become a useful technique for the analysis of mixtures. There are different modes of running LC-NMR experiments. In on-flow mode the mobile phase is flowing continuously through the flow cell installed in the NMR probe. For careful analyses stopped-flow mode is preferred as it permits the acquisition of longer experiments and can improve spectral resolution (under flow conditions the apparent transverse relaxation is faster because excited spins continuously leave the NMR detection region). One method consists in stopping the flow, once the band of solute reaches the flow cell, for as long as is necessary. Another option is to stop every few seconds to measure the NMR spectrum; this allows measurements on compounds that have not been found by the LC detector. It is also possible to collect the different fractions (e.g. into loops of tubing), and once the elution is finished to insert them into the NMR probe for analysis.

6.3. Chromatographically-Ordered Correlation NMR (CHOCO NMR)

One of the main drawbacks of LC-NMR is the time required to acquire the data. The different fractions obtained from the mobile phase are sequentially analysed in the NMR. If they were measured simultaneously the acquisition time could be greatly reduced due to the Felgett advantage, in the same way that FT NMR is faster than CW NMR. It should be possible to do this if the measurements are carried out directly in the column while the mixture is passing through the column; this requires placing the chromatographic column in the probe inside the magnet. The way to distinguish between different signals is by velocity encoding: species with different interactions with the stationary phase will flow at different
average rates through the column. The system consists of a closed circuit with a chromatographic column inside the magnet, and the mixture would be continuously recirculated with the help of a pump. Thus, CHOCO is fundamentally different to LC-NMR as the chromatographic column is inside the magnet, the different chromatographic behaviour is given by the average velocities rather than elution times, the solutes are spread throughout the circuit and they are not separated, and the hardware control and software requirements are simpler. A schematic of the proposed system is presented in Figure 6.2.

![Schematic of CHOCO piping](image)

**Figure 6.2: Schematic of CHOCO piping.** There is continuous pumping of the sample from the vessel through the column in a closed-circuit system. The column is placed inside the active volume of the probe.

Velocity encoding is related to diffusion encoding explained, in section 2.8.1, and the pulse sequences employed are the same: the spins are initially encoded with a gradient obtaining a position-dependent phase, and then decoded with another gradient that will leave all spins with the same phase if they have not changed their positions. Unlike in diffusion encoding, the average displacement is now different from zero, so the ensemble of spins acquires a net phase which is dependent on the distance travelled. The farther away the spins have
moved, the larger the phase change. The phase change is derived from the effects of the gradients (Equation [2.19]). After the action of two gradient pulses of equal duration and opposite strength, the phase change is given by:

$$\Phi = -\gamma \delta G \Delta p \Delta z$$  \[6.5\]

Due to the velocity being actually a distribution of velocities, the signal attenuation with gradient strength can be approximated to a Gaussian decay for a large number of plates. Combining this with equation [2.12], the resulting signal is:

$$s(t,G) = \alpha e^{i\Omega t} e^{-\gamma \delta G \Delta p \Delta z} e^{-G^2/\beta}$$  \[6.6\]

where $\beta$ is a parameter related to the attenuation of the signal caused by the distribution of velocities. As explained in section 2.4.1, the FT of this signal with respect to $t$ will have a real part with absorption (A) lineshapes and a imaginary part with dispersion (D) lineshapes. Another FT, but now with respect to $G^2$ will result in Equation [6.7]:

$$s(\Delta z, \Omega) = \alpha \left( A_1(\Omega) + iD_1(\Omega) \right) \left( A_2(\Delta z) + iD_2(\Delta z) \right)$$  \[6.7\]

The real part is going to be $A_1(\Omega)A_2(\Delta z) - D_1(\Omega)D_2(\Delta z)$, which is a phase-twisted lineshape. It is possible to untangle the absorption and dispersion components by separating the sine and cosine contributions and processing with standard methods such as States-Haberkorn-Ruben (SHR). Positive and negative gradients will cause phase modulations in opposite directions, and its combination produces the cosine and sine modulations used in the SHR method as described by Equations [6.8]:

$$\cos(-i\gamma \delta G \Delta p \Delta z) = e^{i\gamma \delta G \Delta z} + e^{-i\gamma \delta G \Delta z}$$

$$\sin(-i\gamma \delta G \Delta p \Delta z) = e^{i\gamma \delta G \Delta z} - e^{-i\gamma \delta G \Delta z}$$  \[6.8\]
This is the same procedure that can be used in other 2D NMR experiments such as COSY\textsuperscript{29,141}, and the use of 2 datasets with positive and negative gradients is analogue to the so called echo anti-echo method using P and N-type datasets. The explanation of these methods can be found elsewhere.\textsuperscript{29,64}

The change in position $\Delta z$ can be expressed for the simplest case as the product of the average flow velocity $u$ by the time between the applied gradients $A'$ (for the study of more complex cases, other formulas have been derived).\textsuperscript{36,162,317-319} Thus the following expression is obtained for the phase change in a PFG experiment:

$\Phi = \gamma \delta G u A'$ \hspace{1cm} [6.9]

This phase encoding allows Fourier transformation of the data with respect to the gradient strength to obtain the velocity distribution for each chemical shift.\textsuperscript{36,37,162,289}

In contrast to the chromatographic theory presented in section 6.1, the effective length of the column depends on the time $A$ during which the velocity is encoded. The length of the column is then given by $uA'$, and with the HETP given by Equation [6.2], the efficiency is calculated as:

$$n = \frac{uA}{B + \frac{C}{u} + u}$$ \hspace{1cm} [6.10]

which means that the number of plates increases up to an asymptotic value for large velocities. Although intuitively a higher velocity means that the solute molecules can interact with more stationary phase particles, it also implies that the distance between unretained and retained molecules is bigger. This means an increase in the dispersion of the
solute molecules and, consequently, a broadening of the peak bands, hindering the separation of the solutes and reducing the efficiency.

In chromatography the solutes are injected into the eluent, resulting in some diffusion driven by the gradient of concentrations. In CHOCO, there is no such gradient of concentrations because the solutes are being recirculated and remain mixed. Therefore the term B of the equation, related to the longitudinal diffusion, has lower impact as the self-diffusion coefficient is used instead of a gradient concentration diffusion coefficient. A and C are the parameters necessary to be taken into account to design a column with the maximum efficiency, so as in conventional chromatography, desirable features are small particle diameter, large diffusion coefficient of the solutes in the mobile and stationary phases, and homogeneous packing.

The choice of material for the columns in CHOCO is more limited than for standard LC-NMR. The material used must not be ferromagnetic as it must be carefully placed inside the probe under the action of a high magnetic field. The majority of chromatographic columns are manufactured in steel, which is ferromagnetic, although viable alternatives are PEEK (polyether ether ketone) and glass, and commercial columns are difficult to fit inside a standard NMR probe because of their dimensions. However, it is possible to adapt standard NMR tubes by cutting the sealed end and providing suitable sealing at both ends of the tube to allow the flow of the mobile phase and to retain the stationary phase. Another option is machining a suitable material like PEEK to create a chromatographic column that can be placed inside the probe.
The use of a solid phase in the NMR tube offers another challenge in this experiment. The introduction of solids into an NMR sample is a common reason for obtaining very broad peaks in routine $^1$H NMR. Typically the solids have a different magnetic susceptibility than the solvent, thus distorting the magnetic field in the sample. To avoid this problem in CHOCO, the magnetic susceptibility of the solvent used should match or be close to the susceptibility of the stationary phase. Otherwise low chemical shift resolution will be obtained in measurements (unless an experiment that compensates for magnetic susceptibility differences is used$^{287,288}$). Chromatographic packing materials (usually based on silica) do not have the same magnetic susceptibility as the solvents commonly used. If the NMR measurements are to be made directly on the column, it is necessary to change either the stationary or the mobile phase to match the susceptibilities$^{287}$. While stationary phases based on silica are dominant in routine chromatographic separations, there are other options to be considered. Zirconia, apparently more stable than silica in a wide range of conditions$^{321}$, has a magnetic susceptibility approximately equal to that of deuterium oxide$^{322,323}$, making it a suitable candidate for the task. In this experiment, silica gel, being a more widely used chromatographic material, is used as stationary phase with a mixture of deuterated chloroform and diiodomethane as solvent tailored to match its magnetic susceptibility.$^{287}$

### 6.4. Column design

In order to allow insertion of a PEEK (polyether ether ketone) column inside a standard probe, an indirect 8mm NMR probe was adapted by removing the VT air heater from the dewar of the probe. Additionally, a plastic spider supporting a thermocouple was taken out,
requiring the RF coils to be temporarily removed. The 8 mm PEEK tube used as a column was constructed as follows:

This design in 5 main parts allows changing of the packing material and the frits, and can be made with standard tools such as a drill, taps and dies. PEEK frits of 2µm and sizes of 0.188" x 0.062" x 0.250" were used, with O-rings resistant to the solvent used (such as Viton or PTFE o-rings for mixtures in chloroform). PEEK rod of appropriate diameter or

Figure 6.3: Diagram, with sizes, of the column machined from PEEK.
similar is readily available. The wall thickness is determined by the pressure required to flow liquid through the column. The maximum pressure for a tube is given by equation [6.11]:

\[
P = \frac{\text{Wall thickness}}{\text{Outside diameter}} \cdot K
\]  

where \( K \) is a constant related to the tensile strength of the tube material, which in the case of PEEK is about 14,500 p.s.i. (1x10^8 Pa). The pressure drop in the column is calculated using the Ergun equation:

\[
\Delta P = \frac{150 \cdot \eta \cdot u_s \cdot (1 - \varepsilon)^2}{\Psi^2 \cdot d_p^2 \cdot \varepsilon^3} L + \frac{1.75 \cdot \rho \cdot u_s^2 \cdot (1 - \varepsilon)}{\Psi^2 \cdot d_p^2 \cdot \varepsilon^3} L
\]  

where \( \eta \) is the viscosity, \( u_s \) the superficial velocity, \( \Psi \) the sphericity (assumed to be 1), \( L \) the column length, \( \rho \) the density, \( \varepsilon \) the porosity, and \( d_p \) the particle diameter. The most pressure-demanding conditions that may initially be tried would be particles of 5x10^{-6} m, viscosity of 0.4x10^{-3} Pa s, density of 1.5x10^3 kg/m^3, flow of 5 ml/min (about 13x10^{-3} m/s), porosity of 0.5 and a column length of 60x10^{-3} m. This would result in a pressure drop of 3.8x10^6 Pa (about 551 p.s.i), requiring a wall thickness of 0.3 mm. The wall thickness at the unions of the column built is about 0.8 mm, which can withstand up to 1x10^7 Pa.

### 6.5. Experimental part and discussion

Two columns were built following the design of Figure 6.3. One of them was filled with silica gel 200-425 mesh (particle size distribution of 35-75 µm, which will be referred as 55 µm silica gel for simplicity) Sigma Aldrich and the other with silica gel Spherisorb of 5 µm.
The test solution for the system consisted of 20 mL of chloroform-D and diiodomethane in the proportion 78:22 v/v.\textsuperscript{287} TMS, ethanol, acetone, 2-methoxyphenyl acetate, methyl benzoate and 1,3-dimethoxybenzene, all with a concentration around 3 mM. An array of spectra was acquired using the Oneshot sequence with 64 gradient strengths ranging from -12.4 to 12.4 G/cm, a flow rate of 0.75 mL/min, 16 transients, gradient length $\delta$ of 0.4 ms and delay time $\Delta$ of 0.4 s. Data were resolution enhanced in the chemical shift dimension (line broadening of -20 Hz and Gaussian function of 0.027 s) and smoothed in the flow dimension (Gaussian function of 0.432 s). Measurements were carried out non-spinning on a Varian VNMRS 500 spectrometer in an air-conditioned room at approximately 20 °C. Spectra were processed in VnmrJ with the command wft2d(1,0,0,1,0,1,1,0)

Figure 6.4 was obtained after 2D FT of the Oneshot results using 55 µm silica gel. The analytes were flowing at different average speeds. This verifies the principles of CHOCO, allowing the identification of the analytes that pass through a column by their difference in average velocities. The order of speeds obtained matches the octanol-water partition coefficients order, as shown in Table 6.1. As expected, polar solutes interact more with the silica gel and are therefore slowed down, with the ethanol showing a strong binding to the polar silica gel.
Table 6.1: Comparison of average speeds obtained in CHOCO experiment for each analyte with the octanol-water partition coefficients

<table>
<thead>
<tr>
<th>Substance</th>
<th>Average velocity (mm/s)</th>
<th>Octanol-water partition coefficients $K_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>0.1</td>
<td>-0.3</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.7</td>
<td>-0.2</td>
</tr>
<tr>
<td>2-Methoxyphenyl acetate</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Methyl benzoate</td>
<td>2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>1,3-Dimethoxybenzene</td>
<td>2.6</td>
<td>2.2</td>
</tr>
<tr>
<td>TMS</td>
<td>3.7</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Figure 6.4: 2D spectrum of the Oneshot results using 55 µm silica gel at 0.75 ml/min. Resolved signals are coloured, ethanol in light blue, acetone in yellow, 2-methoxyphenyl acetate in red, methyl benzoate in dark blue, 1,3-dimethoxybenzene in green and TMS in purple. All these analytes present different elution speeds. The diiodomethane signal is about 3.9 ppm overlapped with signals from other analytes, it presents negative and positive lobes and is asymmetric.
Although this experiment proves that it is possible to distinguish between species by their different flow rate in a chromatographic column, it suffers from a major problem: lack of resolution, both in chemical shift and in speed. Linewidths of the order of 2 Hz were observed when the column was tried without stationary phase, discarding the possibility that the broadening was caused by the column itself. In experiments in normal NMR tubes the width of the peaks was of the order of 2 Hz, but using the column both in static and in flow experiments the linewidth was of the order of 40 Hz. In other experiments where there were air bubbles in the tube, the linewidths reached hundreds of Hz; this does not rule out the possibility that there were still air microbubbles inside the column. If even degassed the linewidths were not reduced, it would imply that the silica gel suffered a change in magnetic susceptibility either because of the stress applied (perhaps some pores were obstructed with air trapped inside) or because of a chemical change.

The resolution in the flow dimension is related to the efficiency of the column. Its length is determined by $\mu A'$, so once these parameters are optimized the main factors affecting the resolution are the packing material and the experimental conditions. Several experiments were run with different flow rates $\mu$ and delay times $A'$, but no substantial differences were found at the same encoding level. A suitable device to pack the stationary phase in this column was not available, so a packing material with lower particle diameter was tried with the aim to increase the resolution. Figure 6.5 and Figure 6.6 show the resolution achieved in the flow dimension with 55 $\mu$m and 5 $\mu$m silica. Although a minor improvement is observed, this is far below expectations, and suggests that the inhomogeneity of the packing is constraining the achievable resolution.
Figure 6.5: Section of the CHOCO spectrum from Figure 6.4 showing the projections in both dimensions. It can be observed that the resolution in the flow dimension is very poor.

Figure 6.6: Section of the CHOCO spectrum obtained using 5 µm silica gel displaying the projections in both dimensions. The resolution in the flow dimension is slightly better than in Figure 6.5 with 55 µm silica gel.
It should be noted that several previous CHOCO experiments with different mixtures were run with the 55 µm silica gel, so its behaviour might be different from the 5 µm silica, which was used for the first time in the experiment above. The peaks belonging to the 2-methoxyphenyl acetate are not present in this spectrum, but they were present in a simple $^1$H spectrum, although hardly discernible because of their peak width. The same behaviour was observed for the acetone peak. This might be caused by strong adsorption to the silica and it would be a further issue to investigate, but the initial objective of this experiment was not to investigate the chemistry of the chromatographic behaviour, but to evaluate whether better resolution could be achieved using smaller particle size.

6.6. Conclusions

It has been shown that it is possible to distinguish between the components of a mixture on the basis of their different average velocities through a chromatographic column. This represents a potential improvement over DOSY experiments in the sense that the second dimension is obtained by FT and thus the overlap is less problematic. It also represents a potential improvement over LC-NMR experiments because of the Fellgett advantage and the simpler software setup. However, in order to compete with these methods much higher resolution is required. The key to achieve this may lie in reproducing the experiment in a commercial column using a wide bore magnet or with a better engineered column.
Chapter 7

Future work

*Prediction is very difficult, especially about the future*

Niels Bohr
7. Future work

The experiments shown in this thesis have been carried out over the course of three years. Naturally, this has limited the number of experiments carried out and the threads to follow; there are several more experiments that can be considered as a continuation of this work, as well as new lines of research to pursue. Some of them try to overcome the limitations exposed in experiments like $T_1$-DOSY, others are possible variations to further enhance the experiments, or simply applications where the experiments presented may excel. Also, some preliminary work and illustrative examples are described to evaluate the effectiveness and advantages of some research lines.

7.1. Double spin echo Oneshot45

The best choice to avoid $J$-modulation effects would be to convert all magnetization into in-phase magnetization, rather than purging the anti-phase magnetization. This can be done to a certain extent with a double spin echo with a $90^\circ$ pulse between the echoes, as suggested in 1989-1990, but surprisingly this idea was not reported in diffusion measurements until early 2010, in an experiment called $J$-compensated PGSE. Given the superior sensitivity of the spin echo over the stimulated echo, this sequence could be a suitable choice for the analysis of small molecules, where the transverse relaxation losses are not a big concern for the diffusion times required. The $J$-modulation could be reduced even further in this $J$-compensated PGSE by adding the purging $45^\circ$ pulse. The same approach could be used in bipolar pulse stimulated echo sequences, employing this double spin echo element instead of the spin echoes and adding the $45^\circ$ pulse at the end of the stimulated
echo. This would allow further reduction of anti-phase distortions. However, there is one inconvenience that is likely to hinder the usability of this approach: this double spin echo element requires doubling the time for which gradients are on, increasing the amount of heat generated. Standard gradient coils currently in use would be operating at their limits in diffusion measurements of large molecules with BPPSTE sequences, but in the most modern gradient coils it would be possible to double the duty cycle of the gradient coils.

The 45° pulse has already been used in some 2D experiments where $J$-modulation is a problem.\textsuperscript{327,328} There are some experiments that are negatively affected by homonuclear $J$-modulation, and they would benefit from a simple pulse sequence element to reduce $J$-modulation. Further investigation will be needed to evaluate its applicability, but if it is feasible, it will provide advantages such as improved resolution and more accurate $J$ measurements.\textsuperscript{329}

7.2. Heteronuclear DOSY

The pulse sequences presented for $^{13}$C DOSY experiments have been shown to be useful for increasing the signal-to-noise ratio, but they could still be subject to some improvements. The phase cycling of the pulse sequences presented here could be further refined. The substitution of the pair of $^{13}$C 180° pulses by a pair of adiabatic pulses should also be evaluated for a sample where the chemical shifts are spread over about 200 ppm.

These sequences are not exclusive to $^{13}$C, and they could be adapted for other nuclei that would benefit from polarization transfer from $^1$H or other nuclei with high magnetogyric
ratio. Some BPPSTE-INEPT experiments have been carried out in nuclei like $^{31}$P,$^{193}$,$^{330}$, $^6$Li,$^{331}$, $^{29}$Si,$^{136}$,$^{332}$ and $^{19}$F,$^{333}$-$^{335}$; DEPTSE and INEPTSE may be suitable alternatives if the relaxation properties are favourable.

### 7.3. Relaxation Ordered Liquid SpectroscopY (ROLSY)

The main problem of the $T_1$-DOSY experiment was the range of relaxation times for each component. There are different approaches that may help towards achieving the same relaxation time for all the peaks in a component or, at least, for all the peaks in a multiplet.

In EPR, RElaxation filtered hyperFINE spectroscopy (REFINE)$^{255,256}$ allows the identification of different compounds according to their relaxation. In solid state NMR, there is typically fast spin diffusion, meaning that the effective relaxation times of all peaks belonging to a single phase are the same, and thus an inversion recovery experiment followed by cross-polarization can be used to distinguish the components in different phases.$^{336}$ This approach has been used under a different name every time a group redisCOVERS it: Relaxation-Assisted Separation (RAS),$^{337}$ raTe of relaxation Ordered SpectroscopY (TOSY)$^{338}$ or Relaxation Ordered SpectroscopY (ROSY)$^{339}$. In order to continue this tradition, a new experiment designed to distinguish compounds by relaxation in liquids could be named Relaxation Ordered Liquid SpectroscopY (ROLSY). ROLSY consists of an Inversion-Recovery experiment with a fixed time spin-lock following the recovery delay, as pictured in Figure 7.1. During this time spin magnetization will transfer to other spins through spin diffusion.
A ROLSY experiment was carried out with a sample of 1-propanol and 2-pentanol, both at 15 mg mL$^{-1}$, in a Varian VNMRS 500 using 7 recovery delays ranging from 0.2s to 12.8s and a spin lock of 400ms based on the MOCCA-XY16 sequence,$^{340-342}$ which should provide a more efficient magnetization transfer than another widely-used spin-lock sequence, DIPSI.$^{343}$ The $T_1$ analysis is represented in a 2D plot in Figure 7.3, and compared with the results obtained without spin-lock in Figure 7.2. The 2-pentanol triplet at 0.9 ppm does not appear in the plot as it masked by the dominant triplet from the 1-propanol. It can be observed that the apparent relaxation times for the 2-pentanol multiplet at 3.9 ppm have been reduced from 3.5 s to 2 s, thus making the apparent $T_1$ times for 2-pentanol more homogeneous. It should be noted that the apparent $T_1$ variation within the multiplets, discussed in section 5.2.4, remains unchanged.

In small molecules, such as the two alcohols studied, is where the widest range of $T_1$s can be expected, so the separation could be more effective with larger and more rigid molecules using an NOE-type magnetization transfer rather than through scalar coupling. In addition, it is possible to increase spin diffusion using ultra-viscous solvents and low temperatures.$^{344}$ thus increasing the chances of obtaining more uniform $T_1$s for each component.
Figure 7.2: 2D plot of the $T_1$ analysis using an inversion recovery sequence on a mixture of 1-propanol (with $T_1$s around 4.5 s) and 2-pentanol (the other signals).

Figure 7.3: 2D plot of the $T_1$ analysis using the ROLSY sequence with a spin-lock of 0.4s on a mixture of 1-propanol (with $T_1$s around 4.5 s) and 2-pentanol (with $T_1$s around 2 s).
7.4. Pure shift

Another way to get around the problem of different $T_1$s within a multiplet could be to collapse the multiplet structure in the spectrum; this is achieved in pure shift experiments.\textsuperscript{129,130,170} To illustrate the capabilities of the pure shift approach, a sample containing bovine serum albumin (BSA) 0.2 mM and aminoacids at a concentration of around 5 mM each (L-arginine monohydrochloride, L-lysine monohydrochloride, L-valine and L-isoleucine) in pH 7 buffered D$_2$O were analysed with a pure shift sequence\textsuperscript{130} in an experiment with 32 increments and 64 transients. The spectrum obtained is compared with the $^1$H spectrum in Figure 7.4.

Figure 7.4: Comparison of standard $^1$H spectrum (top) with pureshift spectrum (bottom) of a sample with BSA and 4 aminoacids.
The broad protein signals are removed because the selective pulse is long enough (50 ms) to remove virtually all signals with fast transverse relaxation. This can be a convenient simplification for metabolomic studies. Whether these pure shift experiments are suitable for multiway DOSY analysis has yet to be evaluated; there might be systematic deviations that could complicate the analysis or destroy the trilinearity. One of these complications is caused by the non uniformity of the gradients, which in pure shift DOSY experiments cause the apparent diffusion coefficients to be dependent on the chemical shift. This would not represent a problem, in principle, for $T_1$-DOSY because there is already the restriction of analysing only one multiplet for each component at a time, and the calculated diffusion coefficient could be corrected afterwards. However, in timecourse DOSY this represents a fundamental problem that would break the trilinearity of the data.

7.5. Multiway DOSY

The analysis carried out by PARAFAC has some limitations. Sometimes the conditions of the experiment cannot be adequately controlled and there are chemical shift variations over the course of the experiments that cannot be completely corrected by reference deconvolution. This problem is common in other analytical methods. For example, in chromatography the retention time peaks for the same analyte may be shifted between samples; this problem can be partially remedied by applying PARAFAC2 rather than PARAFAC. PARAFAC2 is a less constrained form of PARAFAC, where one of the loading matrices is allowed to vary for each of the datapoints in another mode as long as the product of the loading matrix multiplied by its transpose is constant throughout all the
variations. This characteristic allows taking into account peak shifts during the experiment, because different component spectra will be created for each time or relaxation point.

Another source of problems is when there is linear dependency between components that need to be separated. This is what happens, for example, in timecourse DOSY when two components have the same diffusion coefficient but different concentration profiles; or in $T_1$-DOSY in the analysis of multiplets considering every peak of the multiplet with different $T_1$ as a different component. PARALIND (PARAllel profiles with LINear Dependences) is a constrained form of PARAFAC that allows such linear dependency between components.\textsuperscript{349,350}

### 7.6. CHOCO

It has been established that the main problem found in the experiments carried out was the poor resolution in the velocity dimension. This problem is probably caused by the low efficiency of the column and there are different ways to deal with it. One option would be to increase the length (number of plates) of the column; the actual length of the column used in the experiment is given by $uA'$, so the only way to increase the length of the column is to use a probe with a longer coil length, which is likely to require a different magnet. The alternative is to reduce the plate height, which for this experiment is mainly dependent on particle size and homogeneity of the packing. The effects of reducing the particle size have been seen in the previous chapter, but not the effects of a more homogeneous packing. This could be done with a different packing material, with a special device that could effectively
pack the column or using a commercial column already packed. Given the difficulty of creating a column that can fit in an 8 mm probe, this would likely need to use a custom built probe or a wide bore magnet, such as the ones currently used elsewhere to study chromatographic columns by NMR.\textsuperscript{351,352}

Another aspect to be improved in CHOCO is the chemical shift resolution. This work has been carried out with solvents that match the magnetic susceptibility of the packing material. This may not always be possible. A primitive solution would be to carry out the experiments on a nucleus where there are less chances of overlap, such as $^{13}$C,\textsuperscript{352,353} although the low SNR would hinder its viability for CHOCO experiments. Other alternatives make use of pulse sequences that try to compensate for field inhomogeneity,\textsuperscript{354-359} but under flow conditions their effectiveness can be dramatically reduced.

Although CHOCO has been directed towards mixture analysis, it has a great potential to improve understanding of chromatography and, consequently, industrial processes based on adsorption. Huge amounts of energy are employed to separate components of mixtures in industry using processes like distillation or electrolysis. This has led to the development of less energy intensive processes like adsorption.\textsuperscript{360} The study of adsorption, whether as an industrial process or in the form of chromatography, has traditionally been a complicated field. The use of NMR imaging has advantages over other techniques for providing fluid dynamics information in a noninvasive way in opaque systems.\textsuperscript{351,361} Typically NMR imaging studies in heterogeneous media do not encode chemical shift information, but this additional information could be useful once there is enough chemical shift resolution. By
providing further chemical shift information it will be possible to evaluate the behaviour of species other than the solvent. It has been suggested that $^{13}$C detection to be used for imaging with chemical shift specificity (because it has wider chemical shift range and is less sensitive to susceptibility differences due to the lower magnetogyric ratio),$^{352,353}$ and it has just been recently demonstrated for the analysis of a reaction between acetic acid and ethanol in a fixed bed reactor.$^{362}$

An experiment carried out with the CHOCO setup shown in Figure 6.2 and the column pictured in Figure 7.5 shows a simple example where the chemical shift provides useful information to diagnose problems in a column. A column was designed for zirconia as stationary phase. D$_2$O has a magnetic susceptibility very close to zirconia and will thus be used as solvent.$^{322,323}$ The column was designed using a 5 mm NMR tube with a wall thickness of 0.77 mm (standard medium-walled tube). Special PTFE assemblies were built to hold the packing material in the tube, as can be seen in Figure 7.5. An O-ring inserted in the assembly stops the fluid from coming out. A frit glued to the assembly prevents the packing material flowing into the PEEK tubes. It can be glued by cutting a dip in the outer part of the assembly, where Araldite is placed to hold together the frit and the assembly. A PTFE spacer holds the glue (Araldite) that maintains the position of the assembly in the tube and prevents any possible leaks around the O-ring. The stationary phase is introduced as a slurry and compressed axially by pumping liquid, in order to pack it. This column is held by a spinner in a 5 mm probe with the VT air heater removed from the dewar of the probe, to allow the PEEK tubing inside.
A typical CHOCO experiment with a flow of 0.3 ml/min was tried with this column with an array of 16 gradients ranging from -7.5 G/cm to 7.5 G/cm in steps of 1 G/cm. The spectra obtained are arrayed in Figure 7.6 where a phase change can be observed for all signals except for those located at about 1 ppm.

**Figure 7.5:** Diagram of the chromatographic column built out of a glass NMR tube. The tube is held in position by a spinner placed at the top of the column in such a way that the probe coil detects the active volume.
Figure 7.6: Array of spectra for a CHOCO experiment with a flow rate of 0.3ml/min with zirconia. While other signals change in phase with different gradients, one of them at about 1 ppm remains unchanged.

Another experiment was run with gradients 10 times longer and the spectra obtained are arrayed in Figure 7.7. There are two signals about 1 ppm that only suffer attenuation from diffusion and are not phase modulated. After flushing the column with methanol this signal disappeared. This was identified as a pocket or pockets of hexane trapped inside the zirconia column, unable to get out because of the immiscibility with the solvent. MRI studies with chemical shift specificity would permit locating the zones where the hexane was trapped. This shows that this method can be used to diagnose problems in columns.
Figure 7.7: Array of spectra for a CHOCO experiment with a flow rate of 0.3 ml/min with zirconia using gradients 10 times stronger than in Figure 7.6. The signal at about 1 ppm is merely getting attenuated by diffusion, instead of being phase modulated by the flow.
7.7. Conclusions

This chapter has focused on further development of the methods presented in this thesis. But for the development of new techniques in mixture analysis, it is important to take into account the ongoing research both in NMR\textsuperscript{9,363-365} and in other analytical techniques\textsuperscript{366-373}. New advances will struggle to get incorporated into standard experiments if there are other methods that provide the same results in a more simple or cheaper way; but this situation is not unalterable. NMR technology has been continuously improved; latest spectrometers can provide more resolution, more sensitivity, more capabilities and are more stable. Such is the change that many experiments that are now commonly used, were impossible or very impractical to run in earlier spectrometers. Future advances in the NMR field will improve the prospects of the techniques shown in this thesis. Multivariate methods will be specially benefited by improved spectrometer stability: this includes temperature and frequency lock control. The use of coils that dissipate less heat will allow stronger and longer RF and gradient pulses. The use of hyperpolarization to enhance sensitivity by several orders of magnitude can represent an important advance when it is possible to hyperpolarize the nuclei\textsuperscript{374-377}. This can be especially useful in studies of low spin density systems such as gases. The development of mobile single-sided NMR\textsuperscript{378} may eventually allow the study, using current NMR techniques, of materials that cannot be placed inside a superconducting magnet. This will allow the study of systems on the field and improve its viability as a tool to diagnose problems.
Chapter 8

References

Si perché l'autorità dell'opinione di mille nelle scienze
non val per una scintilla di ragione di un solo.

(In questions of science the authority of a thousand
is not worth the humble reasoning of a single individual.)

Galileo Galilei
8. References


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APPENDICES

An expert is a man who has made all the mistakes which can be made in a very narrow field.

Niels Bohr
APPENDICES

Appendix A: 3 spin system analysis of the effect of a 45° pulse after a spin echo

Rotation of the magnetization into the transverse plane:

\[ \hat{I}_{1x} + \hat{I}_{2x} + \hat{I}_{3z} \xrightarrow{\frac{\pi}{2}(\hat{I}_{1y} + \hat{I}_{2y} + \hat{I}_{3y})} \hat{I}_{1x} + \hat{I}_{2x} + \hat{I}_{3x} \]

For spin \( \hat{I}_{1x} \):

\[ \hat{I}_{1x} \xrightarrow{2\pi \hat{I}_{1y}} \cos(\pi \hat{I}_{1z}) \hat{I}_{1x} + \sin(\pi \hat{I}_{1z}) 2\hat{I}_{1y} \hat{I}_{2z} \]

\[ \cos(\pi \hat{I}_{1z}) \hat{I}_{1x} + \sin(\pi \hat{I}_{1z}) 2\hat{I}_{1y} \hat{I}_{2z} \xrightarrow{2\pi \hat{I}_{1y}} \cos(\pi \hat{I}_{1z}) \hat{I}_{1x} + \sin(\pi \hat{I}_{1z}) 2\hat{I}_{1y} \hat{I}_{2z} \]

The in-phase terms are unaffected, the bilinear antiphase terms get cancelled out, the multiple quantum coherences are unobservable, and so there is a remaining mixture of in-phase terms and trilinear anti-phase terms.
Appendix B: 4 spin system analysis of the effect of a 45° pulse after a spin echo:

Rotation of the magnetization into the transverse plane:

\[
\hat{I}_{1x} + \hat{I}_{2z} + \hat{I}_{3z} + \hat{I}_{4z} \xrightarrow{\pi/2 (I_{1y} + I_{2y} + I_{3y})} \hat{I}_{1x} + \hat{I}_{2x} + \hat{I}_{3x} + \hat{I}_{4x}
\]

For spin \( \hat{I}_{1x} \):

\[
\hat{I}_{1x} \xrightarrow{2\pi d_{1t}I_{1u}} \cos(\pi J_{1x})\hat{I}_{1x} + \sin(\pi J_{1x})2\hat{I}_{y} \hat{I}_{2z}
\]

\[
\cos(\pi J_{12t})\hat{I}_{1x} + \sin(\pi J_{12t})2\hat{I}_{y} \hat{I}_{13} \xrightarrow{2\pi d_{13t}I_{1u}} \cos(\pi J_{12t})\cos(\pi J_{13t})\hat{I}_{1x} + \cos(\pi J_{12t})\sin(\pi J_{13t})2\hat{I}_{y} \hat{I}_{4z}
\]

\[
+ \cos(\pi J_{12t})\sin(\pi J_{13t})2\hat{I}_{y} \hat{I}_{13} \xrightarrow{2\pi d_{13t}I_{1u}} \cos(\pi J_{12t})\sin(\pi J_{13t})\cos(\pi J_{14t})2\hat{I}_{y} \hat{I}_{3z} - \cos(\pi J_{12t})\sin(\pi J_{13t})4\hat{I}_{1x} \hat{I}_{2x} \hat{I}_{4z}
\]

\[
+ \sin(\pi J_{12t})\cos(\pi J_{13t})2\hat{I}_{y} \hat{I}_{2x} \xrightarrow{2\pi d_{13t}I_{1u}} \sin(\pi J_{12t})\cos(\pi J_{13t})\cos(\pi J_{14t})2\hat{I}_{y} \hat{I}_{2z} - \sin(\pi J_{12t})\cos(\pi J_{13t})\sin(\pi J_{14t})4\hat{I}_{1x} \hat{I}_{2x} \hat{I}_{4z}
\]

\[
- \sin(\pi J_{12t})\sin(\pi J_{13t})4\hat{I}_{1x} \hat{I}_{2x} \hat{I}_{3z} \xrightarrow{2\pi d_{13t}I_{1u}} -\sin(\pi J_{12t})\sin(\pi J_{13t})\cos(\pi J_{14t})4\hat{I}_{1x} \hat{I}_{2x} \hat{I}_{3z} - \sin(\pi J_{12t})\sin(\pi J_{13t})\sin(\pi J_{14t})8\hat{I}_{1x} \hat{I}_{2x} \hat{I}_{3z} \hat{I}_{4x}
\]

The product operator transformations are analogous for spins \( \hat{I}_{2x}, \hat{I}_{3x} \) and \( \hat{I}_{4x} \). After the application of a 45° pulse the resulting terms are:
\[
\begin{align*}
\cos(\pi J_{1,t}) \cos(\pi J_{1,t}) \cos(\pi J_{1,t}) \hat{l}_{1x}^{s_1(1x+1x+1x+1x)} & \rightarrow \cos(\pi J_{1,t}) \cos(\pi J_{1,t}) \cos(\pi J_{1,t}) \hat{l}_{1x}^{s_1(1x+1x+1x+1x)} \\
\cos(\pi J_{1,t}) \cos(\pi J_{1,t}) \sin(\pi J_{1,t}) 2 \hat{l}_{1y}^{s_2(1y+1y+1y+1y)} & \rightarrow \\
\cos(\pi J_{1,t}) \cos(\pi J_{1,t}) \sin(\pi J_{1,t}) 2 \hat{l}_{1y}^{s_2(1y+1y+1y+1y)} & \rightarrow \\
\cos(\pi J_{1,t}) \sin(\pi J_{1,t}) \cos(\pi J_{1,t}) 2 \hat{l}_{1y}^{s_3(1y+1y+1y+1y)} & \rightarrow \\
\cos(\pi J_{1,t}) \sin(\pi J_{1,t}) \cos(\pi J_{1,t}) 2 \hat{l}_{1y}^{s_3(1y+1y+1y+1y)} & \rightarrow \\
-\cos(\pi J_{1,t}) \sin(\pi J_{1,t}) \cos(\pi J_{1,t}) 4 \hat{l}_{1z}^{s_4(1z+1z+1z+1z)} & \rightarrow \\
-\cos(\pi J_{1,t}) \sin(\pi J_{1,t}) \cos(\pi J_{1,t}) 4 \hat{l}_{1z}^{s_4(1z+1z+1z+1z)} & \rightarrow \\
-\cos(\pi J_{1,t}) \sin(\pi J_{1,t}) \cos(\pi J_{1,t}) 4 \hat{l}_{1z}^{s_4(1z+1z+1z+1z)} & \rightarrow \\
-\cos(\pi J_{1,t}) \sin(\pi J_{1,t}) \cos(\pi J_{1,t}) 4 \hat{l}_{1z}^{s_4(1z+1z+1z+1z)} & \rightarrow \\
-\cos(\pi J_{1,t}) \sin(\pi J_{1,t}) \sin(\pi J_{1,t}) 8 \hat{l}_{1y}^{s_5(1y+1y+1y+1y)} & \rightarrow \\
-\cos(\pi J_{1,t}) \sin(\pi J_{1,t}) \sin(\pi J_{1,t}) 8 \hat{l}_{1y}^{s_5(1y+1y+1y+1y)} & \rightarrow \\
\end{align*}
\]

All bilinear terms are removed and half of the magnetization from higher order terms is transformed into undetectable multiple quantum coherences.
Appendix C: Macro used in Timecourse DOSY tools to process the maltotriose data.

%% Maltotriose 19dec09 gotadosy
% Adolfo Botana, 5 December 2009
% Macro to run tdparafac with a predefined input
% AB 26dec09: updated with other normalization methods

% tdparafac description:
% PARAFAC analysis with additional plots and fitting data
% for Timecourse experiments (Time And DOSY)
% Displays rate constants for 1st order reactant and product

%% User defined parameters

close all
clear all
format short g

load ma3_8jan_reduced % Data to be loaded
shortdata=reduced; % Data structure should be named shortdata
model=[ ]; % Diffusion encoding levels
mode2=[ ]; % DOSY experiments
range=[1:5300]; % Spectral range to be analysed
% reference=[11251:11410]; % Interval of the spectra where the integral should be constant
reference=[9300:9550]; % Interval of the spectra where the integral should be constant
% Datapoint will be normalized to this region
% If left empty no action is taken
% ORIGINAL DATA WILL BE OVERWRITTEN, and
normalized variable becomes 1
norm_flag=1; % Normalisation type: 0: None
% 1: Normalised on reference integral
% 2: Normalised on the whole spectrum
% 3: Correction for ADC variations in reference region

ncomp=3; % Number of guessed components
integrals=[7 14 21]; % Value of the integrals for each component according to the 1st spectrum until the last spectrum and reaction stoichiometry
options=[0 0 1 0 10 300]; % Parafac options:change 3rd to 0 if memory problems (usually it can be 1)
plotoption=1; % 0:Labelled plots // 1: Scaled plots // 2: Old plots
specshown=[1 48]; % Gradient and dosyexperiment shown in separated plot (if plotoption==2)
constraints=[2 2 2]; % Parafac constraints, set to [2 2 2] for nonnegativity in all three modes
gainflag=1; % If set to 1 displays Integral variation plots
rangeg=[]; % Spectral range to be integrated (all if set to 0)

% Calculation of X AXIS for mode2: xdata as argument of tdparafac
if isempty(mode2)
    mode2=1:size(shortdata.SPECTRA,2);
end
texp=235.69; % time needed for each measurement in seconds
xdata=(mode2-mode2(1))*texp*length(shortdata.Gzlvl); % time in seconds
% It requires a continuous mode2 (e.g. 3:50)
% number of dosy experiment * time experiment * number of gradient levels

%% Processing
if isempty(model)
    model=1:size(shortdata.SPECTRA,1);
end
if isempty(mode2)
    mode2=1:size(shortdata.SPECTRA,2);
end
if isempty(range)
    range=1:size(shortdata.SPECTRA,3);
end
if isempty(rangeg)
    rangeg=1:size(shortdata.SPECTRA,3);
end

%% Gain variation plot
% The gain of the spectrometer may have changed during the time course so
% first check the change in total integral over the timepoints
if gainflag==1
    figure('Color',[1 1 1],...
        'NumberTitle','Off',...
        'Name','Integral comparison for each gradient increment versus DOSY experiment');
    for k=1:length(model)
        subplot(length(model),1, k)
        plot(sum(squeeze(shortdata.SPECTRA(k,:,rangeg))'))
        ylabel(['G ' num2str(k)],'Color',[0.8471 0.1608 0],'FontWeight','bold')
    end
end
%% Integral and ADC of reference
if ~isempty(reference)
    for k2=1:length(mode2)
        for k1=1:length(mode1)
            int_ref(k2,k1)=sum(shortdata.SPECTRA(k1,k2,reference));
        end
    end
    xdata2=[shortdata.dosyconstant shortdata.Gzlvl(1:length(mode1))];
    x0=[1;5E-10];
    optionsr=optimset('TolFun',1e-10);
    [estimates(k2,:),res1,j,sigma]=nlinfit(xdata2,int_ref(k2,:),@fdecaydiffqw,x0,optionsr);
end
figure('Name','Amplitudes of reference')
plot(estimates(:,1))
figure('Name','ADCs of reference')
plot(estimates(:,2))
end

%% Normalization
if norm_flag==1 %normalization on reference
    for k1=1:length(mode2)
        for k2=1:length(mode1)
            shortdata.SPECTRA(k2,k1,range)=squeeze(shortdata.SPECTRA(k2,k1,range))/estimates(k1,1);
        end
    end
elseif norm_flag==2 %normalization on the whole spectrum
    for k1=1:length(mode2)
        grad_one_each_dosy=sum(squeeze(shortdata.SPECTRA(1,k1,range))');
        for k2=1:length(mode1)
            shortdata.SPECTRA(k2,k1,range)=squeeze(shortdata.SPECTRA(k2,k1,range))/grad_one_each_dosy;
        end
    end
elseif norm_flag==3 %normalise the integrals to the average of the integral of the reference peak (TSP)
    if ~isempty(reference) && ~exist('normalized','var')
        for k=1:length(mode1)
            average_ref(k,:)=sum(squeeze(shortdata.SPECTRA(k,:,reference))');
            mean_ref=mean(average_ref');
        end
        for k=1:length(mode1)
            for m=1:length(mode2)
                shortdata.SPECTRA(k,m,range)=squeeze(shortdata.SPECTRA(k,m,range)).*(mean_ref(k)/average_ref(k,m));
            end
        end
    end
end
if gainflag==1
    figure('Color',[1 1 1],'NumberTitle','Off',...
              'Name','Integral comparison after normalisation');

    for k=1:length(model)
        subplot(length(model),1, k)
        plot(sum(squeeze(shortdata.SPECTRA(k,:,:))'))
        ylabel(['G ', num2str(k)],'Color',[0.8471 0.1608 0],'FontWeight','bold')
    end
    normalized=1;
end

%% Parafac
[b,b2,scaling,estimates,xdata1,err,conc]=tdparafac(shortdata,model,mode2,range,ncomp,options,plotoption,xdata,specshown,integrals,constraints);
Appendix D: tdparafac function in Timecourse DOSY tools

function [b,b2,scaling,estimates,xdata1,err,conc]=tdparafac(shortdata,model1,model2,
rangep,ncomp,options,plotoption,xdata2,specshown,integrals,constraints,ol
dmode,fixes)
% Adolfo Botana, 5 December 2009
% PARAFAC analysis with additional plots and fitting data
% for Timecourse experiments (Time And DOSY)
% Displays rate constants for 1st order reactant and product

% 8Jun09 updated gradient attenuation decay to nlinfit

%% Definition of missing arguments
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
format short g
if nargin<12
    oldmode=[];
    fixes=[];
    if nargin<11
        constraints=[];
        if nargin<10
            integrals=[];
        end
    end
end
if isempty(plotoption)
    plotoption=1;
end
if isempty(model1)
    model1=1:size(shortdata.SPECTRA,1);
end
for k1=1:length(model1)
    gzlvl(k1)=shortdata.Gzlvl(model1(k1));
end
if isempty(model2)
    model2=1:size(shortdata.SPECTRA,2);
end
if isempty(rangep)
    rangep=1:size(shortdata.SPECTRA,3);
end
if isempty(xdata2)
    texp=81.6; %time needed for each measurement
    xdata2=(model2-model2(1))*texp*length(shortdata.Gzlvl);
end
if isempty(specshown)
    specshown=[1 1];
end
if options(3)==1
    figure
end

%% PARAFAC

---
[b, it, err, conc] = parafac(shortdata.SPECTRA(model, mode2, rangep), ncomp, options, constraints, oldmode, fixes);

%% Display with labelled axis
ydata = b{1}(:, 1);
xdata1 = gzlvl(1:length(ydata));
figure
subplot(2, 2, 1)
plot(xdata1, b{1})
% set(gca,'YTickLabel','')
title('Diffusion mode', 'FontSize', 10, 'Color',[0.8471 0.1608 0])
xlim([gzlvl(1) gzlvl(end)])
% xlim([0 xdata(size(xdata,2))])
xlabel('Gradient level (T/m)', 'FontSize', 10)

subplot(2, 2, 2)
plot(b{2})
% set(gca,'YTickLabel','')
title('Evolution mode', 'FontSize', 10, 'Color',[0.8471 0.1608 0])
xlabel('DOSY experiment', 'FontSize', 10)

subplot(2, 2, 3)
plot(shortdata.Ppmscale(rangep), b{3})
% set(gca,'YTickLabel','')
xlim([shortdata.Ppmscale(rangep(end)) shortdata.Ppmscale(rangep(1))])
set(gca, 'XDir', 'reverse')
title('Spectral mode', 'FontSize', 10, 'Color',[0.8471 0.1608 0])
xlabel('Chemical shift (ppm)', 'FontSize', 10)

%% Scaling modes
for k1 = 1:ncomp
    [value, maxi(k1)] = max(b{3}(:, k1));
end
b2 = b;

%% Fitting mode 1
modeused = 1;
estimates = zeros(ncomp, 2);
for comp = 1:ncomp
    ydata = b2(modeused)(:, comp);
xdata5 = [shortdata.dosyconstant xdata1];
x0 = [1; 5E-10];
options = optimset ('TolFun', 1e-10);
[estimates(comp, [1 2]), res1, j, sigma] = nlinfit(xdata5, ydata, @fdecaydiffqw, x0, options);
ci2 = nlparci(estimates(comp,:), res1, 'covar', sigma);
ci2(2,:) = xdata6 = linspace(xdata1(1), xdata1(end), 50);
modeused=1;
scaling=zeros(3,ncomp);
for k1=1:ncomp %every component
  scaling(modeused,k1)=1/estimates(k1,1); %scaling factors: rows=modes, columns=components
  for k2=1:size(b{modeused},1) %every increment
    b2{modeused}(k2,k1)=b{modeused}(k2,k1)/estimates(k1,1);
  end
  for k2=1:length(xdata6) %every increment
    diffit(k2,k1)=diffit(k2,k1)/estimates(k1,1);
  end
end

modeused=3;
if isempty(integrals)
  for k1=1:ncomp %every component
    residint=1;
tole=1E-6;
    sum1=sum(b2{modeused}(:,k1));
    while residint>t ole  % Apparently useless?
      b2{modeused}(:,k1)=b2{modeused}(:,k1)/sum1;
      sum1=sum(b2{modeused}(:,k1));
      residint=abs(sum1-1);
    end
    scaling(modeused,k1)=b2{modeused}(maxi(k1),k1)/b{modeused}(maxi(k1),k1);
  end
else
  for k1=1:ncomp %every component
    scaling(modeused,k1)=integrals(k1)/sum(b2{modeused}(:,k1));
    b2{modeused}(:,k1)=b{modeused}(:,k1)*scaling(modeused,k1);
  end
end

modeused=2;
for k1=1:ncomp %every component
  for m1=1:length(mode1)
    for m2=1:length(mode2)
      scaling(modeused,k1)=1/(scaling(1,k1)*scaling(3,k1));
      b2{2}(m2,k1)=b{2}(m2,k1)*scaling(modeused,k1);
    end
  end
end

%% Fitting mode 2 %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Mode 1 fitted in previous section
modeused=2;
for comp=1:ncomp
    ydata=b2{modeused}(:,comp);
    if b2{modeused}(length(mode2),comp)>b2{modeused}(1,comp)
        x0=[0.1;3;1E-4];
        options=optimset('TolFun',1e-10);
        estimatestemp=fminsearch(@fproduct1,x0,options,xdata2,ydata');
        estimates2(comp)=estimatestemp(3);
    else
        x0=[3;1E-4];
        options=optimset('TolFun',1e-10);
        estimatestemp=fminsearch(@freactant1,x0,options,xdata2,ydata');
        estimates2(comp)=estimatestemp(2);
    end
end

scaling estimates estimates2

%% Plots

if plotoption>0
    figure('Name','Scaled')
    subplot(2,2,1)
    plot(xdata1,b2{1},'o')
    hold on
    plot(xdata6,diffit)
    title('Diffusion mode','FontSize',10,'Color',[0.8471 0.1608 0])
    xlim([gzlvl(1) gzlvl(length(gzlvl))])
    xlabel('Gradient level (T/m)','FontSize',10)
    subplot(2,2,2)
    plot(xdata2,b2{2})
    title('Evolution mode','FontSize',10,'Color',[0.8471 0.1608 0])
    xlabel('Time (s)','FontSize',10)
    subplot(2,2,3)
    plot(shortdata.Ppmscale(rangep),b2{3})
    title('Spectral mode','FontSize',10,'Color',[0.8471 0.1608 0])
    xlabel('Chemical shift (ppm)','FontSize',10)
    texty=''
    modeused=1;
for k2=1:ncomp % every component
texty2=['D(' num2str(k2) ')='' num2str(estimates(k2,2))];
texty=strvcat(texty,texty2);
end
modeused=2;
for k2=1:ncomp % every component
texty2=['k(' num2str(k2) ')='' num2str(estimates2(k2))];
texty=strvcat(texty,texty2);
end
texty2=[' '];
texty=strvcat(texty,texty2);
texty2=['Scaling factors:'];
texty=strvcat(texty,texty2);
for modeused=1:2 % 3rd mode information is redundant: scale3=1/scale1/scale2
    for k2=1:ncom
        texty2=['Mode ' num2str(modeused) ' (' num2str(k2) ')='' num2str(scaling(modeused,k2))];
        texty=strvcat(texty,texty2);
    end
end

%% Separated plot
figure('Name','Normalised spectra')
rgborder=get(gca,'ColorOrder');
for k2=1:size(b{3},2)
    subplot(size(b{3},2),1,k2,'FontSize',12)
    if k2>size(rgborder,1)
        plot(shortdata.Ppmscale(rangep),b2{3}(:,k2));
    else
        plot(shortdata.Ppmscale(rangep),b2{3}(:,k2),
        'Color',rgborder(k2,:));
    end
    box('off');
    % set(gca,'YTickLabel',''
    xlim([shortdata.Ppmscale(rangep(end))
    shortdata.Ppmscale(rangep(1))])
    set(gca,'XDir','reverse');
    if k2==size(b{3},2)
        set(gca,'XTickLabel','';
    end
end
xlabel('Chemical shift (ppm)','FontSize',12')

% Old plots

if plotoption==2
figure
subplot(2,2,1)
plot(xdata1,b2{1})
% set(gca,'YTickLabel',''
title('Diffusion mode','FontSize',10,'Color',[0.8471 0.1608 0])
xlim([gzlvl(1) gzlvl(length(gzlvl))])
% xlim([0 xdata(size(xdata,2))])
xlabel('Gradient level (T/m)','FontSize',10)

subplot(2,2,2)
plot(b{2})
% set(gca,'YTickLabel',''
title('Evolution mode','FontSize',10,'Color',[0.8471 0.1608 0])
xlabel('DOSY experiment','FontSize',10)

subplot(2,2,3)
plot(shortdata.Ppmscale(rangep),b{3})
% set(gca,'YTickLabel',''
xlim([shortdata.Ppmscale(rangep(end)) shortdata.Ppmscale(rangep(1))])
set(gca,'XDir','reverse')
title('Spectral mode','FontSize',10,'Color',[0.8471 0.1608 0])
xlabel('Chemical shift (ppm)','FontSize',10)

%Texts
texty=''
k1=1;
for k2=1:ncomp %every component
  texty2=['D(' num2str(k2) ')=' num2str(estimates(k2,2))];
  texty=strvcat(texty,texty2);
end

for k2=1:ncomp
  texty2=['Mode ' num2str(modeused) '( ' num2str(k2) ')=' num2str(scaling(modeused,k2))];
  texty=strvcat(texty,texty2);
end

annotation('textbox',...
'String',{texty},...
'FitHeightToText','off',...
'BackgroundColor',[1 1 1],...
'LineStyle','none',...
'Position',[0.5732 0.01905 0.2482 0.4452]);

figure
for k2=1:size(b{3},2)
 subplot(size(b{3},2),1,k2,'FontSize',12)
 if k2>size(rgborder,1)
     plot(shortdata.Ppmscale(rangep),b2{3}(:,k2));
 else
     plot(shortdata.Ppmscale(rangep),b2{3}(:,k2),'Color',rgborder(k2,:));
 end
 box('off');
 % set(gca,'YTickLabel','')
 xlim([shortdata.Ppmscale(rangep(length(rangep)))
 shortdata.Ppmscale(rangep(1))])
 set(gca,'XDir','reverse');
 if k2==size(b{3},2)
     set(gca,'XTickLabel','');
 end
 end
 if k2~=size(b{3},2)
     set(gca,'XTickLabel','');
 end
 end
 end

 % Used functions

 function sse=fdecaydiffqw(params,Input)
     dosyc=Input(1);
     Input=Input(2:length(Input));
     a=params(1);
     b=params(2);
     sse=a*exp(-b*dosyc*Input.^2)';
 end

 function sse=freactant1(params,Input,Actual_Output)
     b=params(1);
     c=params(2);
     Fitted_Curve=b*exp(-c*Input);
     Error_Vector=Actual_Output-Fitted_Curve;
     sse=sum(Error_Vector.^2);
 end

 function sse=fproduct1(params,Input,Actual_Output)
     a=params(1);
     b=params(2);
     c=params(3);
     Fitted_Curve=a+b*(1-exp(-c*Input));
     Error_Vector=Actual_Output-Fitted_Curve;
     sse=sum(Error_Vector.^2);
 end

%% To do, fit to NUG instead: indata.nugc=[0.0 0.0 0.0 0.0]
% Fitted_Curve = a(1)*exp( - nugc(1)*a(2)*expfactor.*xdata).^1 -...
%     nugc(2)*(a(2)*expfactor.*xdata).^2 -...
%     nugc(3)*(a(2)*expfactor.*xdata).^3 -...
%     nugc(4)*(a(2)*expfactor.*xdata).^4);
Appendix E: Diffusion NMR and trilinear analysis in the study of reaction kinetics (published paper)


**DOI:** 10.1039/b820813a

Measurement of diffusion-weighted NMR spectra as a function of time allows the time-dependence of concentration and the isolated spectrum to be found for each component in a reaction, without prior assumptions about spectra, kinetics or diffusion behaviour, by data decomposition using the PARAFAC algorithm.

Nuclear Magnetic Resonance (NMR) is frequently employed to study reaction kinetics. NMR can provide detailed structural information about (and often identify) the chemical entities involved in a reaction, and as it is non-invasive and non-destructive, the kinetics of an intact mixture can be studied in real time directly in the NMR tube\(^1\) (there are also a number of alternatives for the study of reaction conditions that cannot be duplicated in an NMR probe\(^2\)). Reaction monitoring by NMR works best when each component in a reaction mixture has at least one well-resolved resonance; the change in peak integral can then be used directly to determine the kinetic behaviour\(^3\). When no such resolved peaks are available, as is quite common, the extraction of kinetic data becomes much more challenging, and it is frequently impossible to identify individual reaction components, let alone determine their concentrations. In this investigation, we demonstrate that by adding diffusion information to the NMR experiments, the spectrum, time evolution and diffusion data can be recovered for each component in the reaction mixture. Because the data are trilinear (i.e. they vary independently in three dimensions, here diffusional attenuation, time
evolution and chemical shift) they can be decomposed using a PARAFAC algorithm, and it is therefore possible to analyse the data without the need for fitting to a predetermined model, and without having to constrain the data to fit either the reaction kinetics or the diffusional attenuation.

**Figure 1.** A subset of the raw experimental data. For the time evolution every 16th spectrum is shown, and for the decay with gradient amplitude (caused by diffusion) the first three gradient levels are shown.

The study of reactions is an example of the general case of mixture analysis by NMR. It is well known that it can be frustrating to study intact mixtures by NMR, as it is often difficult to assign resonances unambiguously to given mixture components. It is expensive, tedious and time-consuming to separate components physically (e.g. by chromatography) before submitting them to NMR, and frequently it is the study of the intact mixture itself that is of interest (as for reaction monitoring). Therefore it is highly desirable to develop NMR methods that can recover the required information from intact mixtures. Some of the most powerful NMR methods currently available, commonly referred to as DOSY (diffusion-
ordered spectroscopy) experiments, are based on diffusion\textsuperscript{5-9}; these are most effective where each component in a mixture has a unique rate of diffusion. The diffusion of molecules can be measured by recording the signal attenuation in a pulsed field gradient NMR experiment\textsuperscript{10}, typically by incrementing the gradient strength in a pulsed field gradient spin or stimulated echo. It was recognised early on that the results of such experiments can be used to distinguish the signals from different molecular species\textsuperscript{11}. The decays of individual NMR signals are typically fitted to a model function, and the fitted diffusion coefficient is then used to correlate the signals of individual molecular species. In high resolution DOSY\textsuperscript{6, 5}, this is done by fitting each peak individually (implicitly assuming that there is no spectral overlap), while in multivariate methods the whole bandwidth is fitted simultaneously\textsuperscript{8, 9, 7}. The model function used is typically some form of the Stejskal-Tanner equation\textsuperscript{10}, which describes the effect of pulsed field gradient on signal amplitude for free (unbounded) diffusion; for best results, the equation can be extended to include the effects of imperfect field gradient uniformity\textsuperscript{12, 6}.

Diffusion-ordered spectroscopy and kinetic studies by NMR have a good deal in common: both rely on fitting variations in NMR signal amplitude to suitable model functions, and in both cases it is far easier to analyse experimental data when the NMR signals of individual species are well resolved. DOSY data and timecourse spectra are bilinear: signal intensity $I$ is measured as a function of two variables, frequency and gradient amplitude, and frequency and time respectively. In a bilinear dataset, the theoretical intensity $I_i$ for a given signal $i$ is the product of the signal variation as a function of two different variables, $I_i(p,q) = P_i(p) Q_i(q)$. Thus in DOSY, if the spectrum of component $i$ is $S_i(f)$ and its signals attenuate as a function of gradient amplitude $g$ according to $A_i(g)$, then $I_i(f,g)$ is the product of $S_i(f)$ and $A_i(g)$. The experimental dataset is a tensor of rank 2, and may be represented as a sum over $N$ components $i$ of outer products of two vectors $S_i$ and $A_i$, plus some residual $E$: 
In analysing bilinear data with spectral overlap it is common to employ multivariate methods to help resolve the component spectra (and diffusion/kinetics)\textsuperscript{13, 14, 8, 9, 7}. Unfortunately such analyses suffer from the problem of rotational ambiguity: any linear combination of the true functions $P_i$, or the true functions $Q_i$, gives an equally good fit to the experimental data. For bilinear analysis it is therefore necessary to apply constraints, for example non-negativity and/or known/hypothesised kinetic models, to allow the true solutions to be selected out from the infinite range of linear combinations. This problem can be avoided, and a model-free fit obtained by PARAFAC\textsuperscript{4} decomposition, if trilinear data $I_i(p,q,r) = P_i(p) Q_i(q) R_i(r)$ can be measured. Adding a diffusion dimension to a bilinear dataset can create a trilinear structure\textsuperscript{15}. Recording NMR spectra as a function both of time and of gradient amplitude, i.e. measuring a timecourse of DOSY spectra, gives just such a dataset. No prior knowledge of the component spectra, diffusion behaviour or kinetics is required for its analysis; the only requirement is that the spectrum $S_i(f)$, diffusional attenuation $A_i(g)$ as a function of gradient $g$, and concentration profile $C_i(t)$ of each species be independent of each other, so that the signal intensity $I_i(f,g,t) = S_i(f) A_i(g) C_i(t)$. The experimental dataset is now a rank 3 tensor:

$$I = \sum_{i=1}^{N} S_i \otimes A_i \otimes C_i + E$$  \hspace{1cm} (2)
To demonstrate the value of using diffusion encoding in the NMR study of a reacting mixture we have chosen the well-known acid hydrolysis of maltose to glucose\textsuperscript{16}, in which one maltose is hydrolysed to two glucose units.

A aqueous solution of maltose (5.5 % w/w) in 33 % (w/w) sulfuric acid was prepared, with 0.15 % (w/w) pivalic acid as a reference compound. Hydrolysis was carried out at 50 °C in a thick-walled NMR tube (to prevent convection; i.d. 2.2 mm) in a 400 MHz Varian Inova

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**Figure 2.** Reference spectra of pure materials and spectra obtained from the data of Fig. 1 by PARAFAC for maltose (A) and glucose (B).
instrument, using a 5 mm diameter indirect detection probe equipped with a z-gradient coil allowing gradient pulses up to 30 G cm\(^{-1}\). A series of 98 DOSY experiments was carried out over the course of the reaction, in a total of 41 h 49 min. Each DOSY experiment used the oneshot sequence\(^{17}\) with 32 transients at each of 6 gradient levels, with equal steps in gradient squared, ranging from 3.0 to 27.3 G cm\(^{-1}\). The data were then Fourier transformed, phase corrected, baseline corrected, reference deconvoluted\(^{18}\) using the pivalic acid signal, and the solvent (HOD) peak was removed by digital filtering, all using the manufacturer's VnmrJ software, before export to MATLAB for further analysis. All PARAFAC analysis was performed with the MATLAB N-Way toolbox\(^ {19,20}\). Fig. 1 shows a subset of the experimental spectra as a function of time and gradient level. Small variations in receiver sensitivity over the course of the experiment were corrected for by normalising the integral of the spectrum for each gradient level using the average area of the pivalic acid reference peak.

PARAFAC fitting was carried out, assuming two components, for the spectral region 3.1-5.5 ppm. The resultant fit accounted for 99.8\% of the variance in the data, yielding statistical components \(S_i(f)\), \(A_i(g)\) and \(C_i(t)\) representing the spectrum, signal decay as a function of gradient strength, and time evolution for the reactant and product respectively.

One great advantage of PARAFAC fitting is that, if the assumption of trilinearity holds, the fitted components obtained should have physical relevance, i.e. should in this case be the true spectrum, diffusional attenuation and concentration timecourse. Where prior information exists, therefore, it is possible to assess directly the quality of the PARAFAC decomposition, for example by comparing the spectra of reaction components extracted by fitting the experimental data with the known spectra of the pure materials. As can be seen in Fig. 2, in this case the fitted spectra are virtually identical to the spectra of pure maltose and pure glucose, confirming that trilinear decomposition into two components was successful.
Figure 3. Non-linear least squares fits to first order kinetics for the acid hydrolysis of maltose to glucose. A) relative concentrations of maltose (integral of the inner anomic signal at 5.4 ppm, decaying curve) and glucose (sum of the integrals of the peaks between 4.6 and 5.3 ppm minus the integral of that at 5.4 ppm, rising curve). Estimated rate constant $1.36 \pm 0.02 \times 10^{-5}$ s$^{-1}$. B) Normalised PARAFAC components $C_i(t)$ for maltose (decaying) and glucose (rising); $k = 1.40 \pm 0.01 \times 10^{-5}$ s$^{-1}$.

Because the relative scaling of the three multiplicands in the trilinear model is arbitrary, to obtain true relative concentrations from the analysis it is necessary to ensure that the other two modes in the model, $S_i(f)$ and $A_i(g)$, are normalised. Where, as here, the structures of the reaction components are known this is straightforward: each PARAFAC spectral mode output $S_i(f)$ is normalised to have an integral proportional to the number of protons involved, and each diffusion mode $A_i(g)$ is normalised so that it extrapolates back to unity at zero gradient $g$. Multiplying the remaining raw modes $C_i(t)$ by the normalisation factors by which the $S_i(f)$ and $A_i(g)$ were divided then gives $C_i(t)$ modes which are directly proportional to concentration. The net result for the experimental data of Fig. 1 is shown in Fig. 3B, and as expected gives an excellent fit to first order kinetics. The PARAFAC results can, for this model system in which well-resolved anomic signals are available, be compared with relative concentration profiles obtained by direct integration of the respective anomic signals (Fig. 3A); while there is excellent agreement, the signal-to-
noise ratio for the PARAFAC result is, as expected, superior. Although for this example the full range of chemical shifts, including the well-resolved anomeric signals, was used, essentially identical results were obtained when only the highly overlapped region between 3.1 and 4.1 ppm was fitted, confirming that both fully-resolved spectra and kinetic information can be recovered even when the experimental data contain no resolved peaks. The PARAFAC decomposition is remarkably robust; similar results can be obtained using only two of the six gradient increments measured, and/or with many fewer time points.

From this model study it is clear that by adding diffusion information to an experimental time course study and using multi-way methods to decompose the results into the underlying structure of NMR spectrum, diffusional attenuation and time evolution, it is possible not only to obtain good estimate kinetic data irrespective of whether any resolved signals are available, but also to recover the NMR spectra of individual reaction components. In principle, one could obtain by this method the NMR spectra of intermediates that are difficult or impossible to isolate. The fundamental requirement is that each reaction component shows a different diffusion coefficient and a different time course; even where these requirements are not fully met, a hybrid analysis in which PARAFAC is constrained using prior knowledge (e.g. spectral non-negativity) can still succeed. It should however be stressed that for a trilinear PARAFAC decomposition, no assumptions are needed about the form of the spectra, the diffusional attenuation, or the kinetics. Thus decomposition is just as simple even when no prior information is available, as for example in a heterogeneous reaction where the functional form of the attenuation caused by (bounded) diffusion is unknown.

References

Appendix F: $T_1$-Diffusion-Ordered Spectroscopy: Nuclear Magnetic Resonance Mixture Analysis Using Parallel Factor Analysis (published paper)


DOI: 10.1021/ac901321w

Abstract
DOSY (Diffusion-Ordered Spectroscopy) is one of the most commonly-employed methods for identifying compounds in mixtures by NMR. However, it struggles to resolve component spectra when there is severe signal overlap and/or diffusion coefficients are very similar. In order to improve the ability of DOSY to distinguish between different species, relaxation has here been incorporated into diffusion experiments as a further dimension. This results, to a first approximation, in a locally trilinear dataset which, in contrast with a bilinear dataset (e.g. a standard DOSY dataset), can be decomposed with multivariate statistical methods such as PARAFAC (Parallel Factor Analysis). This enables overlapping multiplets from different species, and by extension whole spectra, to be separated.

Keywords: $T_1$-DOSY, PARAFAC, DOSY, MATLAB, non-uniform field gradients, diffusion, pulsed field gradients (PFG), multi-way, multivariate, trilinear, mixture analysis.
Introduction

The characterization of mixtures is a problem faced frequently both in academic research and in the chemical and pharmaceutical industries. While NMR excels as a powerful tool for the chemical structure elucidation of pure compounds, it is inherently flawed as an analytical tool for mixtures as unambiguous assignment of signals to a particular molecular species is often impossible. Preparing pure components by physical separation is expensive in terms of time and of resources, and is not always feasible (e.g. in the study of interactions between molecules). This has stimulated the development of techniques that allow the extraction of the NMR spectra of different compounds in an intact mixture, with some of the most powerful approaches exploiting self-diffusion properties, which are dependent on the molecular size.

A diffusion NMR dataset is typically acquired by measuring a series of spin or stimulated echo spectra with pulsed field gradients (PFG) of increasing strength. Attenuation of the signal is dependent on the diffusion coefficient, as described by Stejskal and Tanner:

\[ S(G) = S_0 e^{-D\gamma^2\delta G^2 \Delta'} \]  

(1)

where \( S \) is the signal amplitude, \( S_0 \) is the amplitude that would have resulted without diffusion, \( D \) is the diffusion coefficient, \( \delta \) is the gradient pulse width, \( \gamma \) is the magnetogyric ratio, \( G \) is the gradient amplitude, and \( \Delta' \) is the diffusion time corrected for the effects of finite gradient pulse width. In practice the decay deviates somewhat from Eq. [1] as the pulsed field gradients are not uniform over the sample volume, so that the actual experimental signal decay from a monodisperse sample is equivalent to that from a polydisperse sample if the gradients had been perfectly uniform. Fortunately, an empirical expression for the signal decay can be obtained by mapping the gradient and signal profiles, and then parametrized as, for example, the exponential of a power series, an approach that has been used extensively in this laboratory:

\[ S(G) = S_0 \sum_{n=1}^{N} c_n e^{-\frac{D\gamma^2\delta^2 G^2 \Delta'}{n}} \]  

(2)

where the coefficients \( c_n \) are spectrometer/probe/pulse sequence-dependent.
The commonest processing method for PFG NMR diffusion data on mixtures is Diffusion-Ordered Spectroscopy (DOSY)\textsuperscript{5,6} in which the amplitude or area of each peak is fitted to an appropriate decay function and a pseudo 2D plot is generated with the NMR spectrum as the first dimension and diffusion as a second, with the peaks set to a Gaussian lineshape centred on the calculated diffusion coefficient, and a width which is generally determined by the standard error of the fit. In the High Resolution DOSY\textsuperscript{7} approach each signal is fitted to a single exponential function (e.g. Eq. [1] or [2]), assuming that a single molecular species is responsible for each signal. When this assumption holds, high resolution in the diffusion dimension can be obtained, with differences in diffusion coefficient as small as 0.5\% being resolvable; when overlap is present, other processing schemes can be used but with a severe reduction in diffusion resolution (\textit{vide infra}). The majority of interesting mixtures, however, show a \textsuperscript{1}H NMR spectrum with overlapping signals, so considerable ingenuity has gone into devising 2D\textsuperscript{7-9} and 3D\textsuperscript{10-13} DOSY experiments where the overlap of NMR signals is minimized; the use of a different nucleus to the proton can also be very effective at providing spectra with little or no overlap\textsuperscript{8,10,14,15}. However, such experiments usually require long data acquisition times, and the analysis of 3D DOSY data is also much more demanding than 2D.

When NMR signals overlap, the experimental signal decay as a function of gradient strength consists of the superimposed decays for the molecular species involved, and the effect in HRDOSY is typically to give apparent diffusion coefficients that are a compromise intermediate between the true values, making interpretation a frustrating task. The obvious expedient of fitting the data to a sum of decaying exponentials is fraught with difficulties for experimental (noisy) data, and is well-known as an ill-conditioned mathematical problem\textsuperscript{16}. A variety of methods have been used for the analysis of overlapping signal decays in diffusion NMR. These include methods that, like HR DOSY, are univariate and fit each signal individually, such as multiexponential fitting\textsuperscript{3,17}, CONTIN\textsuperscript{18}, maximum entropy\textsuperscript{19}, iRRT\textsuperscript{20}, and Hopfield neural networks\textsuperscript{21}. Other methods, such as DECRA\textsuperscript{22}, MCR\textsuperscript{23}, CORE\textsuperscript{24,25} and SCORE\textsuperscript{26}, use the fact that all signals from a given molecular species shows identical diffusion behaviour (in the absence of
exchange\textsuperscript{27,28} and take advantage of this covariance in the data in a multivariate approach where the entire dataset is fitted simultaneously.

In multivariate processing of diffusion NMR data it is common to describe the raw experimental data as a matrix $X$ in which each row represents the mixture spectrum for a given gradient amplitude. The contribution of each mixture component to $X$ can be described (Eq. 3) as an outer product of a component spectrum ($A_p$) and the transpose of its decay as a function of gradient amplitude ($B_p$). The raw data can thus be described as a sum of $p$ such components and a matrix $R$ containing the residual unfitted data (ideally only experimental noise):

$$X = \sum_{p=1}^{P} a_p \otimes b_p + R \quad (3)$$

Such a bilinear dataset $X$ (varying independently in two dimensions) suffers inherently from rotational freedom\textsuperscript{29} (or rotational ambiguity), meaning that there is a range of solutions for $A$ and $B$ that can be interconverted by linear transformations and still fit the data equally well. In practice this means that it is only possible to obtain physically meaningful decompositions of $X$ by applying constraints (e.g. non-negativity) on the bilinear model that use prior knowledge about the underlying phenomena, as is done for example in MCR, DECRA, CORE and SCORE; a discussion about the different assumptions made can be found in ref \textsuperscript{26}. These methods can give good results when appropriate assumptions are used for the constraints (e.g. pure exponential decay in DECRA), but cross-talk between the spectra $A$ of the different components is common for mixtures of chemical species with similar diffusion coefficients.

By extending the data with an additional independent dimension to obtain trilinear data (varying independently in three dimensions), it can be modelled in a way that does not suffer from rotational freedom\textsuperscript{30}. This is achieved by the PARAFAC model\textsuperscript{31,32} (PARallel FACtor analysis). For a number of components $P$, a PARAFAC model of a three-way array can be described by Eq. 4:

$$X = \sum_{p=1}^{P} a_p \otimes b_p \otimes c_p + R \quad (4)$$
where $A_p, B_p$ and $C_p$ are the $p$\textsuperscript{th} columns, corresponding to the component $p$, of the matrices $A, B$ and $C$, while matrix $R$ contains the residuals. Given the right number of components, the matrices $A, B$ and $C$ contain the evolution of each component along each dimension.

It has been shown previously that a third dimension can be added to a DOSY experiment to obtain such a trilinear data structure by using a number of mixture samples with different relative concentrations\textsuperscript{33-35}, or by following the evolution of concentration during a chemical reaction\textsuperscript{36}. The potential advantages of using trilinear data are important: the contrast added by the third dimension can allow separations of component spectra that are not possible with bilinear data alone (e.g. the extraction of overlapping spectra of molecules with similar diffusion coefficients), and as the PARAFAC decomposition is model-free the only prior knowledge or assumption required (besides trilinearity) is the number of (chemical) components in the mixture.

The combination of relaxation and diffusion has been used previously to obtain trilinear data for a set of samples when no high resolution spectral dimension was available\textsuperscript{37}. In this investigation we propose the use of (longitudinal) relaxation behaviour as a third dimension, in order to separate the spectra of the components in a single intact sample. As relaxation behaviour is in general different for different chemical sites, a complete diffusion-relaxation dataset will not in general be trilinear: while the diffusional attenuation will (in the absence of exchange effects) be the same for every signal in the spectrum of a given component, the $T_1$ values can differ. A dataset therefore has to be partitioned into individual spectral regions, containing a maximum of one multiplet for each component, before it can be decomposed by PARAFAC, but the complete component spectra can then be recovered by concatenating the components obtained from the decomposition of each partition. We show here that adding relaxation contrast to a diffusion experiment can allow clean separation of component spectra where experimental data obtained with diffusion alone did not.
Pulse sequences

In order to combine relaxation encoding with diffusion encoding three sequences were investigated, all which are based on the standard diffusion encoding (DOSY) Oneshot sequence\textsuperscript{38} (Fig. 1). The first two sequences were constructed by concatenating a relaxation encoding segment with the DOSY sequence, and the third by incorporating the relaxation encoding within the existing diffusion delay, as in an iDOSY experiment\textsuperscript{11,39,40}

The first sequence, Inversion Recovery ONeshot (IRON), prefaces the Oneshot sequence with an inversion recovery (Fig. 1a). A conceptually similar sequence (T\textsubscript{1}-weighed spin echo) has been used previously for the determination of water diffusion coefficients\textsuperscript{41}. The advantage of the IRON sequence is that it maximizes the dynamic range available for the relaxation encoding, but it requires a relatively long recovery time d\textsubscript{1} to ensure that the magnetization is at thermal equilibrium before inversion. Reference deconvolution\textsuperscript{42} is generally not applicable to data acquired using the IRON sequence due to the zero crossing of the reference signal. This can be a severe drawback, as such data correction has been shown to important for obtaining the best results in both uni- and multivariate processing of DOSY data\textsuperscript{3,43}.

In the second sequence, relaxation encoding is achieved using saturation recovery to form the SAturation Recovery ONeshot (SARON) sequence (Fig. 1b). This sequence enables more rapid data acquisition, as no recovery time d\textsubscript{1} is needed, and because the signals measured are always positive, reference deconvolution can be used for data correction. This experiment is reliant on very good saturation for a clean result, and the dynamic range is half that of the IRON experiment.
Figure 1. Pulse sequences used for T$_1$-DOSY. a) Inversion Recovery ONeshot (IRON); b) SAuration Recovery ONeshot (SARON); c) Decaying Relaxation ONEshot (DRONE). In a) and b) the effects of relaxation are encoded by varying the recovery delay $\tau$ and diffusion is encoded by varying the gradient strength $G$. In c) relaxation is encoded by varying the recovery delay $\tau$ in sympathy with the gradient strength $G$ so that the product $G^2\tau$ is kept constant for each step of diffusion encoding.

It is also possible to incorporate relaxation encoding within the normal Oneshot sequence by tailoring the parameters to make the relaxation encoding independent of the diffusion...
encoding, in the Decaying Relaxation ONEshot (DRONE) experiment (Fig. 1c). The relaxation delay $\tau$, now located between the second and third 90º pulses, is included within the diffusion time. The effect of $T_1$ relaxation is encoded in the signal amplitude by increasing the diffusion time $\Delta$ while keeping the product $G^2\Delta$ constant for a given level of diffusion encoding, in order to maintain the same diffusional attenuation for different values of $\tau$.

**Experimental**

The $T_1$-DOSY experiments were tested on a mixture of 1-propanol and 3-methyl-3-pentanol with a concentration of 15 mg/mL each in D$_2$O with 4 mg/mL TSP (sodium 3-(trimethylsilyl)-propionate-2,2,3,3-$d_4$) as reference. Reference spectra were measured for solutions of the individual alcohols in D$_2$O. Measurements were carried out non-spinning on a Varian Inova 400 spectrometer in an air-conditioned room at approximately 20 ºC, without spectrometer temperature regulation and with a passive probe air preconditioning system used to minimize temperature gradients.

Standard inversion recovery experiments with 12 different recovery delays were used to determine the $T_1$ values for the signals of the pure compounds, using 8 transients and 16384 complex data points. Diffusion measurements on the mixture sample were acquired in 19 min using the Oneshot sequence with 10 gradient amplitudes ranging from 3 to 27 G cm$^{-1}$ in equal steps of gradient squared using 16 transients, 16384 complex data points, a total diffusion-encoding gradient duration of 2 ms and a diffusion time of 0.2 s.

$T_1$-DOSY data using the IRON sequence were acquired in 20 h, using 8 recovery delays $\tau$ (ranging from 0.25 to 32 s) and 12 gradient amplitudes (ranging from 3 to 27 G cm$^{-1}$ in equal steps of gradient squared) using 16 transients, 16384 complex data points, a delay $d_1$ of 36 s, a total diffusion-encoding gradient duration of 2.5 ms, and a diffusion time of 0.1 s. $T_1$-DOSY data using the SARON sequence were acquired in 6 h using 10 recovery delays $\tau$ (ranging from 0.1 to 51.2 seconds) and 12 gradient amplitudes (ranging from 3 to 27 G cm$^{-1}$ in equal steps of gradient squared) using 16 transients, 16384 complex data points, a delay $d_1$ of 0.5 s, a total diffusion-encoding gradient duration of 2 ms and a
diffusion time of 0.3 s. T$_1$-DOSY data using the DRONE sequence were acquired in only 2 min using 2 recovery delays (0.2 and 3.2 s, selected in order to achieve about 80% attenuation) and 2 gradient amplitudes (3 and 27 G cm$^{-1}$ for the first level of relaxation encoding) using 4 transients, 16384 complex data points, a delay $d_1$ of 5 s and a total diffusion-encoding gradient duration of 2 ms.

All spectra were manually phase corrected, reference deconvoluted with a target lineshape of a 2 Hz Lorentzian (except for the IRON dataset, where a line broadening of 2 Hz was applied without reference deconvolution) and baseline corrected in VnmrJ 2.2C. The data were then imported into Matlab where PARAFAC analysis was carried out using the N-way Toolbox$^{45}$. The DOSY and SCORE plots were produced using the DOSY Toolbox (http://personalpages.manchester.ac.uk/staff/mathias.nilsson/software.htm). The PARAFAC analysis required a few seconds on a standard PC with a dual core 2.33 GHz CPU.

**Results and Discussion**

To demonstrate the potential of the PARAFAC decomposition of a T$_1$-DOSY experiment, data were acquired using a mixture of two alcohols, where signal overlap complicates interpretation of the HR-DOSY$^7$ spectrum (Fig. 2). While the fitted diffusion coefficients for the well-resolved peaks at 1.1 and 3.6 ppm appear at their expected positions, the overlap at 0.9 and 1.5 ppm causes the apparent diffusion coefficients for some multiplet components to adopt compromise values in between those of the overlapping multiplets of the two alcohols. A more powerful approach to resolving the component spectra of a mixture with, as here, a relatively small number of components, is to use a multivariate method and fit the whole dataset simultaneously. A two-component SCORE Speedy Component REsolution$^{36}$ fit of the data shows good agreement between the fitted components and reference spectra (Fig. 3). This method fits the data to a pre-determined decay function; data fitted to the theoretical pure exponential decay (Eq. 1) often show severe cross-talk between the components because gradient non-uniformity causes the experimental decay to deviate from exponential$^{43}$. Prior knowledge about actual
the shape of the experimental decay is therefore needed for best results; Eq. 2 was used for the data of Fig. 3, using parameters $c_n$ determined by gradient and signal mapping$^4$.

**Figure 2.** 400 MHz $^1$H high resolution DOSY spectrum of the mixture of 3-methyl-3-pentanol and 1-propanol in D$_2$O.

**Figure 3.** a) Reference and b) extracted SCORE spectra of 3-methyl-3-pentanol, and c) reference and d) extracted SCORE spectra of 1-propanol. Reference spectra were measured using samples of the individual alcohols, and extracted spectra were obtained by SCORE processing of the data used to produce Fig. 2.
A potentially more powerful alternative method, demonstrated here for $T_1$-DOSY experiments, is to add relaxation information to the experimental data in order to aid the resolution of individual components. The first important advantage of this approach is that differences in relaxation between overlapping multiplet components enhance the separation potential as compared to using diffusion only; the relaxation times $T_1$ measured for the reference samples of the individual alcohols are given in Table 1. The second important advantage is that when decomposing trilinear data with the PARAFAC algorithm, no assumptions are needed as to the form taken by the diffusion evolution or the relaxation evolution. The disadvantages are having to acquire data in an extra dimension (although in practice the total acquisition time needed may actually be shorter, see below), and (because relaxation rates are in general different for different nuclei in a molecule) the need for the dataset to be segmented into individual spectral regions before analysis.

<table>
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<tr>
<th>1-propanol</th>
<th>3-methyl-3-pentanol</th>
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<tr>
<td>Freq. / ppm</td>
<td>$T_1$ / s</td>
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<tr>
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<tr>
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<tr>
<td>0.88</td>
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</tbody>
</table>
Figure 4. $^1$H spectra obtained by PARAFAC decomposition of the results of different $T_1$-DOSY experiments on the mixture of 3-methyl-3-pentanol and 1-propanol. The component spectra (top) constructed from the results of PARAFAC processing using the specified number of components for the four spectral segments indicated, and (bottom) DOSY spectrum constructed from the component spectra and diffusion coefficients obtained for the individual spectral segments. A) IRON (20 h experiment), B) SARON (6 h experiment) and C) DRONE (2 min experiment).

Component spectra, showing good agreement with the reference spectra, were obtained by fitting the $T_1$-DOSY data acquired using each of the three sequences investigated. Prior to analysis by PARAFAC, the datasets were segmented and each segment fitted to an appropriate number of components, as indicated by the horizontal bars in Fig 4. Where the number of components is unknown, it can be very helpful to use tools such as split-half analysis, core consistency diagnostic\textsuperscript{46}, and/or one of the many other methods available\textsuperscript{47}. The outputs from the PARAFAC algorithm for each segment represent the spectral, diffusion and relaxation modes for the appropriate number of components. Diffusion coefficients were estimated by a two parameter fit of diffusion modes to a Gaussian decay (Eq. 2). Complete component spectra were obtained by concatenating individual segment component spectra where the diffusion coefficients were within 5% of the lowest coefficient (Fig 4 (top)). DOSY plots for Figs. 4a and 4b were produced by constructing
the diffusion dimension for each segment using values and estimated standard errors from fitting the diffusion modes (vide supra), as for a standard DOSY plot.

In principle the IRON sequence, which has the largest dynamic range of all the sequences investigated, has the potential to give the best results. In practice, however, the price paid in not having a reference signal available at all \( \tau \) values for reference deconvolution is too heavy, and instrumental problem such as lineshape variation with \( G \) and frequency drifts caused by small temperature fluctuations cause the data to deviate from strict trilinearity, resulting in significant crosstalk between the two component spectra found (see Fig. 4a). Such crosstalk is a common feature of partial breakdown in the assumptions implicit in multivariate analysis of spectral data\textsuperscript{26,43}. Cleaner results were obtained with the SARON sequence (Fig 4b). A number of saturation schemes such as the aperiodic pulse sequence of Roscher et al.\textsuperscript{48} were investigated; in our experiments a pair of long orthogonal pulses (about 700 \( \mu \)s each [12440\(^{\circ}\) flip angle]) worked best, but there is certainly room for improvement.

As noted above, the quality of the experimental data obtainable with IRON and SARON is limited by the inapplicability of reference deconvolution and the difficulty of achieving clean saturation respectively. The DRONE sequence avoids both of these issues by using the diffusion delay to encode relaxation information, and gives the cleanest data and the best results of the three methods described. In principle, only two levels of diffusion and two levels of relaxation encoding should be needed to resolve the spectra of the test sample, as it only contains two components if solvent and reference signals are excluded from the analysis. (Formally, the sample is of chemical rank 2; it should be noted that determining the theoretical minimum (mathematical) rank for a three-way dataset is not as straightforward as for two-way data, where it is equal to the lowest number of rows or columns\textsuperscript{31,49}). Here we show that such an experiment with only two levels each of diffusion and of relaxation encoding can indeed work well in practice, by decomposing a data set acquired using the DRONE sequence in less than 3 min. The result is virtually perfect separation, as can be seen in both the component spectra obtained and the DOSY plot (Fig 4c). One practical problem in a DOSY presentation of the results of the analysis is that no
information is directly available on the statistics of the fit of the diffusion mode, since two parameters are being extracted from two data points. In principle the uncertainty in the value of D determined in this way is given by Eq. 5:

$$\sigma_D = D\sigma_n \sqrt{\frac{1}{I_1^2} + \frac{1}{I_2^2}} \ln \left( \frac{I_1}{I_2} \right)$$

[5]

where $\sigma_n$ is the average noise level of the experimental data and $I_1$ and $I_2$ are the amplitudes of the two data points. In this instance, however, systematic errors probably dominate and a conservative estimate for $\sigma_D$ of five times the calculated value was used in synthesising the DOSY spectrum of Fig. 4c.

In the experiments reported here, the principal limiting factor is systematic error; simulations in which increasing amounts of synthetic noise were added to experimental datasets showed that the PARAFAC decomposition is surprisingly robust with respect to signal-to-noise ratio. (This sensitivity of PARAFAC to systematic error can be turned to advantage in diagnosing instrumental problems, since the systematic variation can often be extracted as an additional component and visual inspection of its spectral mode can be very informative. Thus, for example, signal phase variations during an experiment will lead to an additional component in dispersion mode). One limitation of the $T_1$-DOSY method is the potential for small differences in $T_1$ to arise between different peaks in the same multiplet, where there is cross-correlated relaxation in systems with equivalent spins. For small molecules these effects are generally small, but when present can lead to crosstalk between components and to distorted multiplets in the spectra extracted. In the system studied here, differences in $T_1$ within multiplets are, as shown in Table 1, just above the threshold of statistical significance, but did not prevent a clean PARAFAC decomposition of the experimental data.
Conclusions

It is shown that the addition of a relaxation dimension to diffusion experiments can help in decomposing the overlapping spectrum of a mixture into the spectra of its individual components when combined with appropriate multi-way data processing methods. If the deviations from trilinearity that can arise from instrumental effects such as probe or sample temperature variations are kept to a minimum, PARAFAC analysis is a powerful method for the analysis of three-way, diffusion- and relaxation-encoded data. Experimental data acquired in as little as 2 min for a mixture of two chemical species allows the extraction of spectra that can match or exceed the quality of those obtained using other multivariate methods with longer data acquisition. PARAFAC analysis provides both individual component spectra and values for relaxation times and diffusion coefficients, making T₁-DOSY potentially a powerful tool in the elucidation of structures in complex mixtures.

Acknowledgements

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Appendix G: Reaction Kinetics Studied Using Diffusion-Ordered Spectroscopy and Multiway Chemometrics (published paper)


**DOI:** 10.1021/ac100110m

**ABSTRACT**

Nuclear magnetic resonance (NMR) spectroscopy is frequently used in the monitoring of reaction kinetics, due to its non-destructive nature and to the wealth of chemical information that can be obtained. However, when spectra of different mixture components overlap, as is common, the information available is greatly reduced, sometimes to the point where the identification of individual chemical species is not possible. In such cases the resolution of component spectra and their concentration timecourses can be greatly improved by recording DOSY (Diffusion-Ordered Spectroscopy) data for each time point during the reaction. Adding this additional degree of freedom to the experimental data, allowing the signals of different species to be distinguished through their different rates of diffusion, makes the data trilinear, and therefore susceptible to analysis by powerful multi-way (here more specifically multilinear) model-free decomposition methods such as PARAFAC (parallel factor analysis). This approach is shown to produce high quality data even for species with near-degenerate spectra. Another important limitation of NMR is its inherently low sensitivity. Here we show that the combination of DOSY and PARAFAC is surprisingly robust with respect to input data with low signal-to-noise ratio. High quality component spectra and kinetic profiles are obtained from a dataset in which the signal-to-noise ratios of the reaction components in the spectra for individual time points are below the detection level.
KEYWORDS: NMR, DOSY, PARAFAC, PFGSTE, MATLAB, kinetics, diffusion, pulsed field gradients (PFG), multivariate analysis, multi-way, multilinear, trilinear, chemometrics.

Introduction

The monitoring of chemical reactions is an important task in both industry and academia, for example in process development and degradation studies. The non-invasive and non-destructive method of Nuclear Magnetic Resonance (NMR) can be a particularly effective tool due to the wealth of information it provides, for example on chemical structure, and on intermolecular and intramolecular dynamics. Where the signals of individual species are well-resolved, reaction monitoring is straightforward and peak integrals can be used directly to determine changes in concentration\(^1\). However, a major obstacle in the study of mixtures by NMR is that when component spectra overlap it is often impossible to assign signals unambiguously to a specific molecular species. In such cases, methods that aim to fit entire spectra (i.e. multivariate methods) can often be helpful in resolving component spectra and determining concentrations\(^2\)\(^-\)\(^8\). Much of the power of multi-way (or in this case more specifically multi-linear) methods stems from the fact that multilinearity offers relief from the so-called rotational ambiguity limitation\(^9\) that bilinear methods suffer from (\textit{vide infra}). The combination of diffusion-ordered spectroscopy (DOSY\(^10,\ 11\)) experiments and multivariate multi-way chemometrics\(^12\) has recently been shown to be an excellent tool both for mixture analysis in general\(^13\)\(^-\)\(^15\), and more specifically for reaction kinetics\(^16\). Here the practicalities of this combination of methods are explored, and its remarkable robustness when analysing data with limited signal-to-noise ratio (S/N) is illustrated.

The most powerful general methods for mixture analysis by NMR are LC-NMR\(^17\) and, as used in this investigation, diffusion-based methods such as DOSY\(^10,\ 11\). Although the term DOSY strictly refers only to a processing and display method used with diffusion-weighted NMR data, it is commonly used more loosely to describe both the analysis method and the experimental data, and will be used in that sense here despite actual DOSY spectra not being constructed. A central advantage of DOSY methods is that they do not require any physical separation of a mixture, and therefore are particularly helpful when a reaction is
followed directly in a NMR tube\textsuperscript{16, 18}. The measurement of diffusion-weighted spectra allows the signals from different species to be distinguished, by virtue of their different effective hydrodynamic radii. A DOSY experiment is typically performed by recording a set of NMR spectra in a pulsed field gradient spin or stimulated echo (PFGS[TE]) as a function of gradient amplitude. The NMR signals are ideally attenuated according to the Stejskal-Tanner equation\textsuperscript{19}:

\[
S(G) = S_0e^{-D\gamma^2\delta^2G^2\Delta'}
\]  

(1)

where \(S\) is the signal amplitude, \(S_0\) is the amplitude in absence of diffusion, \(D\) is the diffusion coefficient, \(\delta\) is the diffusion encoding gradient pulse duration, \(\gamma\) is the magnetogyric ratio, \(G\) is the gradient amplitude, and \(\Delta'\) is the diffusion time (\(\Delta\)) corrected for the effects of the non-zero gradient pulse duration. The experimental data are normally fitted either to the Stejskal-Tanner equation or to a version of that equation modified to take into account instrumental imperfections such as non-uniform field gradients\textsuperscript{20}, to obtain the spectra and diffusion coefficients of the mixture components. Most commonly this is done by fitting the decay of each signal individually, using univariate methods\textsuperscript{21-23}, and displaying the result in a 2D DOSY plot. However, as all the signals for each component decay identically (in the absence of exchange\textsuperscript{24, 25}), it is possible to exploit this covariance to separate the component spectra even where individual signals overlap\textsuperscript{24, 26, 27}.

In multivariate methods it is conventional to describe the data analysis in matrix form. For example, in the decomposition of a DOSY dataset into component spectra and attenuation functions for \(N\) different components the experimental data are modeled by the equation

\[
D = AS^T + E
\]  

(2)

where the experimental dataset \(D\) is a matrix in which successive rows contain the measured mixture spectrum for different values of the diffusion-encoding gradient strength. This matrix is represented in Equation (2) as the product of two row matrices containing the spectral amplitude profiles \(A\) (for a DOSY dataset these are the signals decays as a function of gradient strength for the different components) and the component spectra \(S\), where \(T\) denotes the transpose. The error matrix \(E\) contains unfitted data, which ideally consist
solely of unavoidable random experimental errors such as noise. In an alternative notation this can be written as:

\[ D = \sum_{i=1}^{N} s_i \otimes a_i + E \]  

(3)

where \( s_i \) and \( a_i \) are the \( i \)th vectors of the matrices \( S \) and \( A \) and \( \otimes \) is the Kronecker product. The same notation can be used to describe the data obtained in a 'standard' kinetics NMR experiment where a simple proton spectrum is recorded for a succession of time points, with the matrix \( A \) describing the variation in concentration of each of the components with time.

Multivariate methods set out to find the matrices \( A \) and \( S \) that best represent the experimental dataset. Unfortunately this is far from straightforward, as an infinite number of solutions exists in the form of linear combinations of the actual spectra and signal amplitude profiles. This is known as the rotational ambiguity problem for bilinear data.\(^9\) This ambiguity can be resolved by imposing appropriate constraints on the analysis, for example non-negativity, or a predetermined form of concentration profile.\(^2-8, 26, 27\) However, this requires detailed prior knowledge about the system under study, and there is a considerable risk of applying constraints that are not valid. In contrast to the bilinear case, for multilinear data (varying independently in \( n \) dimensions; \( n > 2 \)) rotational ambiguity does not arise. This has the very useful consequence that no prior knowledge about the variation of data in any of the \( n \) dimensions is necessary; fitting data to such a PARAFAC\(^28, 29\) (parallel factor analysis) model, the form of the variation in the different dimensions is obtained directly.

Trilinear data may be obtained by recording a DOSY dataset for each time point in a chemical reaction.\(^16\) In the notation of Eq (3) a trilinear data set can be written as:

\[ D = \sum_{i=1}^{N} s_i \otimes a_i \otimes b_i + E \]  

(4)

where \( b_i \) is the \( i \)th column of a matrix \( B \) containing the relative concentration as a function of time for each of the reaction components. For a given number \( N \) of mixture components the matrices \( S \), \( A \) and \( B \) (often referred to as modes; e.g. \( S \) is the spectral mode) can be
obtained directly from decomposition of $D$ using one of the many algorithms to fit the PARAFAC model$^{30}$; no further assumptions are needed in such 'model free' fitting. In the present investigation the matrices $S$, $A$ and $B$ contain the $N$ spectra, diffusion decays and concentration timecourses, respectively; each column $i$ corresponds to the spectrum, diffusion decay and concentration timecourse for a given mixture component. When the data are truly trilinear, the PARAFAC decomposition is straightforward, but with experimental data it is common to find small (or not so small) additional systematic variation that does not fit a trilinear model. In the present investigation we acquire approximately trilinear data by recording a DOSY spectrum for each time point in the reaction. One obvious source of deviation from trilinearity is that the acquisition of a DOSY spectrum is not instantaneous, so any changes in concentration of the different components during the recording of a given DOSY dataset will cause a non-trilinear variation. In this investigation, however, such effects can safely be ignored as the time taken to record one set of diffusion-weighted spectra is negligible compared to the total reaction time. A second, more insidious, source of deviation from the trilinear model is variation in the component spectra, for example because of composition-dependent chemical shifts. For PARAFAC analysis to succeed, it is vital both that the component spectra remain identical throughout the reaction, and that the behavior of the different components is non-degenerate, i.e. that no two components have one of a spectrum, diffusion coefficient or timecourse in common. (If two modes are the same, the components become indistinguishable). In this work, some common deviations from trilinearity such as frequency shifts and (near-)degeneracy are investigated, and potential solutions to these problems are examined, and the robustness of the combination of DOSY and PARAFAC with respect to signal-to-noise ratio is investigated. The illustrative reactions used are the acid hydrolyses of sucrose and of maltotriose, the former showing the effects on PARAFAC decomposition of chemical shift variation and signal-to-noise ratio, and the latter those of near-degeneracy. (Although these reactions are in principle complicated considerably by the presence of two anomers for most of the species, in practice the kinetics remain simple because anomerisation is rapid compared with hydrolysis under the conditions used.)
Experimental

Four samples were used for the acid hydrolysis experiments, all with D$_2$O as solvent: sample 1 containing maltotriose (Fisher Scientific, 18 mM), pivalic acid (Sigma-Aldrich, 25 mM), and sulphuric acid (90 mM); sample 2 containing sucrose (Silver Spoon, 540 mM), tert-butanol (Fluka, 135 mM), and sulphuric acid (224 mM); sample 3 containing sucrose (50 mM), tert-butanol (105 mM), and sulphuric acid (464 mM); and sample 4 containing sucrose (1.1 mM), tert-butanol (82 mM), and sulphuric acid (592 mM). All hydrolyses were carried out in thick walled 5 mm NMR tubes (i.d. 2.2 mm, to discourage convection) in a 400 MHz Varian Inova spectrometer, using an indirect detection probe equipped with a z-gradient coil allowing gradient pulses up to 30 G cm$^{-1}$. The hydrolysis of sample 1 was carried out at 50 °C, and that of samples 2-4 without temperature control in a room air-conditioned at a nominal 20 °C. All DOSY experiments were recorded using the Oneshot sequence$^{31}$ using 6 gradient levels with equal steps in gradient squared, ranging from 3.0 to 27.3 G cm$^{-1}$. Data were acquired during hydrolysis using the following sample-specific parameters:

sample 1) 130 DOSY experiments with a recycle time of 6 s, a total diffusion-encoding gradient duration of 2 ms and a diffusion time of 0.2 s, using 32 transients of 16384 complex data points in a total time of 52 h;

sample 2) 46 DOSY experiments with a recycle time of 3.6 s, a total diffusion-encoding gradient duration of 3 ms and a diffusion time of 0.1 s, using 16 transients of 16384 complex data points in a total time of 6 h;

sample 3) 110 DOSY experiments with a recycle time of 4 s, a total diffusion-encoding gradient duration of 3 ms and a diffusion time of 0.1 s, using 16 transients of 16384 complex data points, in a total time of 16 h; and

sample 4) 350 DOSY experiments with a recycle time 4 s, a total diffusion-encoding gradient duration of 3 ms and a diffusion time of 0.1 s, using 4 transients of 16384 complex data points in a total time of 15 h.

An illustrative excerpt from the data acquired for sample 2 is shown in Figure 1.
Reference spectra for reactants, intermediates and products were obtained as follows, to allow comparison between the spectra obtained by PARAFAC fitting and the authentic spectra. The reference spectrum for glucose was acquired from sample 1 after the reaction had proceeded to termination, and that for maltotriose was taken as the first spectrum in the hydrolysis timecourse. Because the hydrolysis of maltose is relatively rapid under the experimental conditions used, the reference spectrum for maltose was obtained by back extrapolation of spectra acquired under the same conditions as for the hydrolysis of sample 1, using maltose (Fisher Scientific) as starting material. The reference spectra for sucrose, and for the mixture of glucose and fructose, were acquired using a 0.6 M sucrose solution, and an equimolar 0.6 M solution of glucose (Sigma-Aldrich) and fructose (Alfa Aesar), respectively, in sulphuric acid and D2O.

All spectra were Fourier transformed, phase corrected, baseline corrected, and reference deconvoluted\textsuperscript{32–33} using the pivalic acid (sample 1) or tert-butanol (samples 2–4) signal, all using the manufacturer's VnmrJ software, before exporting to MATLAB (\url{www.mathworks.com}) for further analysis. Reference deconvolution was used to minimise the effects of changes in instrumental lineshape, signal phase, and signal frequency over the course of a reaction, and greatly improves the quality of the final results. PARAFAC analysis was performed with the MATLAB \textit{N}-way Toolbox\textsuperscript{34}. The DOSY Toolbox\textsuperscript{35} (open source free software for DOSY processing), also contains an interface with the \textit{N}-way Toolbox. Small variations in receiver sensitivity over the course of the experiment were corrected for by normalising the amplitude of every individual DOSY dataset to show the
same total reference signal integral for the first gradient level for each DOSY experiment. Where the water signal lay within the spectral region containing signals of interest, it was excluded from analysis by setting the data points to zero for 0.25 ppm either side of the water signal. (Note that this appears at higher chemical shifts than usual because of the effects of high acid concentration and of temperature). The relevant spectral region (3.0-5.7 ppm for sample 1, 3.2-5.7 ppm for sample 2, and 3-5.8 ppm for samples 3 and 4) was then selected and fitted to the PARAFAC model.

PARAFAC analysis produces output modes with arbitrary scaling. Each PARAFAC spectral mode, \( S_i(f) \), was therefore normalized to have an integral proportional to the number of protons involved, and each diffusion mode \( A_i(g) \) was normalized so that the extrapolated amplitude at zero gradient for each component was unity. The remaining mode, concentration \( C_i(t) \), was then multiplied by the factors that \( S_i(f) \) and \( A_i(g) \) were divided by, to make it proportional to the true concentrations of the components. The modes representing the timecourses were fit using the non-linear least squares fitting in the Optimization Toolbox (MATLAB), to first order kinetics. Rate constants are reported with error margins corresponding to twice the standard error of the fit.

**Results and discussion**

One of the central limitations of the DOSY-PARAFAC approach is that degeneracy in one of the modes can cause the clean separation of components to fail. The acid hydrolysis of aqueous maltotriose, which proceeds via maltose to glucose, was therefore investigated as a challenging test case. Here both the spectral modes of maltose and maltotriose are similar, because of the extensive commonality in chemical shifts, and the diffusion modes are similar, because of the relatively small difference in diffusion coefficient. It has been noted previously that imposing physically realistic constraints like non-negativity can be helpful in such cases\(^{28,36}\). An unconstrained three component PARAFAC fit did reproduce the spectra, diffusion decays and timecourses fairly faithfully, but significant deviation from a physically realistic result was evident early in the timecourse, where the ‘maltose’ component showed a small negative initial concentration. These deviations are probably
attributable to small temperature disturbances (see figure caption) causing minor violation of trilinearity. However, when non-negativity constraints were applied to all three modes, no anomalies were seen in any of the modes (explained variance 99.9 %). The fitted component spectra were virtually identical to the reference spectra, and the concentration timecourses followed the form expected for a sequential first order reaction (Figure 2).

In this test case, both the number of components and the component spectra were already known, so definitive validation could be performed by comparing the spectral modes to the model spectra. However split-half analysis, jack-knifing and core consistency checks were all consistent with this prior knowledge; split-half analysis with three components, for example, gave essentially identical results to the full analysis in all three modes. Non-linear least squares fitting of the concentration timecourses to successive first order kinetics gave estimated rate constants of $k_1 = 7.03 \pm 0.15 \times 10^{-5} \text{ s}^{-1}$ for the hydrolysis of maltotriose to maltose under the conditions used, and $k_2 = 1.58 \pm 0.02 \times 10^{-5} \text{ s}^{-1}$ for the hydrolysis of maltose to glucose. Experiments with lower signal-to-noise ratio data were significantly less successful, reflecting the importance of the non-negativity constraints in disentangling the component spectra. Because there are relatively few points in the maltotriose spectrum at which there are no maltose or glucose signals, the decomposition is very vulnerable to interference from noise at these points.
Figure 2. (a) Component spectra obtained from a non-negativity constrained three-component PARAFAC fit of the data acquired for sample 1 (top), and reference spectra (bottom); (b) concentration timecourses (circles) obtained from the PARAFAC fit, together with fits to sequential first order kinetics (solid lines). Estimated rate constants were $k_1 = 7.03 \pm 0.15 \times 10^{-5}$ s$^{-1}$ and $k_2 = 1.58 \pm 0.02 \times 10^{-5}$ s$^{-1}$ for the maltotriose and maltose hydrolyses respectively. The small deviations from the lines of best fit correlate in time with temperature disturbances of the order of 0.1 °C between 0 and 10 h and between 20 and 25 h.

The second reaction studied was the simpler case of the acid hydrolysis of sucrose to glucose and fructose. Although at first sight this is a three-component problem, here the concentration timecourses of glucose and fructose are identical, and the diffusional attenuations very nearly so. The result is that the two products behave in the two-component PARAFAC decomposition (explained variance 99.9%) as a single species with a composite spectrum, so the data may be analysed as a simple two-component problem. At
first sight the fitted spectra obtained by PARAFAC decomposition of the experimental data for sample 2 are quite promising (Figure 3a), but close examination shows the presence of a small number of anomalies at the positions shown by vertical arrows. These anomalies represent cross-talk between the two component spectra, with signals appearing in the sucrose spectrum at the chemical shifts of glucose/fructose signals, and vice versa, and arise from deviations from strict trilinearity in the experimental data. Their “dispersion mode”-like appearance suggests frequency shifts as the origin of the anomalies. Detailed analysis confirms that this is the case; the individual component spectra change subtly as a function of the overall composition of the reaction mixture, with chemical shifts changing by a few parts per billion as the relative proportions of reactant and products change. Interestingly, it is not the case that the signals for which anomalies are seen are the only ones whose chemical shifts change with composition; rather, all the sugar signals shift relative to the tert-butanol reference signal, but the signals that yield anomalies are the few signals that behave differently from the majority.

Figure 3. (a) Component spectra obtained from a PARAFAC fit of the data acquired for sample 2 (540 mM initial sucrose concentration); (b) component spectra obtained by fitting the data acquired for sample 3 (50 mM initial sucrose concentration); (c) reference spectra of sucrose and of an equimolar mixture of glucose and fructose.
The diagnosis that differential medium effects on chemical shifts were the cause of the anomalous signals prompted measurements on sample 3, where the initial concentration was reduced by an order of magnitude and hence the effects of composition on chemical shifts were expected to be much smaller. As predicted, the anomalies were greatly reduced and an excellent match was seen between the fitted spectra yielded by PARAFAC (Figure 3b) and the reference spectra (Figure 3c); the two component fit explained 99.9 % of the variance. The concentration timecourses obtained were fitted to first order kinetics, as shown in Figure 4, yielding a rate constant of $7.48 \pm 0.03 \times 10^{-5} \text{ s}^{-1}$, and the diffusion decays were fitted to the Stejskal-Tanner equation corrected for non-uniform field gradients (Figure 4) giving $D_{\text{sucrose}} = 1.28 \pm 0.01 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and $D_{\text{glucose, fructose}} = 1.37 \pm 0.01 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

**Figure 4.** The diffusional decay and time evolution components obtained from PARAFAC fitting of the data originating from sample 3 (circles), together with (solid lines) non-linear least squares fits to the non-uniform gradient compensated Stejskal-Tanner equation (Eq. 1) and to first order kinetics respectively (a corresponds to fructose + glucose while b corresponds to sucrose). The fitted diffusion coefficients were $1.28 \pm 0.01 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ for sucrose and $D = 1.37 \pm 0.01 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ for the product component (glucose and fructose), while the first order rate constant was $7.48 \pm 0.03 \times 10^{-5} \text{ s}^{-1}$. 
Much larger chemical shift changes can be seen, for example, in the spectra of reactions in which pH changes. In such systems it would be necessary either to exclude itinerant signals from analysis, or to measure spectra under fixed chemical conditions, for example by buffering and/or using diluted sampled aliquots. Differential chemical shift changes cannot corrected by reference deconvolution\textsuperscript{32, 33} (although the latter plays a vital role in minimising the effects of instrumental irreproducibility, as has been shown previously for both uni- and multivariate processing of DOSY data\textsuperscript{23, 37}).

![Figure 5](image)

**Figure 5.** (a) A subset of the experimental data (spectra as a function of pulsed field gradient and reaction time) obtained for sample 4 (1.1 mM initial sucrose concentration) showing data for the start, midpoint, and end of the period monitored, together (b) with the time evolution of the components corresponding to sucrose and to glucose/fructose, and (c) the fitted spectra. The fitted diffusion coefficients were $1.15 \pm 0.05 \times 10^{-10}$ m$^2$ s$^{-1}$ for sucrose and $1.38 \pm 0.03 \times 10^{-10}$ m$^2$ s$^{-1}$ for glucose/fructose, while the first order rate constant was $1.29 \pm 0.02 \times 10^{-4}$ s$^{-1}$.

In order to study the usefulness of the DOSY-PARAFAC approach for low concentration/low signal-to-noise ratio samples, a third hydrolysis of sucrose was carried
out at a very low concentration (1.1 mM; sample 4). Here, the signal-to-noise ratio of approximately 2:1 in the least attenuated (lowest gradient) spectrum (Figure 5a) was below the detection level (normally taken as 5:1). The classical approach of following the intensity changes of individual peaks would here be totally impractical. Remarkably, the PARAFAC decomposition (Figure 5c) produces excellent component spectra, once again showing a very good match with the reference spectra of Figure 3c for sucrose and glucose/fructose (explained variance 16 %, reflecting the extremely low signal-to-noise ratio). The reason why PARAFAC is so successful here is that the entire dataset is fitted, so the signals from all the 350 experiments contribute to the fitted spectra, and all the signals in each spectrum contribute to the fitted timecourses. The latter are also perfectly serviceable (Figure 5b), fitting to first order kinetics yielding a rate constant of $1.15 \pm 0.05 \times 10^{-4}$ s$^{-1}$.

**Conclusion**

The combination of NMR diffusion measurements and trilinear data analysis (in the form of PARAFAC), can be very powerful for the study of reaction kinetics. The method can be used for data with extremely low signal-to-noise ratio, significantly below the level at which conventional methods would fail. Problems with spectral inconsistencies during the reaction that are of instrumental origin can in many cases be alleviated by using reference deconvolution, while changes in spectra due to concentration dependent shifts can be minimised by making measurements at relatively low concentration. While the relatively slow reactions studied here allowed DOSY datasets with many transients and gradient levels to be acquired, where appropriate the method can be used with as little as one transient and two gradient levels per time point, allowing reactions to be studied on timescales as low as 1 min. In systems with near degeneracy in one or several modes (e.g. very similar NMR spectra or diffusion coefficients), accurate kinetic and spectral data can be obtained by imposing physically appropriate constraints such as non-negativity. In contrast to standard NMR methods, the combination of NMR diffusion measurements and trilinear data analysis can be used in situations with severe spectral overlap and at very low concentrations.
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Appendix H: J-modulation effects in DOSY experiments and their suppression: the Oneshot45 experiment (published paper)


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**Abstract**

DOSY (Diffusion-Ordered Spectroscopy) is a powerful NMR method for identifying compounds in mixtures. DOSY experiments are very demanding of spectral quality; even small deviations from expected behaviour in NMR signals can cause significant distortions in the diffusion domain. This is a particular problem when signals overlap, so it is very important to be able to acquire clean data with as little overlap as possible. DOSY experiments all suffer to a greater or lesser extent from multiplet phase distortions caused by J-modulation, requiring a trade-off between such distortions and gradient pulse width. Multiplet distortions increase spectral overlap and may cause unexpected and misleading apparent diffusion coefficients in DOSY spectra. These effects are described here and a simple and effective remedy, the addition of a 45° purging pulse immediately before the onset of acquisition to remove the unwanted anti-phase terms, is demonstrated. As well as affording significantly cleaner results, the new method allows much longer diffusion-encoding pulses to be used without problems from J modulation, and hence greatly increases the range of molecular sizes that can be studied for coupled spin systems. The sensitivity loss is negligible and the added phase cycling is modest. The new method is illustrated for a widely-used general purpose DOSY pulse sequence, Oneshot.
Keywords

NMR, $^1$H DOSY, diffusion-ordered spectroscopy, diffusion, Oneshot, $J$-modulation, phase distortions, purging pulse.

1. Introduction

Diffusion-Ordered Spectroscopy (DOSY [1, 2]), a widely used pulsed field gradient NMR technique, aims to separate the component spectra of mixtures by virtue of the different diffusion behaviour of the molecular species involved. In a DOSY experiment a series of pulsed field gradient spin or stimulated echo spectra is acquired with increased pulsed field gradients, causing a signal attenuation ideally described by the equation

$$S = S_0 e^{-D\gamma^2\delta^2G^2\Delta'}$$  \hspace{1cm} (1)

where $S$ is the signal amplitude, $S_0$ is the amplitude that would have resulted without diffusion, $D$ is the diffusion coefficient, $\delta$ is the gradient pulse width, $\gamma$ is the magnetogyric ratio, $G$ is the gradient amplitude, and $\Delta'$ is the effective diffusion time. For diffusion encoding to be effected, pulsed field gradients have to be applied while magnetization is in the transverse plane, typically either in a spin echo or in a stimulated echo. In such echoes, the chemical shift is refocused but the scalar coupling is not, thus leading to a $J$-modulated spectrum for coupled spin systems [3]. $J$-modulated spectra have peaks with both absorption and dispersive components, the latter leading to increased spectral overlap because of the much slower decay with frequency offset of the dispersion mode. Pulsed field gradient stimulated echo (PFGSTE) sequences [4, 5] are generally preferred to spin echo sequences in DOSY because the magnetization spends less time in the transverse plane, leading to less $J$-modulation (and also often to less signal loss through relaxation). The growing application of DOSY to samples containing large, slowly diffusing species such as micelles [6, 7], hydrocarbon mixtures [8, 9], proteins [10, 11] and DNA [12] is increasing demands for strong diffusion encoding. Gradient coils are limited in the amplitude of the gradient that they can generate, so in order to obtain stronger diffusion
encoding it is typically necessary to increase gradient pulse widths, resulting in greater \( J \)-modulation. The latter phenomenon is frequently the limiting factor in determining the limiting size of species for which useful measurements can be made.

The best diffusion resolution in DOSY is obtained when there is no spectral overlap (the High Resolution DOSY, HR-DOSY [13] case), since each resonance can safely be assumed to belong to a single species and fitted to a two-parameter monoexponential decay. As a consequence, a wide variety of experiments have been devised to minimize overlap either by simplifying spectra [14-18] or by spreading out resonances in more dimensions [19-22]. \(^1\)H HR-DOSY is the most commonly used DOSY technique, because of the relatively simple experimental setup, data processing and interpretation, and because of the high signal-to-noise ratio and efficient diffusion encoding afforded by the high \(^1\)H magnetogyric ratio. However, it is a rare luxury for HR-DOSY to be applied in systems where there is no signal overlap at all; in most practical applications some overlap is present, and is tolerated because the effect on the HR-DOSY spectrum is normally simple and predictable: where two positive signals with different diffusion coefficients \( D_1 \) and \( D_2 \) overlap, the result of monoexponential fitting is to yield a compromise diffusion coefficient intermediate between \( D_1 \) and \( D_2 \). However, in this investigation it is shown that \( J \)-modulation can complicate matters. We analyse how the apparent diffusion coefficient of a signal is altered when it is overlapped by negative signals (typically from dispersive components in \( J \)-modulated spectra), and propose a new method to suppress these dispersive components.

2. \( J \)-modulation and its effects in DOSY experiments

\( J \)-modulation is typically seen in homonuclear spin and stimulated echoes because the effect of the scalar coupling remains while the chemical shift is refocused. In the widely adopted product operator description of spin manipulation [23] the transverse magnetization of an AX system of two spins-½ \( I_1 \) and \( I_2 \) after a spin echo (90° - \( \tau/2 \) - 180° - \( \tau/2 \) -) can be represented by the following operators:

\[
\begin{align*}
M_x & = (j_{12} - j_{21}) \sin \theta \\
M_y & = (j_{12} - j_{21}) \cos \theta \\
M_z & = j_{12} \cos \theta - j_{21} \sin \theta
\end{align*}
\]
\[
M_{xy} = \cos(\pi J_{12} \tau) \hat{I}_{1x} + \sin(\pi J_{12} \tau) 2\hat{I}_{1y} \hat{I}_{2z} \\
+ \cos(\pi J_{12} \tau) \hat{I}_{2x} + \sin(\pi J_{12} \tau) 2\hat{I}_{2y} \hat{I}_{1z} 
\] (2)

The \( \hat{I}_{1x} \) and \( \hat{I}_{2x} \) terms represent in-phase magnetization, and are normally phased to absorption mode in the resultant spectrum. However, the anti-phase terms \( \hat{I}_{1y} \hat{I}_{2z} \) and \( \hat{I}_{1z} \hat{I}_{2y} \) that arise from \( J \)-modulation contribute dispersion mode components to the lineshape. The dispersion mode has positive and negative lobes, and as noted earlier has a much larger frequency footprint than the absorption mode, because the signal intensity decays only hyperbolically with frequency offset at large offsets.

The complications caused by \( J \)-modulation in HR-DOSY arise because of signal overlap, which is increased by the presence of dispersive signal components. Where signals overlap, the signal decay fitted to a monoexponential function is actually biexponential:

\[
S = S_1 e^{-D_1 \gamma^2 \delta^2 G^2 A'} + S_2 e^{-D_2 \gamma^2 \delta^2 G^2 A'} 
\] (3)

where \( S_1 \) and \( S_2 \) are the amplitudes of the overlapping signals in the absence of diffusion and \( D_1 \) and \( D_2 \) are the diffusion coefficients. In the familiar case where both signals are positive, the monoexponential fit to (Eq. 1) will lead to a fitted amplitude \( S_m \) and to an estimated apparent diffusion coefficient \( D_m \) that is intermediate between \( D_1 \) and \( D_2 \), as for example in the illustration of Figure 1a. For similar \( D_1 \) and \( D_2 \) the effect on the quality of fit is very small [24], which makes it difficult to detect deviations from mono-exponentiality, but users of DOSY routinely allow for this effect of overlap when interpreting experimental HR-DOSY spectra.

The first complication caused by \( J \)-modulation is thus an increase in the number and extent of signal overlaps. The second complication is more subtle: where positive and negative signals overlap, the fitted diffusion coefficient \( D_m \) no longer lies between \( D_1 \) and \( D_2 \). For this case where \( S_1 \) and \( S_2 \) in Eq. 3 have opposite sign, assuming that \(|S_1| > |S_2|\), there are 3 cases that can be distinguished (see Appendix A):

- **case 1:** \( D_1 > D_2 \) and \( S_m > 0 \): the fitted \( D_m \) will be greater than both \( D_1 \) and \( D_2 \), as illustrated in Figure 1b.
- **case 2:** \( D_1 < D_2 \) and \( S_m > 0 \): the fitted \( D_m \) will be lower than both \( D_1 \) and \( D_2 \), as illustrated in Figure 1c.
case 3: $D_1 > D_2$ and $S_m < 0$: the fitted $D_m$ will be lower than both $D_1$ and $D_2$, as illustrated in Figure 1d.

Case 3 typically leads to the fitting algorithm returning a large estimated relative error in $D_m$, which may cause a peak to be ignored (as not statistically significant) by programs for synthesising DOSY spectra.

While it might be expected that the presence of a biexponential decay should readily be apparent from an increase in the error in $D_m$ estimated by the fitting algorithm, in practice such increases are often small, and far from diagnostic, as in Figures 1a and 1c. Both missing signals and signals with unexpected apparent diffusion coefficients can easily lead to a misjudgement of the number and nature of species in a sample when DOSY spectra containing overlapping signals are interpreted. The presence of negative signals from the dispersion mode tails of $J$-modulated multiplets can also complicate multivariate analysis of diffusion-weighted data, since such analysis often applies non-negativity constraints [25-28].
Figure 1: Result of monoexponential fitting of a sampled biexponential function resulting from the addition of two exponential functions $A = S e^{-Dx}$ with different amplitudes. In each case the $D_m$ obtained depends on the exact choice of points to sample. Green dashed and red dot-dashed lines show the variation of amplitude $A$ evolution with $x$ for two exponentials with decay constants $D_1$ (green) and $D_2$ (red). Brown dots show sampled points from the sum of the two exponentials and the continuous brown line a monoexponential fitted to the sampled points. On the right side is the simulated DOSY plot that would be obtained with two chemically shifted signals from each of two different species, with the two inner peaks overlapping exactly. a) Monoexponential fitting of the sum of two positive exponentials with amplitudes $S_1 = 1$ and $S_2 = 0.5$ and decay constants $D_1 = 2$ and $D_2 = 1$ yields an apparent diffusion coefficient $D_m = 1.52$, between $D_1$ and $D_2$. b) Monoexponential fitting of the sum of exponentials with amplitudes $S_1 = 1$ and $S_2 = -0.5$ and decay constants $D_1 = 2$ and $D_2 = 1$ yields an apparent diffusion coefficient $D_m = 4.18$, greater than either $D_1$ or $D_2$ (case 1 in text). c) Monoexponential fitting of the sum of exponentials with amplitudes $S_1 = 1$ and $S_2 = -0.5$ and decay constants $D_1 = 1$ and $D_2 = 2$ yields an apparent diffusion coefficient $D_m = 0.70$, less than either $D_1$ or $D_2$ (case 2 in text). d) Monoexponential fitting of the sum of exponentials with amplitudes $S_1 = 1$ and $S_2 = -0.9$ and decay constants $D_1 = 1$ and $D_2 = 0.5$ yields an apparent diffusion coefficient $D_m = 0.07$, less than either $D_1$ or $D_2$ (case 3 in text).
3. Suppression of J-modulation effects

J-evolution is unavoidable in DOSY experiments, as magnetization needs to be in the transverse plane during diffusion encoding, typically in a spin or stimulated echo. For homonuclear couplings, unlike heteronuclear J couplings which can be refocused by applying a 180° pulse to the passive coupled nuclei, there is no general solution that allows the refocusing of scalar couplings (with the exception of pure shift experiments, vide infra), although partial solutions have been reported. Takegoshi et al [29] and van Zijl et al [30] reported that a double spin echo with a 90° pulse orthogonal to the first pulse at the time of the first echo can be used to refocus J-evolution for a two-spin system, but that this refocusing is incomplete for higher order spin systems; this approach has been recently implemented for PFG spin echo experiments [31]. The oscillating-gradient spin echo [32] has been used to obtain pure absorption mode spectra for systems with specific coupling constants. Homonuclear decoupling during spin evolution in principle avoids evolution into anti-phase terms [33], but in practice cannot decouple all spins at once. Homonuclear J-evolution can be refocused by manipulating spins by combined frequency and spatially selective pulses in so-called pure shift experiments [14, 15], but these incur significant sensitivity penalties. In addition, none of the above methods can reliably suppress J-modulation in strongly coupled spin systems.

Given the difficulty of achieving a complete refocusing of homonuclear scalar couplings, other work has focused on methods for purging the unwanted magnetization, but all the methods proposed to date suffer from significant limitations and complications. Torres et al [34] have recently evaluated the performance of three such purging elements in PFG spin and stimulated echo sequences. A spin-lock, or trim, pulse before acquisition removes anti-phase magnetization without affecting the in-phase magnetization [4]. However, the sample is not field-frequency locked while the pulse is applied, which may lead to a deterioration in lineshape, and the radiofrequency power deposited causes sample heating [35, 36], which can be highly detrimental to DOSY experiments if it results in convection. A Longitudinal Eddy Delay (LED) or z-filter element [37], which includes a homospoil or gradient pulse, removes anti-phase magnetization to some extent [38, 39]. The first 90° pulse converts in-phase magnetization into longitudinal, and anti-phase into
multiple and zero quantum coherences. The gradient dephases multiple quantum coherences, but not zero quantum coherences. The second 90° pulse converts longitudinal magnetization back into in-phase and zero quantum coherences into anti-phase magnetization. The last alternative evaluated is the chirp-based z-filter, in which a chirp pulse synchronous with a gradient [40] is included in the LED element. This combination dephases zero quantum coherences, thus leaving only in-phase magnetization. All these three elements lose sensitivity due to relaxation losses, and the latter two require extensive phase cycling for clean results.

3.1. Oneshot45

In this investigation we propose an alternative solution, the addition of a final 45° pulse orthogonal in phase to the preceding 90° pulse. This idea has been used previously for obtaining pure phase homonuclear and heteronuclear 2D spectra, either in the same form [41] or as a pulse pair [42]. It efficiently removes anti-phase magnetization without the drawbacks of previous methods. It is worth noting here that the product operator description of Eq. 2 for transverse magnetization after a spin echo contains in-phase and anti-phase magnetizations with orthogonal phases, and that these can therefore be manipulated independently by radio frequency pulses; the same is true for a stimulated echo. If a 45° pulse is applied along the x-axis immediately after the modulated echo, the in-phase magnetization is unaffected but the anti-phase terms are changed:

\[
\sin(\pi J_{1z}) \frac{\hbar}{2} \left( I_{1y} \frac{\sigma_{A}^{(I_{1x} + I_{2x})}}{2} \right) \cos(\pi/4) \sin(\pi J_{1z}) \frac{\hbar}{2} \left( I_{1y} \frac{\sigma_{A}^{(I_{1x} + I_{2x})}}{2} \right) - \sin(\pi/4) \sin(\pi J_{1z}) \frac{\hbar}{2} \left( I_{1y} \frac{\sigma_{A}^{(I_{1x} + I_{2x})}}{2} \right)
\]

\[
\sin(\pi J_{1z}) \frac{\hbar}{2} \left( I_{1z} \frac{\sigma_{A}^{(I_{1x} + I_{2x})}}{2} \right) \cos(\pi/4) \sin(\pi J_{1z}) \frac{\hbar}{2} \left( I_{1z} \frac{\sigma_{A}^{(I_{1x} + I_{2x})}}{2} \right) - \sin(\pi/4) \sin(\pi J_{1z}) \frac{\hbar}{2} \left( I_{1z} \frac{\sigma_{A}^{(I_{1x} + I_{2x})}}{2} \right)
\]

Since \(\cos(\pi/4) = \sin(\pi/4)\), these terms cancel and the unwanted anti-phase magnetization is removed. The analysis for higher order spin systems is analogous. All bilinear terms (those of the form \(2 \hat{I}_{1x} \hat{I}_{2x}\), with i and j being the coupled spins) cancel out, and half of the coherence from higher order terms is transformed into undetectable multiple quantum coherences. The 45° purging pulse thus allows, in theory, the complete removal of all anti-phase
magnetization from AX systems and, for short echo times, most anti-phase magnetization for more complex spin systems.

The additional pulse requires only a two-step phase cycle for clean results, so the increase in minimum experiment time is modest. The new purging pulse is demonstrated here using the Oneshot [5] pulse sequence, routinely used in DOSY, that provides good spectral quality with a minimum of phase cycling. This combination of the Oneshot sequence and the 45° purging pulse, called Oneshot45, is depicted in Figure 2, and the full phase cycling given in Table 1; the minimum phase cycle for clean results is just two transients. The sequence contains two spin echoes, but any anti-phase magnetization generated in the first spin echo is converted into multiple quantum coherences by the second 90° pulse, and these coherences are not converted back into detectable magnetization. The phase shifts introduced by J-modulation are thus determined solely by the duration $\tau$ of the second echo.

**Figure 2:** Oneshot45 pulse sequence, which consists of Oneshot plus a 45° pulse orthogonal to the preceding 90° pulse.
Table 1: Phase cycling for Oneshot45. Phases are notated as multiples of 90° (0 = 0°, 1 = 90°, 2 = 180°, 3 = 270°), with subscripts denoting repetition; the minimum phase cycle is 2 transients.

<table>
<thead>
<tr>
<th>φ_1</th>
<th>0_41_4+0_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>φ_2</td>
<td>0_1282_128</td>
</tr>
<tr>
<td>φ_3</td>
<td>0_322_32</td>
</tr>
<tr>
<td>φ_4</td>
<td>0_22_2+0_81_82_83_8</td>
</tr>
<tr>
<td>φ_5</td>
<td>0_641_64+0_162_16</td>
</tr>
<tr>
<td>φ_6</td>
<td>φ_1-2φ_2+φ_3-φ_4+2φ_5+1+0_42_4+0_2</td>
</tr>
<tr>
<td>φ_R</td>
<td>φ_1-2φ_2+φ_3-φ_4+2φ_5</td>
</tr>
</tbody>
</table>

4. Experimental

Data were acquired for two mixtures, one of 1-propanol (250 mM) and 2-pentanol (75 mM) in deuterated dimethyl sulfoxide (DMSO-d6) with 27 mM TSP (sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d4) as a reference, and the other camphene (20 mM), geraniol (23 mM) and quinine (19 mM), dissolved in methanol-d4 with TMS (tetramethylsilane) as a reference. Measurements were carried out non-spinning on a Varian VNMRS 500 spectrometer in an air-conditioned room at approximately 20 °C, with probe temperature regulation set at 25 °C and with a passive air preconditioning system used to minimize temperature variations [43].

A series of experiments were carried out with Oneshot and Oneshot45 to evaluate the effects of J-modulation on the corresponding DOSY spectra. Data sets for the mixture of 1-propanol and 2-pentanol were acquired in 5 min with 10 gradient amplitudes ranging from 10 to 30 G cm\(^{-1}\) in equal steps of gradient squared, using 4 transients, 8192 complex
data points, a total diffusion-encoding gradient duration of 1.8 ms, and a diffusion time of 0.2 s. The spin-echo time was varied from 2.8 to 13.8 ms to map out $J$-modulation effects for a range of echo times. Data for the mixture of quinine, camphene and geraniol were acquired in 7 min with 8 gradient amplitudes ranging from 10 to 30 G cm$^{-1}$ in equal steps of gradient squared using 4 transients, 16384 complex data points, a total diffusion-encoding gradient duration of 3.6 ms, an echo time of 19.6 ms and a diffusion time of 0.1 s. The long echo delays used here are to illustrate the effects of long duration of gradient pulses, required for the analysis of large molecules, as for example in the case of a recent paper where 8.5 ms gradient pulses were used for a study of dextran polymers [44]. Nevertheless, problems can arise even for relatively modest echo times and small molecules if large and small signals are close together, as will be shown.

5. Results and discussion

The Oneshot DOSY spectrum for the mixture of 1-propanol and 2-pentanol using an echo time of 13.8 ms shows peaks (Figure 3a) for the two methyl resonances with 3 different apparent diffusion coefficients. The outer peak of the 2-pentanol methyl triplet, at 0.90 ppm (marked with a circle in Figures. 3a and 4a), overlaps with the 1-propanol methyl triplet, and has an apparent diffusion coefficient which is lower than that of the two alcohols. This arises because the small 2-pentanol signal sits on the dispersive tail of the large 1-propanol signal, and is a practical example of the situation illustrated in Figure 1c. In cases like this it would be easy to draw the erroneous conclusion that there is a high molecular weight species in the mixture. In contrast, Figures. 3b and 4b show data from the same sample acquired using the Oneshot45 sequence. In the DOSY spectrum (Figure 3b) peaks in the diffusion dimension all appear at the expected positions, and from the 1D NMR spectrum in Figure 4b it is clear that almost all of the problematic $J$-modulation has been successfully purged.
Figure 3: a) Oneshot and b) Oneshot45 DOSY spectra of 1-propanol (blue) and 2-pentanol (red) with an echo time of 13.8 ms. The signal highlighted belongs to the 2-pentanol but has an apparent diffusion coefficient that is outside the range spanned by the two components.

Figure 4: Detail of the spectrum (a) obtained with Oneshot compared with the spectrum (b) obtained with Oneshot45 for the mixture of 1-propanol and 2-pentanol with an echo time of 13.8 ms. The peak amplitude of the signal highlighted is severely distorted by the dispersive negative tail of a 1-propanol peak, causing a misleading apparent diffusion coefficient (Figure 3).
With these long echo times (outside the range normally used for DOSY experiments on small molecules, but typical of those needed to measure low diffusion coefficients with limited peak gradient amplitude), the spectra obtained with Oneshot45 (Figures 3b and 4b) are largely unaffected by $J$-modulation. Figure 5 shows the apparent diffusion coefficient as a function of echo time for well-resolved and for overlapping (0.90 ppm; highlighted in Figures 3 and 4) peaks, for data measured using the Oneshot and Oneshot45 sequences. As expected, $J$-evolution has no effect on apparent diffusion coefficient for the resolved signals, but the effects of signal overlap are clearly noticeable for data acquired with the standard Oneshot sequence at echo times as low as 4 ms; Oneshot45 suppresses these undesired effects effectively even at an echo time of 14 ms.

The presence of some distortion even at echo times as low as 4 ms shows that problems with $J$ modulation in DOSY are not confined to high molecular mass samples, for which long echo times are needed. The impact of $J$ modulation depends both on the extent of modulation and on the relative intensities of the overlapping signals: a small phase error in a strong signal will cause a big proportionate shift in the intensity of a smaller signal sitting on its dispersion mode tail of the larger.
Figure 5: Dependence of the apparent diffusion coefficients of selected diffusion peaks of the mixture of 1-propanol and 2-pentanol on echo time for a) Oneshot with b) Oneshot45. The apparent diffusion coefficient of the overlapped peak obtained with Oneshot diverges from the true diffusion coefficient with increasing echo time, but for the range of echo times shown Oneshot45 corrects this behaviour.

The mixture of quinine, geraniol and camphene was used to assess the performance of the two sequences with a more complex sample. In both Oneshot and Oneshot45 the severe overlap of small signals between 1 and 2 ppm makes it difficult to resolve the signals with standard HR-DOSY [13, 28], but the rest of the spectrum appears to be resolved. However, J-modulation again is the cause of misleading results. The small doublet at 2.6 ppm appears to belong to geraniol as the apparent diffusion coefficient is consistent with that of other geraniol signals (Figure 6a); in fact, a positive dispersive tail from a quinine multiplet overlaps with this doublet, altering its apparent diffusion coefficient. Using the Oneshot45 sequence (Figure 6b) the dispersive tail is suppressed and the apparent diffusion coefficient now comes out close to the correct value, showing that the doublet actually belongs to camphene.

Figure 6: a) Oneshot and b) Oneshot45 DOSY spectra of quinine (red), geraniol (green) and camphene (blue) with an echo time of 19.6 ms. In the Oneshot DOSY spectrum the
highlighted doublet appears to belong to the geraniol spectrum, but actually originates from camphene as shown in the Oneshot45 spectrum.

6. Conclusions

In DOSY experiments, $J$-modulation can cause signal overlap, resulting in misleading apparent diffusion coefficients. When a peak overlaps with the positive tail of another peak belonging to a different species, the apparent diffusion coefficient calculated in HR-DOSY is intermediate between the diffusion coefficients of the two species. However, when the overlap is from a negative dispersive tail, as caused by $J$-modulation, the effect is counter-intuitive: the apparent diffusion coefficient is outside the range spanned by the two species. The magnetization responsible for this effect of $J$-modulation can be suppressed efficiently, even for relatively long echo times, by adding a 45° purging pulse immediately before acquisition. The Oneshot45 pulse sequence improves the reliability of DOSY data at no significant cost in sensitivity and with only a doubling of the (very short) minimum experiment time, making it ideal wherever rapid and reliable measurements are required, such as for the analysis of unstable mixtures [45] or reactions [46, 47]. In addition, the suppression of distortions caused by J modulation allows much longer gradient-encoding pulses to be used with coupled spin systems. Because of the square law dependence of the Stejskal-Tanner exponent on the gradient pulse width, this translates into an improvement of more than an order of magnitude in the limiting range of diffusion coefficients that can be studied with given hardware. Finally, the relatively small price to pay for the purging of unwanted magnetization also makes Oneshot45 a good candidate for a general purpose DOSY pulse sequence.

Acknowledgements

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Appendix A.

The existence and scope of the different cases for monoexponential least squares fitting of a biexponential curve can be demonstrated analytically. Consider the general biexponential function

$$S = S_1 e^{-D_1 x} + S_2 e^{-D_2 x} \quad (A1)$$

The variance between this and the monoexponential fit function

$$S = S_m e^{-D_m x} \quad (A2)$$

is given by

$$\chi^2 = \int_0^\infty \left( S_1 e^{-D_1 x} + S_2 e^{-D_2 x} - S_m e^{-D_m x} \right)^2 dx$$

$$= \frac{1}{2} \left( \frac{S_1^2}{D_1} + \frac{S_2^2}{D_2} + \frac{S_m^2}{D_m} + \frac{4S_1 S_2}{D_1 + D_2} - \frac{4S_1 S_m}{D_1 + D_m} - \frac{4S_2 S_m}{D_2 + D_m} \right) \quad (A3)$$

The turning points in $\chi^2$ can be found by solving the simultaneous equations

$$\frac{d\chi^2}{dS_m} = 0 = \frac{S_m}{D_m} - \frac{2S_1}{D_1 + D_m} - \frac{2S_2}{D_2 + D_m} \quad (A4),$$

$$\frac{d\chi^2}{dD_m} = 0 = \frac{1}{2} \left[ \frac{4S_1 S_m}{(D_1 + D_m)^2} + \frac{4S_2 S_m}{(D_2 + D_m)^2} - \frac{S_m^2}{D_m^2} \right]$$

yielding four roots.

Figure A1 shows contour plots of the relative values $D_m/D_1$ and $S_m/S_1$ that minimize Eq. A2 (for $|S_1| > |S_2|$). A clear discontinuity is seen in both plots running down from the degenerate case of equal amplitudes and diffusion coefficients towards the line $D_2/D_1 = 0$. 

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On one side of the discontinuity the best fit $D_m$ rises sharply as it is approached, on the other it falls to a low value, corresponding to a change between the best fit monoexponential being fast and positive to being slow and negative. The three cases identified in the text correspond to (1) the top halves of the two plots, where $D_m < D_1$, $D_2$ and $S_m > 0$; (2) the lower right hand parts of the plots, where $D_m > D_1$, $D_2$ and $S_m > 0$; and (3) the lower left hand parts of the plots, where $D_m < D_1$, $D_2$ and $S_m < 0$. 
**Figure A1:** Contour plots, as a function of the ratios $S_2/S_1$ and $D_2/D_1$ of relative amplitudes and decay constants of the components of a biexponential function, of (a) the best fit relative monoexponential rate constant $D_m/D_1$ and (b) the best fit relative monoexponential amplitude $S_m/S_1$. 
Appendix B.

In a system with 3 spins $\hat{I}_1$, $\hat{I}_2$ and $\hat{I}_3$ where magnetization is initially longitudinal, the effect of the spin echo will be as described by the following product operator transformations. For spin $\hat{I}_1$:

$$\hat{I}_{1x} \xrightarrow{\pi/2(l_{1x})} \hat{I}_{1x} \xrightarrow{2\pi(l_{1x}l_{2x})} \cos(\pi J_{12}) \hat{I}_{1x} + \sin(\pi J_{12}) 2\hat{I}_{1y} \hat{I}_{2x}$$

The product operator transformations are analogous for spins $\hat{I}_{2x}$ and $\hat{I}_{3x}$. After the application of a $45^\circ$ pulse the operators experience the following transformations.

$$\cos(\pi J_{12}) \cos(\pi J_{13}) \hat{I}_{1x} \xrightarrow{\pi/4(l_{1x}l_{2x}l_{3x})} \cos(\pi/4) \cos(\pi J_{12}) \cos(\pi J_{13}) \hat{I}_{1x}$$

$$\cos(\pi J_{12}) \sin(\pi J_{13}) 2\hat{I}_{1y} \hat{I}_{3x} \xrightarrow{\pi/4(l_{1x}l_{2x}l_{3x})} \cos(\pi/4) \cos(\pi J_{12}) \sin(\pi J_{13}) 2\hat{I}_{1y} \hat{I}_{3x} - \sin(\pi/4) \cos(\pi J_{12}) \sin(\pi J_{13}) 2\hat{I}_{1y} \hat{I}_{3y}$$

$$\sin(\pi J_{12}) \cos(\pi J_{13}) 2\hat{I}_{1y} \hat{I}_{2x} \xrightarrow{\pi/4(l_{1x}l_{2x}l_{3x})} \cos(\pi/4) \sin(\pi J_{12}) \cos(\pi J_{13}) 2\hat{I}_{1y} \hat{I}_{2x} - \sin(\pi/4) \sin(\pi J_{12}) \cos(\pi J_{13}) 2\hat{I}_{1y} \hat{I}_{2y}$$

$$- \sin(\pi J_{12}) \sin(\pi J_{13}) 4\hat{I}_{1x} \hat{I}_{2x} \hat{I}_{3x} \xrightarrow{\pi/4(l_{1x}l_{2x}l_{3x})} - \cos(\pi/4) \sin(\pi J_{12}) \sin(\pi J_{13}) 4\hat{I}_{1x} \hat{I}_{2x} \hat{I}_{3x} - \sin(\pi/4) \sin(\pi J_{12}) \sin(\pi J_{13}) 4\hat{I}_{1x} \hat{I}_{2y} \hat{I}_{3y}$$

The in-phase terms are unaffected, the bilinear anti-phase terms get cancelled out with the bilinear anti-phase terms of the other spins and the trilinear anti-phase magnetization gets partially converted into unobservable multiple quantum coherences. The resulting observable terms are a thus a mixture of in-phase and reduced trilinear anti-phase
magnetization. These trilinear terms are a function of a product of two $\tau$ dependent sinusoids, and so can be neglected for short $\tau$ echo times.

References