SOME ASPECTS OF POLARISATION TRANSFER IN NMR SPECTROSCOPY

HARRI KOSKELA

Faculty of Science,
Department of Chemistry,
University of Oulu

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Abstract

Modern NMR methods in liquids are based on the transfer of polarisation from nucleus to nucleus to generate multidimensional heteronuclear correlation spectra. The factors that influence the efficiency of heteronuclear polarisation transfer in multi-pulse experiments, and in that way the quality of spectra are the subject of this thesis. The flow of coherence through pulse sequences can be designed and analysed with the aid of product operator calculations. Results of the study include improvements to quantitative two-dimensional shift-correlated experiments, and demonstration of the benefits of closely spaced 180° pulse trains in polarisation transfer steps in long-range correlation experiments and isotope editing filters.

Keywords: Carr-Purcell-Meiboom-Gill sequences, Hartmann-Hahn transfer, Quantitative 2D NMR, RF pulse imperfections
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Helsinki, August 2005

Harri Koskela
## Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>$^{13}$C</td>
<td>Carbon-13</td>
</tr>
<tr>
<td>$^{15}$N</td>
<td>Nitrogen-15</td>
</tr>
<tr>
<td>1D</td>
<td>One-dimensional</td>
</tr>
<tr>
<td>$^1$H</td>
<td>Proton</td>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>$^{31}$P</td>
<td>Phosphorus-31</td>
</tr>
<tr>
<td>AQ</td>
<td>Acquisition period in pulse sequence</td>
</tr>
<tr>
<td>ASTM</td>
<td>American Society for Testing and Materials, an international standards development organisation</td>
</tr>
<tr>
<td>$B_0$</td>
<td>Static magnetic field parallel to $z$ axis</td>
</tr>
<tr>
<td>$B_1$</td>
<td>Radio frequency field perpendicular to $B_0$</td>
</tr>
<tr>
<td>$B^{\text{o}}_1$</td>
<td>Optimum radio frequency field strength</td>
</tr>
<tr>
<td>$\vec{B}^r$</td>
<td>Effective magnetic field vector in rotating frame</td>
</tr>
<tr>
<td>BBI</td>
<td>Broad Band Inverse, a probe head type</td>
</tr>
<tr>
<td>BIRD</td>
<td>Bilinear rotational decoupling; a method for selective inversion of protons bound to $^{13}$C</td>
</tr>
<tr>
<td>CAGEBIRD</td>
<td>CPMG And Gradient-Enhanced BIRD; a pulse cluster for isotope-editing</td>
</tr>
<tr>
<td>CAHSQC</td>
<td>CPMG-Adjusted HSQC</td>
</tr>
<tr>
<td>COLOC</td>
<td>COrelation Spectroscopy via LOng-range Coupling, a 2D long-range correlated experiment</td>
</tr>
<tr>
<td>CPMG</td>
<td>An evenly spaced 180° pulse train named after its inventors, Carr, Purcell, Meiboom and Gill</td>
</tr>
<tr>
<td>CPMG-INEPT</td>
<td>INEPT cluster modified with CPMG pulse train</td>
</tr>
<tr>
<td>CW</td>
<td>Chemical Weapons Convention</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless Enhancement by Polarisation Transfer</td>
</tr>
<tr>
<td>$F_1$</td>
<td>First indirectly detected dimension in n-dimensional spectroscopy</td>
</tr>
<tr>
<td>$F_2$</td>
<td>Directly detected dimension in two-dimensional spectroscopy</td>
</tr>
<tr>
<td>GBIRD</td>
<td>Gradient-BIRD; a pulse cluster for isotope editing</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation, a 2D long-range correlated experiment</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Coherence, a 2D chemical shift correlated experiment</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence, a 2D chemical shift correlated experiment</td>
</tr>
<tr>
<td>$I_s$</td>
<td>Spin operator</td>
</tr>
<tr>
<td>INEPT</td>
<td>Insensitive Nuclei Enhancement by Polarisation Transfer</td>
</tr>
<tr>
<td>$^{n}J_{jk}$</td>
<td>Spin-spin coupling constant of nuclei $j$ and $k$ over $n$ bonds</td>
</tr>
<tr>
<td>$J^\circ$</td>
<td>Average coupling constant, used for the calculation of the polarisation transfer delay</td>
</tr>
<tr>
<td>LR</td>
<td>Long-Range, a prefix for long-range correlated experiments</td>
</tr>
<tr>
<td>$M_\alpha$</td>
<td>Magnetisation vector aligned to axis $\alpha$, where $\alpha = x, y$ or $z$</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>Q-CAHSQC</td>
<td>Quantitative CAHSQC</td>
</tr>
<tr>
<td>Q-HSQC</td>
<td>Quantitative HSQC</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>$T_1$</td>
<td>Longitudinal relaxation time</td>
</tr>
<tr>
<td>$t_1$</td>
<td>First indirect evolution period in multidimensional NMR</td>
</tr>
<tr>
<td>$T_2$</td>
<td>Transverse relaxation time</td>
</tr>
<tr>
<td>$t_2$</td>
<td>Acquisition period in 2D NMR</td>
</tr>
<tr>
<td>TANGO</td>
<td>Testing for Adjacent Nuclei with a Gyration Operator, a method for selective excitation of protons bound to $^{13}$C</td>
</tr>
<tr>
<td>$V_C$</td>
<td>Cross peak volume in 2D NMR spectrum</td>
</tr>
<tr>
<td>XY-16</td>
<td>A variant of CPMG sequence with super cycle of 16 pulses</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>Polarisation transfer delay in pulse sequences, optimised corresponding to $J^\circ$</td>
</tr>
<tr>
<td>$\Delta_{max}$</td>
<td>Maximum polarisation transfer delay in Q-HSQC</td>
</tr>
<tr>
<td>$\Delta B_0$</td>
<td>The $z$ component of $\vec{B}$</td>
</tr>
<tr>
<td>$\Delta B_z(r)$</td>
<td>Spatial dependence of $B_0$ field strength during $z$ gradient pulse</td>
</tr>
<tr>
<td>$\Delta \nu$</td>
<td>Chemical shift difference of coupled nuclei in hertz</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Radio frequency pulse/chemical shift offset</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Pulse angle</td>
</tr>
<tr>
<td>$\delta$</td>
<td>Multiplicity editing period in 2D INEPT</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Pulse phase</td>
</tr>
<tr>
<td>$\gamma_i$</td>
<td>Gyromagnetic ratio of nucleus $i$</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Overhauser enhancement factor</td>
</tr>
<tr>
<td>$\nu_{mod}$</td>
<td>Modulation frequency of $J$ coupling during CPMG sequence</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Density operator</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Inter-pulse delay in CPMG sequences</td>
</tr>
<tr>
<td>$\tau_{grad}$</td>
<td>Gradient pulse duration</td>
</tr>
<tr>
<td>$\tau_m$</td>
<td>Isotropic mixing time</td>
</tr>
<tr>
<td>$\theta$</td>
<td>The tilt angle of $\vec{B}$ with respect to $z$ axis</td>
</tr>
<tr>
<td>$\omega_0$</td>
<td>$B_0$ field frequency</td>
</tr>
<tr>
<td>$\omega_i$</td>
<td>Larmor frequency of nucleus $i$</td>
</tr>
<tr>
<td>$\omega_{RF}$</td>
<td>RF field frequency</td>
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List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


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Introduction

Over four decades pulsed-Fourier NMR spectroscopy has evolved from a simple excitation–acquisition scheme to embrace a number of complex pulse sequences [1]. The advance in methodology that has given rise to numerous experimental methods and applications has its roots in the basic NMR concept – the polarisation transfer.

The need to detect insensitive nuclei within a reasonable recording time inspired signal enhancement through the manipulation of thermodynamic spin populations. The first applications in the early fifties, exploited the nuclear Overhauser enhancement (NOE) obtained with a broadband saturation of sensitive nuclei, usually protons [2-5]. The utilisation of cross-polarisation in the rotating frame [6] demonstrated an alternative approach, and some applications in liquids were reported [7-9].

In 1979, Morris and Freeman [10] published a new method where coherence was transferred during $J$ coupling evolution under spin-echo conditions. Since it relied on the use of non-selective RF pulses, the experimental demands were less constraining than with cross-polarisation transfer, and the method offered a higher signal enhancement factor, $\eta$, than the optimal Overhauser enhancement. The method, abbreviated INEPT (Insensitive Nuclei Enhanced by Polarisation Transfer), soon became widely used in multinuclear magnetic resonance spectroscopy [11-13]. A major shortcoming of the method, namely the anti-phase multiplet structure of resonance and consequent prohibition of proton decoupling during acquisition, was soon addressed to allow multiplicity editing [14-17].

A rival method that offered some advances over INEPT was published in 1982 by Doddrell and co-authors. This method, called DEPT (Distortionless Enhancement by Polarisation Transfer), [18], used fewer RF pulses than refocused INEPT, which was of some importance at a time when $B_1$ fields of probes were rather inhomogeneous. The polarisation was transferred from proton to heteronucleus through multiple-quantum coherence states. DEPT offered a better tolerance to heteronuclear $J$ coupling mismatch in multiplicity editing than INEPT [19] and became established as the primary method for multiplicity editing in one-dimensional multinuclear resonance spectroscopy.

The introduction of two-dimensional techniques [20,21] in the 1970s was a major event in the history of NMR. It took NMR to a new level and led to an active development of methods [22-31]. The significance of the discovery and the subsequent
research of the most prominent contributor, Richard R. Ernst [32-45], was recognised in 1991 with the Nobel Prize in Chemistry.

In 1980, soon after the introduction of INEPT, Bodenhausen and Ruben published a new two-dimensional shift-correlated experiment designated HSQC (Heteronuclear Single Quantum Coherence) [46]. The method employed inverse detection, i.e., the proton polarisation was transferred to a heteronucleus by INEPT, and after an evolution period $t_1$ the polarisation was returned to the proton for observation with a reverse-INEPT block [47]. HSQC thus offered superior sensitivity to the two-dimensional methods employing heteronuclear detection [48]. Moreover, the previous inverse detected method, HMQC (Heteronuclear Multiple Quantum Coherence) [49], relied on multiple-quantum coherence transitions during the pulse sequence, which caused undesirable cross peak broadening in the $F_1$ dimension due to the $J_{HH}$ evolution [50,51]. The homonuclear coupling evolution contribution to cross peak shape was effectively suppressed in HSQC due to single-quantum coherence states.

Afterwards, HSQC, and hence INEPT, was successfully implemented in many other multidimensional methods as the basic building block of polarisation transfer. As a result of this development, NMR has become a major method for the structural analysis of biological macromolecules, most notably proteins [52]. The significance of the work was underlined by the awarding of the Nobel Prize in Chemistry to Kurt Wüthrich [53-57] in 2002.

The CPMG (Carr–Purcell–Meiboom–Gill) sequence [58-60], routinely used for $T_2$ relaxation measurements, has recently been implemented in multidimensional experiments. Some applications have exploited the conventional CPMG function – mostly in the analysis of protein backbone dynamics from $^{15}$N relaxation times – by introducing either a $T_2$ relaxation weighting in the multidimensional spectrum [61,62] or a separate $T_2$ relaxation dimension [63,64]. The CPMG sequence has also been used in novel contexts. CPMG-INEPT has been demonstrated to improve the sensitivity in studies of fast exchanging spin systems [65-67]. The most intriguing application to date has been in the sequential assignment of RNA oligonucleotides. The cross peak phase twists caused by $J_{HH}$ evolution in $^1$H-$^{31}$P polarisation transfer steps have been effectively suppressed by use of CPMG-INEPT with a fast 180° pulsing rate [68]. This discovery has solved problems of many NMR methods, as will be demonstrated in the pages to follow.

This thesis consists of three parts: , , and . Chapter 1, , commences with a theoretical discussion of factors that contribute to the efficiency of polarisation transfer, and concludes with an examination of CPMG sequences, emphasising the $J$ evolution suppression. Chapter 1, , describes how the use of CPMG-INEPT for polarisation transfer improves heteronuclear coupling evaluation in LR-HSQC-type experiments, and how the GBIRD isotope editing filter can provide a much better parent signal suppression level when the BIRD sandwich is modified with CPMG sequences. Factors relevant to the use of heteronuclear shift-correlated 2D NMR methods for quantification are discussed. A summary of the research is presented in Chapter 1, , along with some potential applications for the future.
1 Aims of the study

The starting point of this study was to clarify the mechanisms of polarisation transfer that are exploited in modern NMR pulse sequences for the study of small organic molecules and to identify possible sites for improvements. One of the aims was to develop two-dimensional NMR methods that, while offering superior resolution to the one-dimensional methods, were able to simultaneously produce reliable quantitative information about sample components. The analysis of coherence flow also offered a way to improve the quality of spectra in certain NMR experiments.

The emphasis of the study was on the evolution of heteronuclear ($J_{CH}$) and homonuclear ($J_{HH}$) couplings, but some aspects of RF pulse imperfections were addressed as well. A fast pulsing rate during CPMG results in a homonuclear isotropic mixing condition, leading to in-phase Hartmann–Hahn transfer in the $^1$H–$^1$H-coupling network. One fruitful area of study was applications of CPMG sequences to polarisation transfer steps for homonuclear $J$ evolution suppression in experiments, where $J_{HH}$ evolution can compromise the quality of spectrum.
2 Basic concepts

This part of the thesis describes the basic concepts of polarisation transfer efficiency with respect to heteronuclear and homonuclear coupling. The problems associated with the polarisation delay mismatch with respect to the actual heteronuclear coupling, and the polarisation leak due to homonuclear couplings are demonstrated in Section 2.1 with product operator formalism. Some of the properties of RF pulses are discussed and demonstrated using the Bloch equation description. Section 2.2 presents a theoretical and experimental survey of CPMG sequences and some of their properties with fast pulsing rates.

2.1 Efficiency of heteronuclear polarisation transfer

A number of mathematical methods can be used to describe the behaviour of magnetisation. The Bloch equations \[69,70\] effectively present the evolution of bulk magnetisation, but they have only limited ability to handle $J$ couplings and multiple-quantum coherence. The spin dynamics can be analysed rigorously using density matrices \[71-74\], but the arithmetic treatment of large spin systems during multi-pulse sequences tends to be laborious and demands computer-aided calculations. Product operator formalism \[72,75-78\] is a concise version of density matrix computations, which provides tools to follow multiple-quantum coherence evolution by simple transformations of spin operators. The principles of spin operator calculations are illustrated below.

The density operator of magnetisation in the thermodynamic equilibrium is

$$\sigma_0 = I_z$$

Eq. 1

where $I_z$ is the Cartesian spin operator corresponding to a magnetisation of arbitrary $\frac{1}{2}$-spin $I$ parallel to the $z$ axis. A radio frequency pulse with arbitrary pulse angle $\beta$ and phase $\phi$ perturbs the state as follows:
\[ I_z \xrightarrow{\beta \phi} I_z \cos(\beta) + I_x \sin(\beta) \sin(\phi) - I_y \sin(\beta) \cos(\phi) \]

Eq. 2

The transverse magnetisation will evolve during time \( t \) with a rate referred to as the chemical shift:

\[ I_x \xrightarrow{\Omega t} I_x \cos(\Omega t) + I_y \sin(\Omega t) \]

Eq. 3

Likewise, \( J \) coupling evolution can be described as

\[ I_x \xrightarrow{\frac{\pi r}{2J} I_x'} I_x \cos(\pi J t) + 2I_y I_z' \sin(\pi J t) \]

Eq. 4

For simplicity, relaxation and RF pulse offset effects are often omitted in product operator calculations. The magnetisation evolution can then be delineated in pulse sequences in a straightforward manner.

Section 2.1.1 briefly describes the transfer of polarisation in INEPT through heteronuclear coupling evolution, and shows, through product operator calculations, how the transfer efficiency is dependent on the matching of heteronuclear coupling and polarisation transfer delay. The polarisation leak due to homonuclear couplings is discussed in Section 2.1.2. The properties of RF pulses are briefly illustrated with the Bloch equations in Section 2.1.3.

### 2.1.1 Effect of heteronuclear \( J \) mismatch

INEPT is the basic building block in many pulse sequences (Fig. 1). It also provides an illustrative example of the usefulness of product operator formalism. Furthermore, it demonstrates some key factors that can contribute to polarisation transfer efficiency.
We take a simple case where a single proton is coupled to a carbon. This is the AX spin system with $A = ^1H$, $X = ^{13}C$, $J_{CH} \neq 0$. Hereafter we denote this spin system simply by HC. The initial state of magnetisation is assumed to be a proton (H) magnetisation in thermodynamic equilibrium, which is denoted with the symbol $H_z$. The evolution of magnetisation through the INEPT pulse sequence can be presented in detail with product operators. However, for the sake of brevity, in most cases through the text, only the final result is presented. Consequently, the final state of magnetisation after INEPT is

$$H_z \xrightarrow{\text{INEPT}} H_y \cos(\pi J_{CH} \Delta) - 2H_z C_y \sin(\pi J_{CH} \Delta)$$

Eq. 5

$H$ and $C$ are the spin operators for proton and carbon magnetisation, respectively. Below, for the sake of brevity, the normalisation factor 2 in the heteronuclear anti-phase term $2H_z C_y$ is omitted.

At this point we are only interested in the carbon term(s), and so in this case the term $H_z C_y$. This term represents transverse carbon magnetisation that is in anti-phase with respect to the proton coupling. In the spectrum this would appear as a doublet, where the lines have 180° phase difference with respect to each other [17]. The efficiency of the transfer from the proton magnetisation to the term $H_z C_y$ is governed by the trigonometric part, $\sin(\pi J_{CH} \Delta)$. This part has two important factors: the magnitude of $J_{CH}$ and the polarisation transfer delay $\Delta$. The efficiency of the polarisation transfer is maximal when

$$|\sin(\pi J_{CH} \Delta)| = 1$$

Eq. 6
The coupling constant $J_{CH}$ is spin system dependent, and we have no way to alter it. Hence, Eq. 6 must be solved for with consideration of the polarisation transfer delay $\Delta$. The solution with shortest $\Delta$ would minimise the relaxation losses. Thus, we get:

$$\Delta = \frac{1}{2|J_{CH}|} \Rightarrow \sin\left(\pi \left|J_{CH}\right| \frac{1}{2|J_{CH}|}\right) = 1$$

Eq. 7

Since the $J_{CH}$’s cancel one another, the absolute value notation is omitted below. Variation in $J_{CH}$’s creates a problem with respect to polarisation transfer optimisation. One-bond proton–carbon couplings, for example, vary typically in the range of 115–220 Hz, depending on the orbital hybridisation of the carbon and the electronegativity of substituent(s). In a normal INEPT experiment, the delay $\Delta$ is set before the acquisition to correspond to the average coupling constant $J^\circ$, as shown in Eq. 7. If we examine the efficiency of the polarisation transfer with respect to the mismatch between the average coupling $J^\circ$ and the true coupling $J_{CH}$, we can reformulate Eq. 5 as

$$H_z \xrightarrow{\text{INEPT}} H_z C_y \sin\left(\frac{\pi}{2} \frac{J_{CH}}{J^\circ}\right)$$

Eq. 8

The sine type dependence of the polarisation transfer with respect to the $J_{CH}/J^\circ$ ratio is illustrated in Fig. 2. If the polarisation transfer period is optimised for $J_{CH}$ of 125 Hz, the typical coupling of aliphatic hydrocarbons, the aromatic carbon with $J_{CH}$ of 175 Hz will acquire only 81% of the maximum transferable polarisation. For qualitative studies this loss of intensity makes no difference, but for quantitative analysis the mismatch between $J_{CH}$ and $J^\circ$ can be a significant source of error.
Fig. 2. Theoretical polarisation transfer efficiency of INEPT for an AX spin system (HC spin system) with respect to the $J_{CH}/J^0$ ratio, where $J_{CH}$ is the real coupling constant and $J^0$ is the average coupling constant used for calculation of the polarisation transfer period.

2.1.2 Effect of homonuclear $J$ couplings

Next, we consider a situation when a proton is coupled both to a carbon and to another proton. This is the AMX spin system, where $A = ^1H$, $M = \text{remote} ^1H$, and $C = ^{13}C$, $J_{CH} \neq 0$, $J_{CH'} = 0$. In the following this spin system is denoted by $HH'C$. A product operator calculation gives the following result for the final state of the magnetisation after INEPT:

$$
\begin{align*}
H_z & \xrightarrow{\text{INEPT}} -H_z C_x \sin \left( \frac{\pi}{2} \cdot J_{CH}/J^0 \right) \cos \left( \frac{\pi}{2} \cdot J_{HH}/J^0 \right) \\
& \quad + H_y \cos \left( \frac{\pi}{2} \cdot J_{CH}/J^0 \right) \cos \left( \frac{\pi}{2} \cdot J_{HH}/J^0 \right) \\
& \quad + H_x H_y \cos \left( \frac{\pi}{2} \cdot J_{CH}/J^0 \right) \sin \left( \frac{\pi}{2} \cdot J_{HH}/J^0 \right) \\
& \quad + H_x H_y C_y \sin \left( \frac{\pi}{2} \cdot J_{CH}/J^0 \right) \sin \left( \frac{\pi}{2} \cdot J_{HH}/J^0 \right)
\end{align*}
$$

Eq. 9
Eq. 9 shows, that the magnitude of term $H_zC_y$ does not only depend on the evolution of the $J_{CH}$ coupling, but also on the evolution of the $J_{HH}$ coupling. When the optimised $J^\circ$ is close to the proton-proton coupling values, the original magnetisation of the $H_z$ term will be transferred effectively to the single- and multiple-quantum coherence terms shown in the parenthesis of Eq. 9, while the desired transfer to the term $H_zC_y$ will become smaller (Fig. 3).

Fig. 3. Theoretical polarisation transfer efficiency of INEPT for an AMX spin system (HH'C spin system, $J_{HH'}, J_{CH} \neq 0, J_{CH'} = 0$) with respect to the $J_{CH}/J^\circ$ ratio, where $J_{CH}$ is the proton-carbon coupling constant, $J_{HH}$ is the proton-proton coupling constant and $J^\circ$ is the average coupling constant used for calculation of the polarisation transfer period.

The INEPT transfer for a spin system bearing a proton with a single coupling to a carbon and $n$ couplings to remote protons can be presented in the form given in Eq. 10:

$$H_z \xrightarrow{\text{INEPT}} -H_zC_y \sin\left(\frac{\pi}{2} \cdot \frac{J_{CH}}{J^\circ}\right) \prod_{i=1}^{n} \cos\left(\frac{\pi}{2} \cdot \frac{J_{HH}}{J^\circ}\right)$$

Eq. 10

In the cases where $J^\circ >> J_{HH}$, the polarisation leak to non-desired coherences evolved through homonuclear coupling evolution can be neglected. This approximation is valid when INEPT is optimised for $^1J_{CH}$ couplings. When INEPT is used for $^2J_{CH}$ transfer, however, $J^\circ \approx J_{HH}$, and considerable leak is inevitable.
2.1.3 RF pulse imperfections

With use of product operator formalism it is convenient to assume ideal RF pulses, through, no RF pulse is perfect, of course. Two factors may compromise the pulse performance: 1) non-optimal RF coil characteristics result in variation on the $B_1$ field strength with respect to the optimal field strength, $B_1^{\circ}$, referred to as $B_1$ inhomogeneity, and 2) the offset dependent nutation of the effective $B_1$ field vector towards the $z$ axis gives rise to off-resonance effects.

With recent progress in the probe head design, present RF coils can generate a spatially uniform $B_1$ field. Thus, modern NMR spectrometers rarely suffer from $B_1$ inhomogeneity. Often the primary concern is mis- or non-calibrated pulses. The off-resonance effects have become a more serious concern, however, due to the higher $B_0$ field strengths, since the spectral range of a given nucleus is proportional to the $B_0$ field strength. For example, an RF power of 20 kHz on the $^{13}$C channel, which corresponds to a $90^\circ$ pulse duration of 12.5 $\mu$s, is sufficient to excite the natural carbon chemical shift range of 200 ppm on an NMR spectrometer with $B_0$ strength of 9.4 T (corresponds to $\gamma B_0 \approx 100$MHz). The same RF power is too low for that task on an NMR spectrometer with $B_0$ strength of 18.8 T (which corresponds to $\gamma B_0 \approx 200$MHz).

The perturbation of magnetisation with an RF pulse can be illustrated with a classical description. The Bloch equations [69,76] in the rotating frame are a convenient way to estimate the efficiency of an RF pulses. The behaviour of the magnetic moment $\overrightarrow{M}'(t)$ under the magnetic field $\overrightarrow{B}'(t)$ is

$$\frac{d\overrightarrow{M}'(t)}{dt} = \overrightarrow{M}'(t) \times \gamma \overrightarrow{B}'(t)$$

Eq. 11

where

$$\overrightarrow{B}' = B_1 \cos(\phi)\overrightarrow{i}' + B_1 \sin(\phi)\overrightarrow{j}' + \Omega \overrightarrow{k}'$$

Eq. 12

and $\Omega = -\gamma B_0 - \omega_{RF} = \omega_0 - \omega_{RF}$ is the offset between the static field frequency $\omega_0$ and RF field frequency $\omega_{RF}$, and $\overrightarrow{i}'$, $\overrightarrow{j}'$, $\overrightarrow{k}'$ are unit vectors in the rotating frame. The effective field strength $B'$ is
\[ B' = \sqrt{B_1^2 + \Delta B_0^2} = B_1 \sqrt{1 + \left(\tan(\theta)\right)^2}, \]

\[ \Delta B_0 = \frac{\Omega}{\gamma}, \]

\[ \tan(\theta) = \frac{B_1}{\Delta B_0}. \]

Eq. 13

where \( \Delta B_0 \) is the reduced static field, which corresponds to the \( z \) component of the effective field \( B' \), and \( \theta \) is the tilt angle of \( B' \) with respect to the \( z \) axis. According to Eq. 12 and Eq. 13, in on-resonance condition, the magnetisation experiences only the \( B_1 \) field with phase \( \phi \). As \( \Omega \) increases, the effective field vector turns towards the \( z \) axis. These off-resonance condition would result in a situation where, for example, steady-state magnetisation does not experience a complete inversion after the \( 180^\circ \) pulse, but the magnetisation vector lies somewhere in the lower hemisphere of the rotating frame (Fig. 4). Imperfect performance of RF pulses due to the off-resonance effects causes incomplete transfer of coherence and loss in signal intensity, and it can even produce spurious artefacts in the spectrum [79].

Fig. 4. Trajectories of magnetisation vectors during \( 180^\circ \) pulse when the RF pulse offset is incremented from 0 Hz to 16 kHz in 4 kHz steps. \( B_1 \) field strength was 20 kHz. The simulation was carried out with Bruker NMR-Sim [80].
Fig. 5. Contour plots demonstrating the efficiency of normal pulses and some composite pulses as a function of the $B_1$ inhomogeneity ratio $B_1/B_0$ and the pulse offset $\Omega/\gamma B_1$. On the left are the contour plots of normal RF pulses for excitation and inversion of magnetisation and refocusing of magnetisation either parallel or perpendicular with respect to the pulse
phase. On the right are the excitation [81], inversion [85] and refocusing efficiency plots of two typical composite pulses. The contour curves are drawn for efficiency levels of 98, 90, 80, 60, 40, 20 and 0%. The Bloch equations for the efficiency plots were calculated with Mathematica [86], and the contour plots were produced with MATLAB [87].

Performance deficiencies are more attenuated with composite pulses [81-83]. A composite pulse consists of a number of RF pulses applied in succession with different pulse phases and duration. Most of the composite pulses compensate with good efficiency either $B_1$ inhomogeneity or off-resonance effects, but there have also been some attempts to produce dual performance compensating pulses [84]. Composite pulses are also designed for specific tasks, i.e., for excitation, inversion or refocusing of magnetisation. The efficiency of composite pulses or of any pulse train for arbitrary $\pm M_\alpha \rightarrow \pm M_\beta$ transfer ($\alpha, \beta = x, y, z$) can be illustrated with contour plots that present the magnetisation with respect to the $B_1$ inhomogeneity ratio $B_1/B_0$, and pulse offset $\Omega/\gamma B_1$ (Fig. 5).

A number of options are available for compensating the imperfections of pulses in pulse sequences. One option is to replace normal pulses that play a critical role, with suitable composite pulses [88-90]. Another is to practice global compensation [83,91], where every pulse in the pulse sequence behaves as an individual pulse element, and the pulse sequence as a whole produces the compensation for pulse imperfections. The best choice of method is often found through trial and error due to the characteristics of spectrometers.

### 2.2 Properties of CPMG sequences

In 1954, Carr and Purcell [58] introduced an experiment able to reveal the intrinsic transverse relaxation time $T_2$. The method was based on measurement of 1D $^1$H NMR spectra with a varying $T_2$ evolution period (180° pulse train duration). The $T_2$’s of individual protons could be iterated by applying an exponential fitting to peak intensities. Shortly thereafter, Meiboom and Gill [59] improved the method by shifting the phase of the excitation pulse by 90 degrees to compensate for the cumulative effects of the inhomogeneous $B_1$ field. This improved method was called the Carr–Purcell–Meiboom–Gill (CPMG) experiment. Gullion and co-authors [60] compensated the rapid dephasing of magnetisation non-parallel to the axis of 180° pulses through introduction of an XY-16 phase cycle. CPMG has withstood the test of time as the method for $T_2$ relaxation measurements, and it has been applied lately to multidimensional pulse sequences [62,63].

The suppression of $J$ coupling modulation during a fast spin echo pulse train was quickly examined in many theoretical studies [92-94]. However, only a few experimental data exist to confirm the $J$ coupling suppression, and applications that exploit it are few. Section 2.2.1 focuses on the theoretical aspects of $J$ coupling suppression with CPMG type sequences. In addition, some experimental results with CPMG-modified two-dimensional $J$-resolved NMR spectroscopy are presented. Theoretical aspects of Hartmann–Hahn transfer are discussed in Sections 2.2.2 and 2.2.3.
2.2.1 J evolution suppression

The spin–spin interaction mediated through bonding electrons, the \( J \) coupling, produces characteristic fine structure in resonances [95]. Interpretation of these multiplet patterns gives valuable information for structure elucidation. The \( J \) coupling evolution can be exploited in multi-pulse experiments, for example to give higher sensitivity of insensitive nuclei through heteronuclear polarisation transfer [10,14,17,18]. During the past few decades, multi-pulse experiments have been used as building blocks in the development of more versatile multidimensional methods [46,96,97].

In some experiments, \( J \) coupling evolution between certain nuclei is not desired, since it can introduce unfavourable contributions to the spectrum. In the case of heteronuclear couplings, the evolution is easily refocused, but the homonuclear coupling evolution is difficult to avoid.

One example of the homonuclear coupling evolution during a pulse sequence is a simple spin-echo experiment, \( 90^\circ(x) – \tau – 180^\circ(x) – \tau – AQ(x) \). While the 180\(^\circ\) pulse refocuses the chemical shift and the heteronuclear coupling evolution, the homonuclear coupling evolution evolves intact during the spin-echo period, giving a twisted lineshape to resonances that have homonuclear coupling partner(s).

During the 1950s new pulse sequence CPMG [58,59] was proposed for transverse relaxation analysis. The experiment consisted of a 90\(^\circ\) excitation pulse and 180\(^\circ\) pulse train: \( 90^\circ(y) – [\tau – 180^\circ(x) – \tau]_n – AQ(y) \). Subsequent studies indicated that with a fast
pulsing rate during the 180° pulse train the homonuclear $J$ evolution can be avoided. This result is illustrated in Fig. 7.

Fig. 7. Expansion of the 500 MHz $^1$H NMR spectrum of 20 mg sarin in 1 ml CDCl$_3$ (on top), and the corresponding CPMG spectrum (on bottom) with delays $\tau$ of 100 $\mu$s and $2n\tau$ of 71 ms. The spectra were recorded with Bruker DRX 500 with 5 mm $z$ gradient BBI probe at 300 K, with one scan without dummy scans. The spectra were weighted with an exponential window function with line broadening of 0.1 Hz.

According to the homonuclear two-spin study of Wells and Gutowsky [92], the signal intensity after the CPMG train is

$$S(2n\tau) = \cos \left( \frac{2\pi}{2n\tau} \right) \left( 1 - \frac{\sin \left( \frac{2\pi}{2n\tau} \sqrt{J^2 + \Delta \nu^2} \right)}{\frac{2\pi}{2n\tau} \sqrt{J^2 + \Delta \nu^2}} \right) \exp \left( -\frac{2\pi}{T_2} \right)$$

$$J/\sqrt{J^2 + \Delta \nu^2} \ll 1,$$

or

$$2\pi\tau \sqrt{J^2 + \Delta \nu^2} \ll 1$$

Eq. 14

where $n$ is the number of consecutive spin-echo cycles, $J$ is the scalar coupling constant between the spins, $\Delta \nu$ is the shift difference between coupled nuclei, and $\tau$ is the delay on either side of the 180° pulses. The signal not only decays by the $T_2$ relaxation, but it is
modulated by the cosine term of Eq. 14. If the $T_2$ relaxation is omitted, the modulation frequency of the signal intensity can be expressed in simplified form with Eq. 15:

$$
\nu_{mod} = J(1 - \sin \phi(x))
$$

$$
x = 2\pi \tau \sqrt{J^2 + \Delta \nu^2}
$$

Eq. 15

When $x$ is very large, $\nu_{mod}$ equals $J$. This corresponds to the normal spin-echo situation for a homonuclear spin system, where the 180° pulses applied during the CPMG train will refocus the chemical shift evolution and the $J$ coupling will evolve intact. When $x$ approaches zero, however, $\nu_{mod}$ also tends to zero, which means that the $J$ evolution is suppressed during the CPMG train. This condition can be achieved for homonuclear spin systems with use of a sufficiently short $\tau$ value. The $J$ coupling suppression is feasible if the required $\tau$ values lie within a feasible time-scale from the point of view of hardware. In the case of protons, $J$ and $\Delta \nu$ are usually small enough for the $J$ coupling suppression.

![Fig. 8. 2D $J$-resolved experiment with CPMG-type pulse train in $t_1$ period. H and G represent the channels for proton RF pulses and gradient pulses, respectively. Narrow and wide bars represent the 90° and 180° RF pulses, respectively; the pulse phase is $x$ if not stated otherwise. The test was performed using the XY-16 phase cycle [60], which, unlike the normal CPMG, conserves the magnetisation on all Cartesian axes (see Fig. 9). The pulse phases of the XY-16 super cycle are $[x, y, x, y, x, y, x, -x, -y, -y, -y, -y, -x, -y, -x, -x, -x]$. The $t_1$ period increment was conducted by increasing the $n$. The $N$ is set to be the total number of points in $t_1$ dimension. The length of a single XY-16 defines the dwell time of $t_1$ and, consequently, the spectral width of the indirect $J$ evolution dimension. A number of $N-n$ cycles are added at the beginning of the sequence to keep the heat delivery to the sample constant during the increment of $t_1$. The spoiler gradient pulse $sg$ (36.6 G/cm) is executed before the 90° pulse in order to defocus any transverse magnetisation produced by the compensating XY-16 cycles.](image)
Fig. 9. Contour plots demonstrating the efficiency of CPMG-4 (\(\tau - 180^\circ\)) and XY-16 as a function of the \(B_1\) inhomogeneity ratio \(B_1/B_0\) and the pulse offset \(\Omega/\gamma B_1\). The \(\tau\) was set for 10 ms. On the left are the contour plots of CPMG-4 for 360° rotation of transverse magnetisation either parallel or perpendicular to the pulse phase, and for 360° rotation of longitudinal magnetisation. On the right are the corresponding XY-16 efficiency plots. The contour curves are drawn for efficiency levels of 98, 90, 80, 60, 40, 20 and 0%.

The effect of the \(\tau\) delay on the evolution of \(J\) couplings can be demonstrated by carrying out a \(J\)-resolved experiment employing a CPMG-type sequence (Fig. 8) with various \(\tau\) values, as suggested by Ernst and co-authors [98]. The CPMG pulsing was modified to employ an XY-16 phase cycle [60], which has significantly better tolerance on \(B_1\) inhomogeneity and resonance offset. The conventional CPMG sequence rapidly dephases
magnetisation not aligned with the pulse phase (Fig. 9), and any anti-phase term that might evolve due to $J$ evolution would not be seen.

The results of the $J$-resolved experiments show that the $J$ evolution is indeed suppressed with small $\tau$ values (Fig. 10). Moreover, the theoretical results obtained with Eq. 15 are in good agreement with the detected $J$ evolution (Fig. 11). If the $J$ evolution is to be suppressed during the CPMG pulse train, the following condition must hold:

$$\tau < \frac{1}{2\sqrt{J^2 + \Delta \nu^2}} \Leftrightarrow \nu_{\text{mod}} < J$$

Eq. 16

For a weakly coupled spin system the approximated condition $\tau < 1/(2\Delta \nu_{\text{max}})$, where $\Delta \nu_{\text{max}}$ is the largest shift difference of the coupled spins encountered, is sufficient. The lower limit of $\tau$ is defined by the duty cycle of the spectrometer.

![Fig. 10. Methyl resonance expansions from 2D $J$-resolved CPMG spectra recorded with Bruker DRX 500 with 5 mm $z$ gradient BBI probe at 300 K from 20% ethanol in CDCl$_3$ ($J_{\text{HH}} = 7.2$ Hz): $\tau = 100$ $\mu$s (A) and $\tau = 850$ $\mu$s (B). On top of the spectra are $F_2$ projections of the corresponding spectral region. The spectra were measured with 16 scans per increment and a total of 64 increments. The number of dummy scans was 64. The spectra were processed in magnitude mode, and weighted with a squared sine bell window function in both dimensions. No spectrum tilt was conducted along the $F_1$ dimension. Note that after the measured multiplet splitting in $F_1$ dimension has reached the maximum $J$ value due to the increment of $\tau$, the $\nu_{\text{mod}}$ stays constant. This is contrary to the theoretical $\nu_{\text{mod}}$, which oscillates with decreasing amplitude around the maximum $J$ (Fig. 11). The failure of Eq. 14 is due to exceeding of the condition of validity. The theory is sufficiently accurate within the $J$ suppression region defined by Eq. 16, however.](image-url)
Fig. 11. Measured multiplet line separations ($\nu_{mod}$) in $F_1$ dimension for 20% ethanol in CDCl$_3$ ($J_{HH} = 7.2$ Hz) from 2D $J$-resolved CPMG experiments, and the theoretical values calculated with Eq. 15 as a function of delay $\tau$. The $\tau$ values covered the range from 50 $\mu$s to 1 ms in 50 $\mu$s steps. Multiplet line separations in $F_1$ dimension were measured for both resonances, and average $\nu_{mod}$ values were plotted against corresponding $\tau$. Identical experiments were conducted at two different $B_0$ field strengths at 300 K on Bruker DPX 200 with 5 mm $z$ gradient BBI probe and on Bruker DRX 500 with 5 mm $z$ gradient BBI probe. The $^1$H frequencies were 200.13 MHz and 500.13 MHz, respectively, giving shift separation $\Delta \nu$ of 488 Hz and 1220 Hz.

When considering CPMG-type sequences in terms of the ease of $J$ suppression, low $B_0$ field strength can be considered beneficial. Lower $\Delta \nu$ enables efficient suppression of $J$ evolution without the need for very short $\tau$ values, leading to a reduced duty cycle.

2.2.2 Homonuclear Hartmann–Hahn transfer

The $J$ coupling evolution suppression presented in the preceding section is not a result of a suppression of interactions between the nuclei in question. In fact, the reason is another interaction that takes place. A fast RF pulsing rate during CPMG sequences leads to an isotropic mixing condition [6,99-101], bringing about homonuclear Hartmann–Hahn transfer of magnetisation through $J$ couplings. According to Braunschweiler and Ernst [99], Hartmann–Hahn polarisation transfer between protons of an AX spin system ($A = ^1$H, $X = $ remote $^1$H, $J_{HH'} \neq 0$; hereafter we denote this spin system simply by HH') can be described with Eq. 17:
where $\overline{H}_{J}$ is the isotropic mixing Hamiltonian and $\tau_{m}$ is the mixing time. The transfer of magnetisation to various terms with respect to mixing time according to Eq. 17 is illustrated in Fig. 12.

**Eq. 17**

$$H_{x} \xrightarrow{\overline{H}_{J} \tau_{m}} \frac{\gamma}{2} H_{x} \left[ 1 + \cos(2\pi J_{H'H'} \tau_{m}) \right] + \frac{\gamma}{2} H_{x}' \left[ 1 - \cos(2\pi J_{H'H'} \tau_{m}) \right] + \left( H_{x}' H_{z}' - H_{z} H_{x}' \right) \sin(2\pi J_{H'H'} \tau_{m})$$

The transfer due to isotropic mixing occurs at twice the rate of COSY-type transfer (see Eq. 4). Inspection of the results of Eq. 17 from the perspective of both spins gives us the following result:

**Eq. 18**

$$H_{x} + H_{x}' \xrightarrow{\overline{H}_{J} \tau_{m}} \frac{\gamma}{2} H_{x} \left[ 1 + \cos(2\pi J_{H'H'} \tau_{m}) \right] + \frac{\gamma}{2} H_{x} \left[ 1 - \cos(2\pi J_{H'H'} \tau_{m}) \right] + \left( H_{x}' H_{z}' - H_{z} H_{x}' \right) \sin(2\pi J_{H'H'} \tau_{m})$$

The in-phase magnetisation for the H and H’ spins is constant due to the interchange of polarisation between the nuclei while the anti-phase terms cancel out. Eq. 18 describes the Hartmann–Hahn transfer for homonuclear spin systems during CPMG sequences with...
a fast pulsing rate, and explains the seemingly homonuclear $J$ evolution suppression described in Eq. 15. When the pulsing rate is not fast, the Hartmann–Hahn transfer ceases, and $J$ couplings evolve according to the normal spin-echo condition. The results discussed here open the way to the study on spin systems below, where some of the nuclei have a shift difference that does not fulfill the condition described in Eq. 16.

### 2.2.3 CPMG sequences on a heteronuclear spin system

Here we extend the examination of coherence flow during CPMG sequences to the HH'C spin system ($J_{CH}, J_{HH'} \neq 0, J_{CH'} = 0$). The experimental set-up for the heteronuclear spin system is achieved by introducing the CPMG pulsing on both nuclei, as illustrated in Fig. 13.

![Fig. 13. Principle of a CPMG sandwich for a heteronuclear HC spin system. The CPMG pulsing on both RF channels was modified with the XY-16 phase cycle [60] to preserve the magnetisation on all Cartesian axes (see Fig. 9). Wide bars represent 180° RF pulses. The pulse phases of the XY-16 super cycle are $[x, y, x, y, x, y, x, y, x, y, x, y, x, y]$. The total duration of the sequence is $t = 16*2n\tau$.](image-url)

When $\tau$ is set according to Eq. 16 for H, H’ spins, normal homonuclear $J$ evolution is suppressed. We take our starting point from Eq. 17, which describes the Hartmann–Hahn transfer of magnetisation between protons H and H’. The heteronuclear Hartmann–Hahn transfer [7,43,102,013] between proton H and carbon C does not occur unless the Hartmann–Hahn condition for heteronuclear transfer, $\gamma_H B_{HH} = \gamma_C B_{HC}$, happens to hold. For CPMG experiments on heteronuclear spin systems the fulfilment of the $\gamma_H B_{HH} = \gamma_C B_{HC}$ condition should preferably be avoided. Furthermore, the condition for $J$ evolution suppression (see Eq. 16) for H, C spins does not hold, and the $J_{CH}$ evolution between H, C spins is active. A rigorous analysis of the spin dynamics would require the use of density matrices, but, with good approximation, the magnetisation evolves as follows:
The terms involving transverse H magnetisation produce heteronuclear anti-phase magnetisation with respect to $J_{CH}$, with the consequence that the $J_{CH}$ evolution causes oscillating exchange of polarisation betw een proton H and carbon C, while the homonuclear coupling evolution between H, H’ spins evolves according to isotropic mixing condition. This kind of spin dynamics can be exploited for example, for heteronuclear polarisation transfer with significantly less homonuclear coupling contribution. If the polarisation transfer period $\Delta$ in INEPT (Fig. 1) is replaced with the pulse scheme depicted in Fig. 13, the resulting CPMG-INEPT [65] facilitates heteronuclear polarisation transfer in a normal manner. The isotropic mixing condition on protons emphasises the in-phase $^1\text{H}-^1\text{H}$ transfer; and consequently the anti-phase proton terms play only a minor role. Some examples of the benefits of this approach are demonstrated below.

\begin{equation}
\begin{aligned}
H_z \xrightarrow{\pi/2_t} & \quad \pi/2_t H_{t, C} \quad \xrightarrow{\pi} \quad \frac{1}{2} H_z \left[1 + \cos(2\pi J_{HH^\prime} t)\right] \cos(\pi J_{CH} t) \\
& + \frac{1}{2} H_z C_z \left[1 + \cos(2\pi J_{HH^\prime} t)\right] \sin(\pi J_{CH} t) \\
& + \frac{1}{2} H'_{z^\prime} \left[1 - \cos(2\pi J_{HH^\prime} t)\right] + H_z H'_{z^\prime} \sin(2\pi J_{HH^\prime} t) \cos(\pi J_{CH} t) \\
& - H_z H'_{z^\prime} C_z \sin(2\pi J_{HH^\prime} t) \sin(\pi J_{CH} t) - H_z H'_{z^\prime} \sin(2\pi J_{HH^\prime} t)
\end{aligned}
\end{equation}

Eq. 19
In the following sections, some of the practical applications of the analysis of coherence flow and CPMG pulse trains in polarisation transfer steps are discussed. The XY-16 phase cycle is assumed for CPMG pulsing if not explicitly stated otherwise. Section 3.1 reviews some problems associated with the use of LR-HSQC for heteronuclear coupling evaluation, and demonstrates how these problems can be avoided with implementation of a CPMG-INEPT step. The analysis of parent signal suppression efficiency in a GBIRD (Gradient-BIRD) isotope-editing filter is described in Section 3.2, and the improved method, CAGEBIRD (CPMG And Gradient-Enhanced BIRD), is presented. Section 3.3 deals with the application of 2D NMR methods for quantification.

### 3.1 2D heteronuclear long-range correlation spectroscopy

Two-dimensional correlation spectroscopy gives valuable insight into the connectivities between atoms in a molecule [104]. The first applications to determine $^1$H-$^{13}$C long-range connections in small molecules, like COLOC (CORrelation Spectroscopy via LOng-range Coupling) [105], were based on the detection of a heteronucleus and suffered from poor sensitivity. Later on, inverse detection methods, like the HMBC (Heteronuclear Multiple Bond Correlation) experiment [106,107], were employed to overcome this problem. The wide variation in long-range couplings that makes it difficult to optimise polarisation transfer steps has been addressed with use of the Accordion principle [108-110]. The $J_{CH}$ correlation interference has been attenuated through improvements in low-pass filters [111,112]. As well, a number of methods have been presented where the $J_{CH}$ cross peaks have been suppressed with a differential principle using selective inversion for one-bond correlations [113-115].

Many of the 2D methods express the long-range couplings qualitatively, but the actual magnitude of the coupling that provides important information about bond angles is difficult to extract from the spectra. As methods generally rely on multiple-quantum polarisation transfer, the $J_{HH}$ evolution during the pulse sequence produces highly distorted cross peak shape. Thus, the spectra are generally processed in magnitude mode without further attempt to analyse the couplings. To some degree, however, the
magnitude of the heteronuclear coupling can be estimated even from the cross peak volumes [116].

Several attempts have been made to evaluate the long-range coupling constants from the $F_2$ projection of cross peaks with lineshape fitting by using a reference spectrum that has the same level of $J_{HH}$ distortion [117-119]. Other studies have been reported where heteronuclear coupling splitting has been projected onto the $F_1$ dimension of either homonuclear correlation spectra [120-121] or heteronuclear spectra [122,123].

Recently, the HSQC sequence has been applied for the evaluation of heteronuclear long-range coupling constants [124,125]. Unlike normal HSQC, the sequence of LR-HSQC (Fig. 14) has a low-pass filter at the beginning of the sequence to suppress the $^1J_{CH}$ cross peaks. The reverse-INEPT period is omitted to minimise relaxation losses. As well, composite pulse decoupling is omitted to preserve the observed heteronuclear anti-phase magnetisation.

![Fig. 14. Simplified presentation of the LR-HSQC pulse sequence.](image)

In contrast to the traditional HMBC sequence, the long-range versions of HSQC have only a little peak phase distortions in cross peaks caused by $J_{HH}$ evolution. Thus, a normal $^1H$ spectrum has been considered to be sufficient to construct the reference spectrum for evaluation of the coupling constants from the $F_2$ trace of cross peak by lineshape fitting [126]. The splitting due to $J_{HH}$ is considered to be in-phase, while the $^2-^3J_{CH}$ evolution produces anti-phase splitting in the cross peak (Fig. 15). In the ideal case, the heteronuclear coupling constant can be evaluated from the $F_2$ trace of a cross peak simply by superimposing the trace on the sum spectrum of two $^1H$ NMR spectra that have a 180° phase difference and that are shifted with respect to each others by the amount of the
$^2-3J_{CH}$ coupling, and by minimising the difference between the $F_2$ trace and the reference spectrum through changes to the trial $^2-3J_{CH}$ coupling magnitude.

However, $J_{HH}$ evolution during the INEPT period jeopardises the polarisation transfer efficiency in LR-HSQC [II]. This can be demonstrated by considering the HH'C spin system evolution ($J_{HH}$, $J_{CH} \neq 0$ and $J_{CH'} = 0$). Product operator calculations with POMA [127] show that the magnetisation evolves during LR-HSQC as follows:

$$
\begin{align*}
H_z \xrightarrow{\text{INEPT}} & -H_z C_y \cos(\pi J_{HH} \Delta) \sin(\pi J_{CH} \Delta) \\
+ H_z H' x C_y \sin(\pi J_{HH} \Delta) \sin(\pi J_{CH} \Delta) & \xrightarrow{t_1\text{-period}} 90^\circ(H_z C_y) \\
- H_z C_z \cos(\pi J_{HH} \Delta) \sin(\pi J_{CH} \Delta) \cos(\Omega C t_1)
\end{align*}
$$

Eq. 20

where $\Delta$ is the duration of the INEPT period. The trigonometric term $\cos(\pi J_{HH} \Delta)$ arises from the $J_{HH}$ coupling evolution during INEPT. If $J_{HH}$ approaches $J^*$, where $J^* = (2\Delta)^{-1}$, the magnetisation will diminish close to zero as the polarisation is transferred to the term $H_z H' x C_y$, and thus the intensity of the corresponding long-range correlation peak will be significantly reduced. Further, additional protons coupled to the observed proton with $J_{HH}$ similar to $J^*$ may result in the development of more complex magnetisation, which could become observable and contribute to the correlation peak shape. The evolution of a
HH’H”C spin system \( (J_{HH'}, J_{HH''}, J_{CH} \neq 0 \text{ and } J_{CH}, J_{CH'} = 0) \), is a good example to demonstrate the source of lineshape distortion. The magnetisation for the HH’H”C spin system after the INEPT step is described in Eq. 21:

\[
\begin{align*}
H_z & \xrightarrow{\text{INEPT}} \\
- H_z C_y \cos(pJ_{HH}-\Delta)\cos(pJ_{HH''}-\Delta)&\sin(pJ_{CH}+\Delta) \\
+ H_y H'' C_x &\sin(pJ_{HH'}-\Delta)\cos(pJ_{CH}+\Delta) \\
+ H_y H'' C_x &\sin(pJ_{HH'}-\Delta)\sin(pJ_{CH}+\Delta) \\
+ H_y H'' C_x &\sin(pJ_{HH'}-\Delta)\sin(pJ_{CH}+\Delta)
\end{align*}
\]

Eq. 21

The term \( H_z C_y \) is the desired coherence. The evolution of this term after INEPT is shown in Eq. 22:

\[
\begin{align*}
H_z C_y & \cos(pJ_{HH}-\Delta)\cos(pJ_{HH''}-\Delta)\sin(pJ_{CH}+\Delta) \\
& \xrightarrow{t \text{-period}} 90(H_y,C_y) \\
H_z C_y & \cos(pJ_{HH}-\Delta)\cos(pJ_{HH''}-\Delta)\sin(pJ_{CH}+\Delta)\cos(\Omega_{c}t_1)
\end{align*}
\]

Eq. 22

The term \( H_z C_y \) is responsible for the long-range cross peak in the spectrum. If we look back at Eq. 21, we can see that the term \( H_y H', H'' C_y \) is of considerable intensity after the INEPT step. The evolution of this term during the period \( t_1 \) is presented in Eq. 23:

\[
\begin{align*}
H_z & H', H'' C_y \sin(pJ_{HH}-\Delta)\sin(pJ_{HH''}-\Delta)\sin(pJ_{CH}+\Delta)\xrightarrow{t \text{-period}} \\
&\left[ - H_z H', H'' C_y \cos(pJ_{HH}-t_1)\cos(pJ_{HH''}-t_1) \right] \\
&- H_y H', H'' C_y \sin(pJ_{HH}-t_1)\cos(pJ_{HH''}-t_1) \\
&- H_y H', H'' C_y \cos(pJ_{HH}-t_1)\sin(pJ_{HH''}-t_1) \\
&- H_y H', H'' C_y \sin(pJ_{HH}-t_1)\sin(pJ_{HH''}-t_1) \\
&\times \sin(pJ_{HH}-\Delta)\sin(pJ_{HH''}-\Delta)\sin(pJ_{CH}+\Delta)\cos(\Omega_{c}t_1)
\end{align*}
\]

Eq. 23

The 90° pulse pair will convert the term \( H_y H', H'' C_y \) into observable magnetisation:

\[
\begin{align*}
- H_z H', H'' C_y & \sin(pJ_{HH}-\Delta)\sin(pJ_{HH''}-\Delta)\sin(pJ_{CH}+\Delta) \\
& \times \sin(pJ_{HH}-t_1)\sin(pJ_{HH''}-t_1)\cos(\Omega_{c}t_1) \\
& \xrightarrow{90(H_y,C_y)} \\
H_y H', H'' C_y & \sin(pJ_{HH}-\Delta)\sin(pJ_{HH''}-\Delta)\sin(pJ_{CH}+\Delta) \\
& \times \sin(pJ_{HH}-t_1)\sin(pJ_{HH''}-t_1)\cos(\Omega_{c}t_1)
\end{align*}
\]
The final, observable magnetisation after LR-HSQC arising from the HH'H''C spin system is presented in Eq. 25:

\[-H_y C_z \sin(\pi J_{CH} \Delta) \cos(\pi J_{HH'} \Delta) \cos(\pi J_{HH''} \Delta) \cos(\Omega_c t_1)\]

\[+ H_y H'_z H''_z C_z \sin(\pi J_{CH} \Delta) \sin(\pi J_{HH'} \Delta) \sin(\pi J_{HH''} \Delta) \sin(\pi J_{HH'} \Delta) \sin(\Omega_c t_1) \]

\[\times \sin(\pi J_{HH'} t_1) \sin(\pi J_{HH'} t_1) \cos(\Omega_c t_1)\]

Eq. 25

When \(J_{HH'} \approx J_{HH''} \approx J\), the term \(H_y H'_z H''_z C_z\), which appears as an anti-phase multiplet with respect to both carbon and proton couplings, may contribute significantly to the cross peak shape in the spectrum, while the desired magnetisation described by the term \(H_y C_z\) will approach zero. These effects may severely compromise the spectral quality and result in a spectrum with distorted lineshape.

The lineshape distortion can be alleviated by replacement of the INEPT period with CPMG-INEPT to form the LR-CAHSQC experiment (Long-range CPMG-adjusted HSQC) [II]. During the CPMG-INEPT step, as discussed in Section 2.2, the isotropic mixing in the \(^1\text{H}-^1\text{H}\) network mainly results in in-phase transfer, and the residual anti-phase transfer should not interfere with the cross peak lineshape. The magnetisation evolution of the HH'C spin system during LR-CAHSQC is presented below.

The polarisation transfer step CPMG-INEPT leads to the isotropic mixing condition for protons, and the polarisation transfers between protons accordingly:

\[-H_y \overset{\pi J_{HH} \Delta}{\longrightarrow} \]

\[-\frac{1}{2} H_y \left[1 + \cos(2\pi J_{HH} \Delta)\right] \]

\[-\frac{1}{2} H' y \left[1 - \cos(2\pi J_{HH'} \Delta)\right] \]

\[+(H_y H'_z - H'_z H'_y) \sin(2\pi J_{HH'} \Delta)\]

Eq. 26

The \(J\) evolution suppression condition for proton–carbon coupling does not hold, since \(\Delta \nu\) is normally tens or even hundreds of MHz. Thus, the polarisation is transferred from proton to carbon through \(J_{CH}\) coupling evolution. Since the carbon C has \(J\) coupling only to proton H, the terms in Eq. 26 involving transverse H' spin magnetisation are omitted:
The 90° pulse pair transfers carbon magnetisation terms before the $t_1$ evolution period:

$$
-\frac{1}{2} H_y \left[ 1 + \cos \left( 2\pi J_{HH^1} \Delta \right) \right] + H_z H_y^* \sin \left( 2\pi J_{HH^1} \Delta \right)
$$

$$
-\frac{1}{2} H_y \left[ 1 + \cos \left( 2\pi J_{HH^1} \Delta \right) \right] \cos \left( \pi J_{CH} \Delta \right)
$$

$$
+ \frac{1}{2} H_z C_z \left[ 1 + \cos \left( 2\pi J_{HH^1} \Delta \right) \right] \sin \left( \pi J_{CH} \Delta \right)
$$

$$
+ H_z H_y^* \sin \left( 2\pi J_{HH^1} \Delta \right) \cos \left( \pi J_{CH} \Delta \right)
$$

$$
+ H_z H_y^* C_z \sin \left( 2\pi J_{HH^1} \Delta \right) \sin \left( \pi J_{CH} \Delta \right)
$$

**Eq. 27**

During the following $t_1$ period and the second 90° pulse pair, the $H_y C_y$ term evolves to produce the observable magnetisation for LR-CAHSQC:

$$
+ \frac{1}{2} H_y C_y \left[ 1 + \cos \left( 2\pi J_{HH^1} \Delta \right) \right] \sin \left( \pi J_{CH} \Delta \right)
$$

$$
+ \frac{1}{2} H_z C_z \left[ 1 + \cos \left( 2\pi J_{HH^1} \Delta \right) \right] \sin \left( \pi J_{CH} \Delta \right)
$$

**Eq. 28**

The transfer efficiencies of CPMG-INEPT (Eq. 28) and INEPT (Eq. 20) for the HH'C spin system are illustrated in Fig. 16. The most distinct difference between the methods is the transfer to undesired term $H_y H_y^* C_y$. The evolution rate is twice as fast for CPMG-INEPT as for INEPT, and the term $H_y H_y^* C_y$ is close to zero for CPMG-INEPT when the $J_{HH^1}/\Delta^p$ ratio is close to 1. The same term is at its maximum for INEPT. This finding is also applicable to spin systems with a larger number of protons. The possibility that a more complex anti-phase proton-proton term evolves to observable magnetisation later on during the sequence and contributes to lineshape is much smaller for CPMG-INEPT than INEPT. As well, the possibility of self-cancellation due to the anti-phase contribution is less likely (Fig. 17).

**Eq. 29**
Fig. 16. Plot showing the proton magnetisation transfer efficiencies of the HH’C spin system to terms \( H_xC_y \) and \( H_yH’xC_y \) for CPMG-INEPT and INEPT as a function of the \( J_{HH’}/J^0 \) ratio. \( J_{HH’} \) is the homonuclear coupling constant, and \( J^0 = (2\Delta)^{1/4} \), where \( \Delta \) is the polarisation transfer delay. In both cases, \( H_xC_y \) is zero when the \( J_{HH’}/J^0 \) ratio equals 1. For INEPT, the sign of the \( H_xC_y \) term is dependent on the ratio, but for CPMG-INEPT it is always positive. The term \( H_yH’xC_y \) for INEPT reaches a maximum when the ratio \( J_{HH’}/J^0 \) equals 1, the same term for CPMG-INEPT, however, reaches zero.

The efficiency of transfer \( Hz \rightarrow H_xC_y \) should approach zero for both CPMG-INEPT and INEPT when \( J_{HH’} = J^0 \) (Fig. 16). However, the sign of the term in INEPT depends on the \( J_{HH’}/J^0 \) ratio and changes when the ratio passes value 1. For CPMG-INEPT the sign of the term remains the same regardless of \( J_{HH’}/J^0 \). The sign of the term correlates with the phase of the cross peak in 2D spectra. For LR-HSQC the phase will be shifted by 180° if the \( J_{HH’}/J^0 \) ratio is larger than 1. For LR-CAHSQC, the phase of the cross peak should be the same regardless of the \( J_{HH’}/J^0 \) ratio (Fig. 17).
Fig. 17. Comparison of selected expansions of long-range correlated spectra of 34 mg strychnine nitrate in 1.3 ml D$_2$O recorded with LR-CAHSQC (A) and LR-HSQC (B) when $J^0$ was 10 Hz. Notice the missing cross peaks $^3J(H^{23a/b},C^{21})$ and very weak $^2J(H^{23a/b},C^{22})$ in the LR-HSQC spectrum due to the self-cancellation. Cross peaks of $^2J(H^{20a},C^{21})$, $^3J(H^{20a},C^{22})$ have 180° phase difference between spectra (A) and (B) because the geminal coupling on H$^{20a}$ is larger than 10 Hz. The spectra were recorded with Bruker DRX 500 with 5 mm z gradient BBI probe at 300 K, and measured with 16 scans per increment and a total of 400 increments. The number of dummy scans was 64. The spectra were processed in phase sensitive mode using gradient-based echo-antiecho method for quadrature detection, and weighted with a squared cosine bell window function in both dimensions. The spectra were phased according to $^3J(H^{16},C^{21})$, where H$^{16}$ has only small homonuclear couplings, to give the same phase of cross peaks for the two spectra.

The evaluation of heteronuclear long-range coupling constants with lineshape fitting from LR-CAHSQC is straightforward due to the improved cross peak lineshape characteristics, and the results are therefore more accurate. This is illustrated in Fig. 18.
Fig. 18. Comparison of heteronuclear long-range coupling constant evaluation with lineshape fitting from $F_2$ traces. (A) $F_2$ traces of $^3J(H^{III},C^1)$ (3.2 Hz) cross peak and (B) $^3J(H^4,C^6)$ (3.5 Hz) cross peak of sucrose. The corresponding protons have one homonuclear coupling ($^3J(H^{III},H^{IV}) = 7.8$ Hz) and two homonuclear couplings ($^3J(H^4,H^{3,5}) \approx 9.4$ Hz). The traces are from LR-HSQC (broken line), LR-CAHSQC (dotted line) and reference spectra (solid line). The reference spectra were constructed from a normal $^1$H NMR spectrum by subtracting the spectrum from itself after shift of the number of hertz corresponding to the heteronuclear coupling constant. The long-range experiments were optimised for long-range couplings of 10 Hz. The sample was prepared by dissolving 68 mg sucrose in 0.5 ml $D_2O$. The spectra were recorded with Bruker DRX 500 with 5 mm $z$ gradient BBI probe at 300 K. Acquisition and processing of the spectra are described in detail elsewhere [II].

In summary, LR-CAHSQC offers improved $J_{CH}$ coupling evaluation from cross peak shape through better lineshape characteristics [II].
3.2 Isotope editing in multi-pulse experiments

With the application of inverse detection in multidimensional NMR, the need has arisen for selective filtering of proton magnetisation involving a specific isotope. Owing to the carbon isotope distribution, only 1.1% of the proton magnetisation in $^1$H-$^{13}$C shift-correlated experiments contributes to the required coherence. The rejection of undesired magnetisation yields considerable benefits in dynamic resolution and $T_1$ noise level suppression [128,129]. In long-range correlation experiments, on the other hand, the cross peaks of proton directly bonded to $^{13}$C nucleus is considered as artefacts. In response to this, several “low-pass filters” have been proposed to eliminate this proton magnetisation [111,112].

The filter sequence GBIRD, reported by Emetarom and co-authors [128], ingeniously combines the BIRD sandwich [130], which delivers 180° phase difference to protons depending on the isotope of the involved carbon nucleus, with a gradient based selection (Fig. 19). The GBIRD sequence was originally applied for isotope editing, i.e., for selection of $^{13}$C-bound proton magnetisation, but it is also an equally valid method for isotope filtering, i.e., for rejection of $^{13}$C-bound proton magnetisation [115]. This section describes the isotope editing with GBIRD and proposes some modifications that result in better suppression of the parent signal ($^{12}$C-bound protons) and increases the available $^{13}$C-bound proton magnetisation after filtering.

![Fig. 19. Pulse sequence for a single GBIRD filter. H, C and G represent the channels for proton RF pulses, carbon-13 RF pulses and gradient pulses. The narrow and wide bars represent 90° and 180° RF pulses, respectively. The pulse phase is $\pi$ if not stated otherwise. The polarisation transfer delay $\Delta$ is set to $1/(2J)$, where $J$ is the average $^{1}J_{CH}$ coupling constant. The acquisition (AQ) is also with phase $\pi$ if not stated otherwise. Gradient pulses (G1) are shown with half-ellipses.](image)
The original GBIRD sequence has two drawbacks implicit in the properties of the BIRD sandwich. The first drawback is the inability of BIRD to refocus the evolution of the $^{1}J_{CH}$ coupling, which leads to distortions in the spectrum. According to POMA calculations [127], the magnetisation of the HC spin system is in the following state after single GBIRD:

\[-H_y \sin \left( \frac{\pi}{2} J_{CH} \Delta \right) \cos \left( \frac{\pi}{2} J_{CH} 2 \tau_{\text{grad}} \right) + H_y C_z \sin \left( \frac{\pi}{2} J_{CH} \Delta \right) \sin \left( \frac{\pi}{2} J_{CH} 2 \tau_{\text{grad}} \right)\]

\[\text{Eq. 30}\]

where $\Delta$ is the duration of BIRD delay, and $\tau_{\text{grad}}$ is the duration of the gradient pulse $G1$ (Fig. 19). The result of Eq. 30 indicates that the evolution of the heteronuclear coupling is active during gradient pulse periods, causing a mixture of in-phase proton magnetisation and anti-phase proton magnetisation with respect to carbon coupling. This leads to a problem, when single GBIRD is applied to a pulse sequence to deliver isotope editing, because NMR experiments normally start with in-phase magnetisation. The amount of magnetisation usable in the subsequent stages of a pulse sequence is

\[I_{\text{SingleGBIRD}} \propto \cos \left( \frac{\pi}{2} J_{CH} 2 \tau_{\text{grad}} \right)\]

\[\text{Eq. 31}\]

This loss of magnetisation is shown as a reduction of S/N in the spectrum. A second drawback of the GBIRD sequence is that the evolution of homonuclear coupling can introduce a significant parent signal leak to the spectrum. For a HH' spin system, the magnetisation is in the following state after single GBIRD:

\[-\frac{1}{2} H_z \left[ \cos \left( \frac{\pi}{2} J_{HH'} 2 \tau_{\text{grad}} \right) \right] - \cos \left( \frac{\pi}{2} J_{HH'} \Delta \right) \cos \left( \frac{\pi}{2} J_{HH'} 2 \tau_{\text{grad}} \right)\]

\[\frac{1}{2} H_z H'_{z} \left[ \sin \left( \frac{\pi}{2} J_{HH'} 2 \tau_{\text{grad}} \right) \right] - \cos \left( \frac{\pi}{2} J_{HH'} \Delta \right) \sin \left( \frac{\pi}{2} J_{HH'} 2 \tau_{\text{grad}} \right)\]

\[+ \frac{1}{2} H'_{y} \left[ \sin \left( \frac{\pi}{2} J_{HH'} \Delta \right) \sin \left( \frac{\pi}{2} J_{HH'} 2 \tau_{\text{grad}} \right) \right] \sin \left( \omega_H - \omega_{H'} \right) \tau_{\text{grad}}\]

\[+ \frac{1}{2} H'_{x} \left[ \sin \left( \frac{\pi}{2} J_{HH'} \Delta \right) \cos \left( \frac{\pi}{2} J_{HH'} 2 \tau_{\text{grad}} \right) \right] \sin \left( \omega_H - \omega_{H'} \right) \tau_{\text{grad}}\]

\[-\frac{1}{2} H_x H'_{y} \left[ \sin \left( \frac{\pi}{2} J_{HH'} \Delta \right) \cos \left( \frac{\pi}{2} J_{HH'} 2 \tau_{\text{grad}} \right) \right] \sin \left( \omega_H - \omega_{H'} \right) \tau_{\text{grad}}\]

\[\text{Eq. 32}\]

where $\omega_H$, $\omega_{H'}$ are the Larmor frequencies of the nuclei. When $\tau_{\text{grad}}$ is very small, Eq. 32 can be approximated to

\[-\frac{1}{2} H_z \left[ 1 - \cos \left( \frac{\pi}{2} J_{HH'} \Delta \right) \right]\]

\[+ \frac{1}{2} H_z H'_{z} \left[ \sin \left( \frac{\pi}{2} J_{HH'} \Delta \right) \right]\]

\[\text{Eq. 33}\]
The COSY-type transfer term $H_J H'$ during BIRD delay $\Delta$ is a source of considerable parent signal leak, thus compromising the performance of isotope editing with single GBIRD. For example, a HH' spin system with $J_{HH}$ of 7 Hz transfers 7.6% of the magnetisation to the anti-phase relay term $H_J H'$, if BIRD delay is optimised for the 145 Hz $J_{CH}$ couplings. When the desired $^{13}$C-bound proton magnetisation has an intensity of 1.1% of the total magnetisation, the parent signal is fairly prominent in the spectrum (Fig. 20).

In the improved method, designated CAGEBIRD [III], a CPMG sequence with XY-16 phase cycle (Fig. 13) is incorporated to the BIRD polarisation transfer delay $\Delta$ (Fig. 19). Because of a fast pulsing rate, the polarisation between protons is transferred according to isotropic mixing condition as follows.

For the HH' spin system, the magnetisation is in the state described in Eq. 34 before the CPMG pulsing. Chemical shift and homonuclear coupling evolution are omitted during $\tau_{\text{grad}}$ for the sake of simplicity:

$$
\begin{align*}
H_z & \xrightarrow{90^\circ H_x} H_J H' \Delta H_x \tau_{\text{grad}} H_z \\
& \xrightarrow{-H_z \cos(\gamma H \Delta B_z (r) \tau_{\text{grad}}) + H_x \sin(\gamma H \Delta B_z (r) \tau_{\text{grad}})} \\
& \xrightarrow{90^\circ H_x} \\
\end{align*}
$$

Eq. 34
The term $\gamma_B \Delta B_\Delta (r)$ in Eq. 34 presents the spatial dependence of the $B_0$ field strength during the gradient pulse. For brevity, the symbol $G$ is used in place of $\gamma_B \Delta B_\Delta (r) \tau\text{grad}$. The Hartmann–Hahn transfer during CPMG pulsing results in the following:

$$\Delta \text{term} = \frac{G}{B_0} \sin(G)$$

Eq. 35

Delay $\Delta$ is the duration of the BIRD delay modified with CPMG sequences [III]. As discussed in Section 2.2.2, the interchange of polarisation through the Hartmann–Hahn transfer between coupled spins leads to a seemingly stable state (Eq. 18). When we take both $H$ and $H'$ spins into account, the result at the end of $\Delta$ period is

$$\Delta \text{term} = \frac{G}{B_0} \sin(G)$$

Eq. 36

As can be seen, the anti-phase terms cancel out and only the in-phase terms remain. After we apply the remaining 90° RF pulse, which phase must be shifted by 180° [III], and then the gradient pulse, we get the final state of the HH’ spin system magnetisation for CAGEBIRD, as follows:
The trigonometric terms involving gradient pulse effects can be simplified through integration. The $z$ gradient pulse $G$ produces spatial dephasing of transverse magnetisation over all possible angles in the $xy$ plane. The integration of Eq. 37 from 0 to $2\pi$ with respect to $G$ gives the very simple result

$$\int_0^{2\pi} \left[ -(H_y + H_x') \cos(G) + (H_x + H_x') \sin(G) \right] dG = 0$$

Eq. 38

This means that, in theory, for the HH' spin system, the parent signal can be completely removed. In practice, some leakage is always present due to the coherence evolution during gradient pulses, incomplete $J$ evolution suppression during CPMG pulsing, additional coupled protons, RF and gradient pulse imperfections and so on. The level of suppression is still superior to that of the single GBIRD filter, however (Fig. 21).

The incorporation of CPMG pulsing to $\Delta$ period offers yet another advantage. According to POMA simulations [127] for the HC spin system, the evolution of the $^1J_{CH}$ coupling can be refocused during CPMG pulsing due to the even number of 180° pulses, and the heteronuclear anti-phase term evolving during gradient pulse periods is eliminated:

$$H_z \xrightarrow{CAGEBIRD} H_y \sin^2\left(\frac{\pi}{2} J_{CH} \Delta\right)$$

Eq. 39

As a consequence, single CAGEBIRD produces pure in-phase magnetisation for the HC spin system, as illustrated in Fig. 21. This gives a clear advantage over the GBIRD filter, as the $^{13}$C-bound proton magnetisation is attenuated only by the polarisation transfer period mismatch and relaxation during the filter.
Fig. 21. 1D single CAGEBIRD spectrum of 63 mg 1,1-dichloroethane in 0.5 ml CDCl₃. Notice the pure phase of ¹³C satellite signals and low parent signal intensity compared to 1D single GBIRD spectrum (Fig. 20). The spectrum was recorded with Bruker DRX 500 with 5 mm z gradient BBI probe at 300 K. Acquisition and processing of the spectrum are described in detail elsewhere [III].

The modified GBIRD filter CAGEBIRD offers substantial benefits over its predecessor. These improved isotope-editing properties can be readily utilised in multidimensional NMR to produce higher S/N and lower $t_1$ noise level. The results for isotope-edited HSQC experiments are presented elsewhere [III].

3.3 Quantification with 2D NMR

The basic use of NMR spectroscopy in structural analysis is determination of the number of protons in a molecule. Similarly, the number of carbons can be conveniently determined by $¹³$C{$¹$H} NMR spectroscopy, where inverse-gated proton decoupling [131] is employed to avoid the uneven NOE contribution to the signal intensity [132]. Analytical chemists have applied these methods to quantify both relative and actual amounts of compounds in mixtures [133]. Quantification with $¹$H NMR is straightforward from the point of view of the experimental set-up. With a 90° excitation pulse, the repetition rate of the scans must be equal to or more than five times the longest $T_1$ of protons of the sample in order to ensure sufficient relaxation of the magnetisation before the next scan [133]. A standard procedure for quantification with inverse-gated $¹³$C{$¹$H} NMR spectroscopy is to use a shorter excitation pulse angle (30-45°) pulse so that adequate relaxation can be achieved in a shorter time period than five times the longest $T_1$ of carbon-13. This approach provides no significant advantage in total acquisition time for a required S/N, as discussed by Traficante [134]. However, since the excitation range of the RF pulse is the reciprocal of its length, the use of short pulses is valuable for nuclei with a wide spectral range [135].

Inverse-gated $¹³$C{$¹$H} NMR lacks sensitivity due to the absence of NOE. As a means of gaining more sensitivity, polarisation transfer experiments such as DEPT and INEPT have been adapted for quantification [136-138]. Moreover, since these methods detect proton polarisation transferred to carbon, the repetition rate is dictated by the $T_1$ relaxation of protons, not carbons. Thus the acquired signal-to-noise ratio per time unit is superior to that in inverse-gated $¹³$C{$¹$H} NMR. The drawback of all polarisation transfer methods is that quaternary carbons are not visible in the spectrum. With solids, the
application of cross-polarisation transfer methods with magic-angle spinning has demonstrated reasonable accuracy for quantitative analysis [139].

The overlapping of signals often hampers reliable quantification with 1D techniques. This is critical in solid-state NMR, where resonances are broad due to the fast spin-spin relaxation. In liquids the resonances are narrow due to the isotropic medium, but the complexity of the sample may still result in insufficient resolution of signals. Some experimental set-ups are reported to diminish the instrumental contribution of $^{13}$C{^1H} NMR linewidths below 0.003 Hz [140], but questions have risen as to whether these claims can truly be delivered in high $B_0$ fields [141]. Several processing methods for the analysis of crowded spectra, such as deconvolution [142,143] and singular value decomposition [144], in some degree help in resolving overlapped resonances, but their reliability is compromised in highly crowded spectra.

Multidimensional NMR methods offer superior resolution to 1D methods, and are a promising alternative for the quantification of complex mixtures. Quantification by 2D NMR is not straightforward, however, as the factors that contribute to the volume of a correlation peak are numerous. The key issue in the use of 2D methods in quantification is the uniform transfer of polarisation between spins, regardless of chemical shifts, relaxation rates, magnitude of the heteronuclear coupling constants, multiplicity or additional couplings to remote spins (e.g., homonuclear couplings). Recently, some studies have addressed these problems in the application of heteronuclear correlated 2D NMR methods for quantitative analysis [I,IV]. The following two sections present the background and some aspects of these methods. Section 3.3.1 focuses on the analysis of concentrated samples by a 2D INEPT-based method, where carbon-13 detection yields good results within reasonable recording time. Section 3.3.2 describes the analysis of dilute samples by the HSQC method, which is based on inverse detection. The problems arising from the wide carbon-13 spectral width are discussed.

### 3.3.1 2D INEPT-based method

The oil industry relies on traditional chemical quantities for characterising the product properties of base oil fractions, e.g., pour point [145], and viscosity index [146], the latter describing the temperature dependency of viscosity [147]. Although the control of oil refinery processes has evolved through time and a multitude of process parameters are now adjusted to achieve oil fractions with the best possible product properties, engineers are still unclear as to why a particular oil fraction has good properties while another does not. Help from analytical chemistry has been required to obtain fuller knowledge of the compounds in oil fractions and to establish the relationship between compound structures and product properties.

NMR has been used in studying oil products [148], to characterise the average structural parameters of conventional oil fractions such as normal paraffin, iso-paraffin, naphthene, olefinic and aromatic ratios [149]. Present environmental regulations encourage refineries to favour non-aromatic and non-olefinic base oil fractions, and the dominant factor to the product properties is now the type and the amount of branches in the aliphatic oil fraction components. This should be a straightforward task to determine
by NMR analysis, but the usefulness of 1D techniques in this matter is, however, compromised due to heavy overlapping of resonances. While the range of interest in the $^{13}$C spectrum of the conventional oil fraction was 200 ppm, the resonances of presently favoured oil fractions fall within a range of 50 ppm. The reduction of spectral width has been even more dramatic in $^1$H NMR; the spectral width of proton resonances has dropped from 10 ppm to 1 ppm.

Oil fraction samples are normally highly concentrated, so that sensitivity is not an issue. Thus, the carbon-detected 2D methods, which offer better resolution in the $F_2$ dimension than do proton-detected methods, provide a convenient tool for the quantification. Given the narrow spectral widths in proton and carbon dimensions, the off-resonance effects can be neglected even at higher $B_0$ field strengths. The prerequisite for quantitative work is to ensure a sufficient relaxation of magnetisation between acquisitions. While the observed nucleus is carbon-13, the transferred polarisation is from protons, and the repetition rate can be set according to $T_1$ of protons. The mismatch between $J_0$ and $J_{CH}$ leads to an uneven polarisation transfer efficiency between spins. The main influences on the cross peak volume are multiplicity and the variation of the $J_{CH}$ couplings. The 2D INEPT experiment [1] has two parameters dependent on the aforementioned factors: the polarisation transfer delay $\Delta$ and the multiplicity-editing period $\delta$ (Fig. 22).

**Fig. 22.** Pulse sequence for 2D INEPT. H, C and G represent the channels for proton RF pulses, carbon-13 RF pulses and gradient pulses. The narrow and wide bars represent 90° and 180° RF pulses, respectively. The pulse phase is $x$ if not stated otherwise. The delays $\Delta$ and $\delta$ represent the polarisation transfer delay and multiplicity editing period, respectively. Composite pulse decoupling is used during acquisition. Spoiler gradients (G1, G2) are shown with half-ellipses.
The polarisation transfer delay $\Delta$ is set to $1/(2J^\circ)$ to give an optimum signal-to-noise ratio (Section 2.1.1). The multiplicity-editing period is normally set to $1/(3J^\circ)$ in order to obtain positive peak phase regardless of its multiplicity (Fig. 23).

Fig. 23. The dependence of multiplicity editing delay $\delta$ of 2D INEPT on $J_{CH}$ constant and the number of protons attached to carbon-13.

With use of the aforementioned constrain for delays $\Delta$ and $\delta$, calculations with POMA [127] gives the following intensity response over $J_{CH}/J^\circ$ variation for each CH$_n$ group:

$$CH = \sin\left(\frac{\pi}{2}, \frac{J_{CH}}{J^\circ}\right) \sin\left(\frac{\pi}{3}, \frac{J_{CH}}{J^\circ}\right)$$

$$CH_2 = \sin\left(\frac{\pi}{2}, \frac{J_{CH}}{J^\circ}\right) \sin\left(\frac{2\pi}{3}, \frac{J_{CH}}{J^\circ}\right)$$

$$CH_3 = \frac{3}{4} \sin\left(\frac{\pi}{2}, \frac{J_{CH}}{J^\circ}\right) \left[\sin\left(\frac{3\pi}{3}, \frac{J_{CH}}{J^\circ}\right) + \sin\left(\frac{\pi}{3}, \frac{J_{CH}}{J^\circ}\right)\right]$$

Eq. 40

The application of 2D INEPT requires prior knowledge of the $J_{CH}$ couplings of the sample. The $J_{CH}$ couplings for an oil fraction are very similar between samples, so that the coupling evaluation is needed only once. The correction coefficients for the cross peak volumes can be calculated with use of Eq. 40 and the relative amounts of
components can be obtained with good accuracy. The standard deviation remains well below 5%, if the ratio the components does not exceed 1:10 [1].

3.3.2 HSQC-based method

Biopolymers are of great current interest. Medicine has exploited NMR in the diagnosis of diseases from both tissue preparations and body fluids [150,151]. With the help of NMR, pharmaceutical chemists are exploring potential drug chemicals [152,153]. Some branches of hard industry have also started to pay interest to their raw material of biological origin. For example, the characterisation of wood lignin is of interest to the paper industry given the important place of lignin in chemical and mechanical pulping [154,155]. Biopolymers are complex in nature and thus challenging materials for qualitative and quantitative analysis. As well, the material available for study is typically scarce and becomes a limiting factor for NMR investigations. Methods like HSQC, which employ inverse detection and place fewer demands on sample size, are thus highly advantageous for 2D NMR studies.

An important prerequisite for quantitative work is sufficient relaxation of magnetisation between acquisitions. Five times the longest $T_1$ of protons leads to a state where at least 99% of the magnetisation has reached thermodynamic equilibrium. The multiplicity, i.e., the number of protons, has a clean impact on the volume of the cross peaks, which should be taken into account in quantification. Uneven polarisation transfer efficiency between spins occurs if there is a mismatch between $J^*$ and $J_{CH}$. It should be possible to correct polarisation transfer imperfections of HSQC in a similar manner as with 2D INEPT because the volume of the cross peak is dependent on the $J_{CH}/J^*$ ratio as described in Eq. 41:

$$V_c \propto \sin^2 \left( \frac{\pi \cdot J_{CH}}{2 \cdot J^*} \right)$$

**Eq. 41**

The requirement for prior knowledge of the $J_{CH}$ couplings is sometimes a very inconvenient one, in particular when the composition of samples in a sample series varies. Heikkinen and co-authors [156] have proposed an ingenious method, Q-HSQC, to avoid the problem of $J_{CH}$ variation: they use several polarisation transfer delay $\Delta$ values per transient, to obtain a sufficiently uniform transfer efficiency over the range of natural $^1J_{CH}$ couplings. In a discussion of their method, the authors noted, that the evolution of homonuclear coupling(s) during rather long constant-time INEPT periods can compromise the quantitivity with a factor

$$\prod_{i=1}^{n} \cos^2 \left( \pi J_{HHi} \Delta_{\text{max}} \right)$$

**Eq. 42**
where $\Delta_{\text{max}}$ is the duration of constant-time INEPT. This $J_{\text{HH}}$ evolution is readily visible from the twisted lineshape of the $F_2$ trace of cross peaks. The use of CPMG-INEPT instead of INEPT for the polarisation transfer steps in the modified Q-HSQC experiment, Q-CAHSQC [IV], significantly improves the lineshape of resonances bearing multiple $J_{\text{HH}}$ couplings, as shown in Fig. 24, yielding easier phasing and integration of spectra.

Fig. 24. $F_2$ traces from Q-HSQC and Q-CAHSQC spectra of 60 mg strychnine in 0.5 ml CDCl$_3$ at correlations H-11a,C-11 and H-11b,C-11 [IV]. The spectra were recorded with Bruker DRX 500 with 5 mm z gradient BBI probe at 300 K. Acquisition and processing of the spectra are described in detail elsewhere [IV].

The contribution of homonuclear couplings is in fact minor relative to the off-resonance contribution to the quantification. The volume of cross peaks that are on-resonance with respect to the carbon transmitter frequency is much greater than the volume of cross peaks that are off-resonance. In high $B_0$ fields, where the chemical shift range is wide, the poor off-resonance performance of RF pulses has a dramatic effect on cross peak volumes. The same has been observed in application of inverse-gated $^{13}$C{${^1}$H} NMR for quantitative studies [133]. Although high power 90° carbon pulses are generally considered to have adequate off-resonance performance (Fig. 5), their efficiency may be inadequate in quantitative work. The 180° pulses are even more vulnerable in this respect. In contrast, the off-resonance performance of the CPMG pulsing should not be a problem in CPMG-INEPT. Although the normal CPMG sequence with a single pulsing phase is of limited usefulness for compensating of off-resonance and $B_1$ inhomogeneity, the XY-16 phase cycle has very good characteristics both for inversion and for refocusing of magnetisation (Fig. 9). The actual source of offset dependency in quantitative HSQC experiments is 90° carbon pulses.

The volume profile of the Q-CAHSQC cross peak relative to carbon-13 offset resembles $\text{sinc}^2(3x)$-type tendency, where $x$ is the relative offset $\Omega\gamma B_1$ [IV]. Thus, accurate quantification with standard deviation well below 10% is achievable if the results are corrected with the relative offset-dependent coefficient. If the carbon chemical shift range is reasonable with respect to the $B_1$ field strength, the substitution of appropriate composite pulses for key carbon pulses can yield results with standard deviation as low as 5.0%.

When the offset compensation is carried out with composite pulses, care should be taken that the pulses truly effectively produce the needed transformation. For example,
90° composite pulses do not work as universal 90° pulses. Rather, their efficiency depends on whether they are applied on longitudinal or transverse magnetisation (Fig. 25). Combined theoretical and experimental verification of the performance gives the best assurance of the quantitativity of the chosen method.

Fig. 25. Contour plots demonstrating the efficiency of some composite excitation pulses as a function of $B_1$ inhomogeneity ratio $B_1/B_0$ and pulse offset $\omega/\gamma B_1$. On the left are contour plots of $360(\pm x) - 270(\pm x) - t$ ($t = (2\gamma B_1)^{-1}/\pi$) [157] composite pulse efficiencies for $M_z \rightarrow -M_y$ and $M_y \rightarrow M_z$ transfer. On the right are the contour plots of $385(\pm x) - 320(\pm x) - 25(\pm x)$ [88] composite pulse efficiencies for $M_y \rightarrow -M_x$ and $M_x \rightarrow M_y$ transfer. The contour curves are drawn for efficiency levels of 98, 90, 80, 60, 40, 20 and 0%.
4 Conclusions

This work has demonstrated the possibility of sufficiently accurate quantification of organic compounds by heteronuclear shift-correlated 2D NMR [I,IV]. Certain conditions must be met to ensure the accuracy of results: i) The main factor that compromises the accuracy of quantitative 2D spectrum is the off-resonance effects. These effects can be compensated either with a proper correction coefficient derived from off-resonance profiles or with the use of composite pulses exhibiting good off-resonance performance.

ii) The variation of natural $J_{CH}$ couplings decreases the efficiency of polarisation transfer steps. This can be taken into account with correction coefficients derived from product operator calculations. Also other means have been devised [156] to overcome this problem.

iii) The distortion of cross peaks caused by homonuclear evolution during pulse sequences hampers the phasing and integration of quantitative 2D spectra, and reliable results are difficult to obtain. Homonuclear coupling evolution is readily suppressed with the application of CPMG sequences on polarisation transfer steps.

iv) Concentration requirements are quite high for the 2D INEPT method, but the analysis of less concentrated samples is feasible with Q-CAHSQC. With very dilute samples, however, total acquisition time becomes unreasonable due to the requirements for slow repetition rates. Actual detection limits for quantitative HSQC methods have not been determined.

The use of CPMG sequences significantly improves the efficiency of the GBIRD isotope-editing filter [III]. The parent signal leakage is much lower with the new method, CAGEBIRD, and phase distortion of $^{13}$C-bound proton resonance is avoided. The benefits of applying CPMG-INEPT in LR-CAHSQC are also evident in the better lineshape properties observed in evaluation of the heteronuclear long-range coupling constants [II].

For the current work, the maximum duration of CPMG pulsing on high power was several hundreds of milliseconds with inter-pulse delay on the order of tens of microseconds. Although no hardware problems were encountered, a fast CPMG pulsing rate with long duration can strain the electronics of the spectrometer, and care should be taken that the safety limits are not exceeded. Since heat delivery to the sample is increased with the use of CPMG pulsing, a sufficient number of dummy scans is recommended before the acquisition to confirm thermally steady-state conditions.
The methods presented in this thesis were applied to small organic molecules, and polarisation transfer was employed between proton and carbon-13. Other heteronuclei than carbon-13 are also feasible for these methods, as demonstrated by Luy and Marino [68]. A potential application utilising phosphorus is the screening of chemical warfare agents in compliance with the Chemical Weapons Convention (CWC) [158]. Most of the agents are organophosphorus compounds, such as sarin (see Fig. 6). The verification of agents in environmental samples by $^1$H NMR tends to be hampered by the heavy background in the spectrum. This background is effectively suppressed by 1D $^1$H-$^{31}$P HSQC methods [159], which facilitate selective observation of proton resonances of organophosphorus chemicals. Structural analysis of the alkyl chain bound to phosphorus is of particular importance, as the alkyl chain largely determines the relevance of a chemical to CWC. Preliminary results have indicated that the application of CPMG-INEPT in 1D $^1$H-$^{31}$P HSQC methods for Proficiency Test screening [159] helps to minimise the homonuclear evolution distortions on resonances, thus facilitating the determination of alkyl sidechain structure from the resonance fine structure.

Another experiment that could benefit from the use of CPMG is the isotope-editing filter TANGO (Testing for Adjacent Nuclei with a Gyration Operator) [160], which in principle resembles the BIRD sandwich. The TANGO sequence effectively delivers a 90° pulse to $^{13}$C-bound protons, while $^{12}$C-bound protons are left on the $z$ axis. Preliminary simulations suggest that homonuclear coupling evolution during polarisation transfer period can introduce parent signal leakage. Substitution of CPMG pulsing for the polarisation transfer period as demonstrated with CAGEBIRD [III], may be suitable for homonuclear evolution suppression and thereby improve the parent signal suppression. The benefits of this approach have yet to be tested.
References


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146. ASTM International, Standard D2270-04. Standard Practice for Calculating Viscosity Index From Kinematic Viscosity at 40 and 100°C.