

# Improved 3D DOSY-TOCSY experiment for mixture analysis†

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**With respect to best currently available pulse sequences, a 10-fold reduction in minimum experiment time together with significant resolution enhancement can be achieved in 3D DOSY homonuclear experiments by means of Hadamard encoding, as illustrated here for the 3D DOSY-TOCSY experiment.**

Analysis of complex mixtures currently represents an active field of research. In this context, a commonly used analytical method is hyphenation, *i.e.* the sequential combination of chromatography with single or multiple spectroscopic techniques such as UV, mass, or NMR spectrometry.<sup>1</sup> Impressive applications of hyphenated and related techniques on a large variety of complex matrices have already been reported.<sup>2–4</sup> Alternatively, diffusion ordered NMR spectroscopy (DOSY) appears as an attractive technique for mixture analysis because it allows substances to be discriminated according to their molecular size<sup>5,6</sup> and, for isomers, according to their molecular shape.<sup>7</sup> Unlike hyphenated techniques such as, for instance, LC-NMR, which require specific instrumentation, DOSY can be easily implemented on routinely available NMR instruments, the only prerequisite being the availability of a gradient coil and amplifier, which both accompany nowadays most high resolution NMR spectrometers. Basically, DOSY requires <sup>1</sup>H spectra to be recorded at increasing diffusional attenuation, resulting for a given frequency in an exponential decay that must be inverted to yield its corresponding diffusion coefficient (*D*). A pseudo 2D spectrum can then be constructed, showing NMR chemical shifts on one dimension and diffusion coefficients on the other. The NMR spectra of the mixture components can thus be resolved, thereby easing the overall structural assignment without the need for any preliminary physical separation.

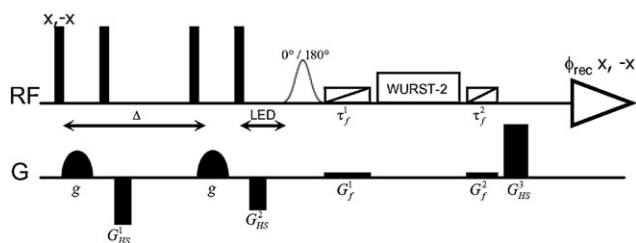
The data inversion step undeniably represents the key to DOSY. When the resonances are completely separated in the frequency dimension, high resolution in the diffusion dimension can be achieved with a simple two-parameter monoexponential fitting, an approach referred to as high resolution DOSY.<sup>8</sup> In contrast, when significant spectral overlap occurs, the methodology fails. In this case, unless the diffusion coefficients of the overlapping resonances differ significantly, incorrect *D* values will be obtained, especially in the presence of noise. Without *a priori* knowledge of the system, this data

processing step may end up in an intractable problem.<sup>5</sup> As emphasized by Nilsson and Morris,<sup>9</sup> two main strategies can hence be adopted to circumvent this difficulty. The first relies on sophisticated post-processing and data analysis schemes,<sup>5,10</sup> which may lead to significant resolution enhancement in the diffusion dimension.<sup>11,12</sup> The second consists of simplifying the data analysis by minimizing signal overlap. This is classically achieved through additional frequency dimensions by combining a diffusion encoding step with a 2D correlation building block, which can split a crowded spectral region into distinct sub regions on which exponential fitting procedures can be subsequently applied. This has resulted in numerous 3D DOSY pulse sequences.<sup>5,9,13–18</sup> Another promising technique was independently proposed by two research groups,<sup>19,20</sup> who used conceptually different methods to suppress multiplet fine structures to achieve simplified homodecoupled <sup>1</sup>H spectra. Although a significant loss in sensitivity must be deplored in both cases, exciting developments may already be anticipated.

Typically, bringing an additional frequency dimension to DOSY experiments leads to a considerable increase in experimental time, and hence a few methods were proposed to minimize this time penalty. Vitorge and Jeannerat suggested computer optimized spectral aliasing of the indirect dimension in <sup>1</sup>H–<sup>13</sup>C HSQC experiments in order to profit as much as possible from the gain in resolution afforded by <sup>13</sup>C spectra.<sup>21</sup> Morris and coworkers proposed a series of efficient pulse sequences in which the diffusion-encoding step is internally incorporated into the 2D correlation block, giving rise to the so-called<sup>9</sup> IDOSY pulse sequences family (HMQC-IDOSY,<sup>14</sup> 2J-IDOSY,<sup>9</sup> COSY-IDOSY,<sup>16</sup> DQFCOSY-IDOSY<sup>18</sup>). In this case, the time saving is mostly achieved through phase cycling reduction. Another method used for minimizing experimental time relies on Hadamard-encoded NMR spectroscopy, which has been extensively described by Freeman and coworkers,<sup>22</sup> and where the evolution time in the indirect dimension of the 2D block is replaced by phase-encoded multisite selective excitation, in order to focus the experimental time on the signal-containing spectral regions. Steinbeck and Chmelka demonstrated the use of *heteronuclear* Hadamard-encoded NMR diffusion measurements to allow overlapping signal decays to be resolved with substantially shorter measuring times.<sup>23</sup> Interestingly, however, no application to *homonuclear* 3D DOSY experiments has been reported so far, although the experimental time saving offered by Hadamard encoding in homonuclear 2D correlations has long been realized.<sup>22</sup>

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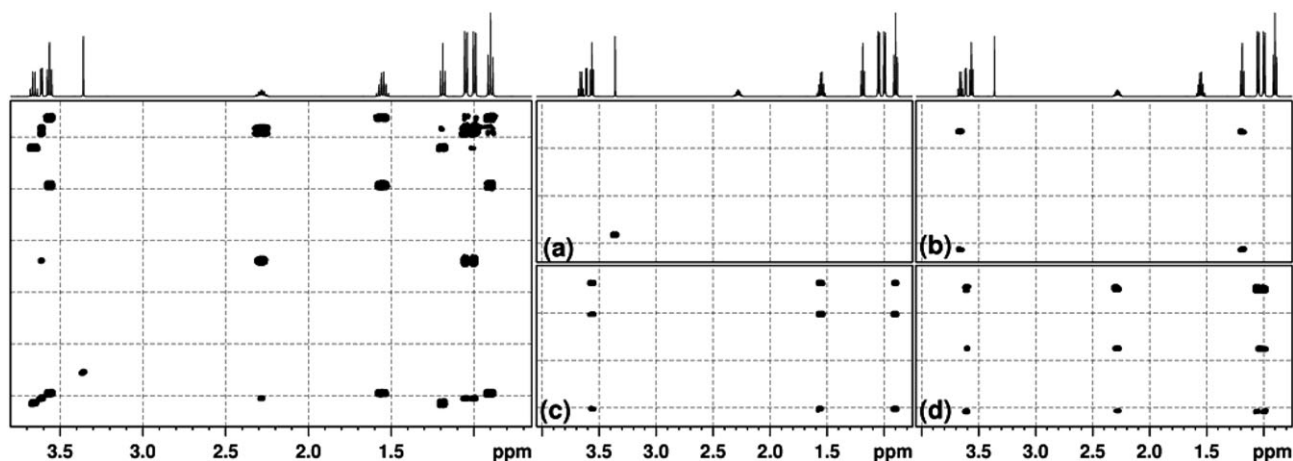


**Fig. 1** Pulse sequence of the 3D Hadamard-encoded DOSY-TOCSY experiment (unless specified, all pulses have phase  $x$ ).

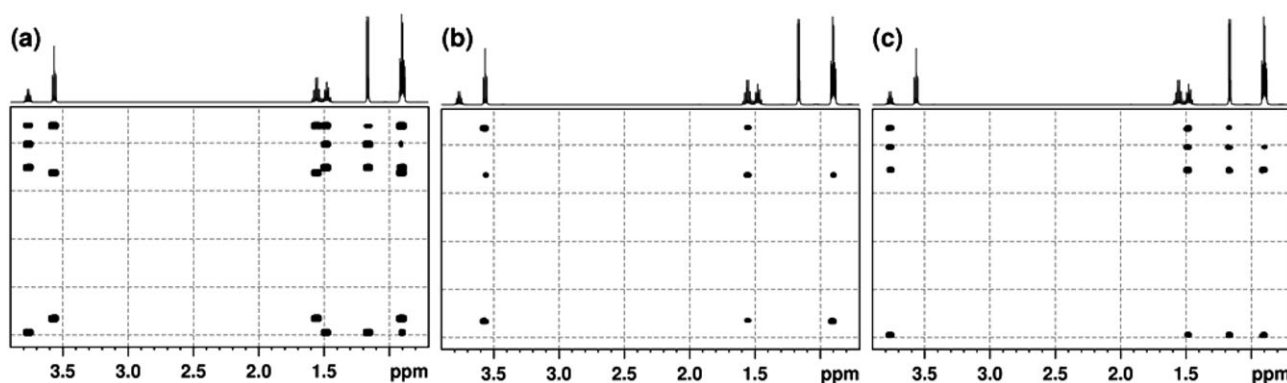
In this communication, we illustrate the potential of this methodology and propose a pulse sequence based on Hadamard encoding that yields, under favourable circumstances, the TOCSY spectra of each mixture component in a matter of minutes, thereby affording a large reduction in experimental time with respect to currently available experiments. Because TOCSY is traditionally regarded as a tool of choice for mixture analysis<sup>24</sup> and offers, with respect to COSY, enhanced spectral resolution due to in-phase off-diagonal cross-peaks, this preliminary report focuses on the DOSY-TOCSY experiment. A few groups have already combined diffusion encoding with TOCSY (DETOCSY, PFG-TOCSY, DO-TOCSY, and DECODES).<sup>5</sup> While 3D displays were not systematically synthesized, three of these implementations could (directly or indirectly) reveal the TOCSY spectrum of the individual mixture components. However, the considerable time requirements of the corresponding pulse sequences (several hours) make them totally unsuitable for routine analysis. Fig. 1 shows the 3D Hadamard-encoded DOSY-TOCSY pulse sequence<sup>‡</sup> proposed in this work, where the diffusion encoding period precedes the Hadamard-encoded TOCSY block in agreement with the nomenclature clarified by Nilsson *et al.*<sup>9</sup> This encoding is achieved by monopolar gradients, and the sequence incorporates a  $z$  filter delay (LED)<sup>5</sup> and a homospoil gradient, to allow eddy currents to dissipate and residual transverse magnetization to dephase, respectively. Next, the Gaussian polychromatic pulse is applied, according to the on/off

( $0^\circ/180^\circ$ ) excitation scheme advocated by Freeman and coworkers,<sup>22</sup> and this is followed by an 80 ms isotropic mixing period employing WURST-2 adiabatic pulses used for reducing RF field inhomogeneity artefacts.<sup>25</sup> In order to improve spectral quality and reduce phase cycling, single-scan ZQC filters were applied before and after the mixing period.<sup>26</sup> Finally, a strong spoiler gradient was applied prior to signal acquisition to remove any residual transverse magnetization. All NMR experiments were carried out at 300 K on a BRUKER AVANCE 500 MHz spectrometer fitted with a 5 mm triple broad band inverse  $^1\text{H}/^31\text{P}/\text{X}$  BRUKER probe equipped with a  $56 \text{ G cm}^{-1}$  gradient coil. A simple mixture was first adopted for this proof of principle (Fig. 2). The irradiation frequencies of the 90 ms phase-encoded Gaussian pulses used for Hadamard encoding were selected automatically by running a peak picking on a  $^1\text{H}$  spectrum processed with a 10-Hz line broadening. Ten irradiation frequencies were obtained, while a  $16 \times 16$  Hadamard matrix was chosen due to computer constraints. Fifteen gradient values from 9.0 to 33.9  $\text{G cm}^{-1}$  in equal steps of gradient squared were used. A simple two-step phase cycle was employed, giving a total experimental time of  $\sim 20$  min.<sup>†</sup>

In Fig. 2, the TOCSY spectrum of the mixture acquired with a conventional 2D TOCSY experiment based on the pulse sequence by Thrippleton and Keeler<sup>26</sup> is compared to the sub TOCSY spectra achieved with the pulse sequence in Fig. 1, showing that all expected correlations can be clearly detected. In most 3D DOSY experiments published so far, the minimum experimental time was in the range of several hours, especially when many gradient pulses were used. This is to be compared to the pulse sequence reported in Fig. 1, which gave satisfactory sub TOCSY spectra in a few minutes. Furthermore, to preliminarily evaluate the impact of signal overlapping on the outcome of the proposed 3D Hadamard-encoded DOSY-TOCSY experiment, a two-component mixture (propanol and 2-butanol), whose  $^1\text{H}$  spectrum shows complete overlapping of the terminal  $\text{CH}_3$  resonances (at 0.9 ppm), was analyzed. Fig. 3 compares the 2D Hadamard-encoded



**Fig. 2** [Left] TOCSY spectrum of a mixture of methanol, ethanol, propanol, and valine. [Right] Sub TOCSY spectra from the 3D Hadamard-encoded DOSY-TOCSY experiment on the same mixture at (a) 1.32, (b) 1.05, (c) 0.89, and (d)  $0.63 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ , corresponding to methanol, ethanol, propanol, and valine, respectively. These  $D$  values equalled within  $\pm 2\%$  those determined by conventional DOSY experiments. The 1D  $^1\text{H}$  spectrum is shown at the top.



**Fig. 3** (a) 2D Hadamard-encoded TOCSY spectrum of a mixture of propanol and 2-butanol. (b and c) Planes from the 3D Hadamard-encoded DOSY-TOCSY experiment recorded on the same mixture at (b) 9.5 and (c)  $8.1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ , corresponding to propanol and 2-butanol, respectively. These  $D$  values equalled within  $\pm 2\%$  those determined by conventional DOSY experiments. The 1D  $^1\text{H}$  spectrum is shown at the top.

TOCSY spectrum of this mixture to the TOCSY planes obtained for each substance with the pulse sequence of Fig. 1 (total experimental time  $\sim 13$  min). As can be seen, each diffusion-encoded TOCSY plane exhibits all the expected cross-peaks of the respective mixture component, showing that the cluster of peaks at 0.9 ppm has been totally resolved. Similarly to Nilsson *et al.*,<sup>16</sup> the only absent peak is the diagonal (0.9; 0.9) ppm peak, whose weighted-average diffusion coefficient falls outside the diffusion ranges of the mixture components.

Importantly, in Hadamard NMR spectroscopy, the spectral resolution in the indirect dimension is approximately inversely proportional to the length of the selective excitation or inversion pulse. Steinbeck and Chmelka estimated the time saving accessible by Hadamard encoding,<sup>23</sup> and showed that it is inversely proportional to the Hadamard matrix dimension, which relates to the number of irradiated sites. Clearly, as the number of excited frequencies increases, this time saving will be scaled down correspondingly. In addition, with respect to currently available experiments such as COSY-IDOSY and DQFCOSY-IDOSY, the 3D Hadamard-encoded DOSY-TOCSY experiment outlined in this work bears two sensitivity penalties because more RF pulses are used (including the long, selective ones) and the sequence is built on the stimulated echo, which is intrinsically less sensitive than the Hahn echo (although this may be somewhat compensated for by the predominance of  $T_1$  vs.  $T_2$  relaxation). However, it has the advantage of yielding in-phase cross-peaks. Data are hence processed in the phase sensitive mode, which represents a substantial resolution enhancement compared to the above mentioned sequences, which require magnitude mode processing to compensate for the phase evolution that occurs during the spin echo. Overall, our pulse sequence is mostly adapted to the study of small molecules, as the rather long excitation pulses used for Hadamard encoding make it probably unsuitable for the investigation of large molecular weight species. Finally, while this preliminary report strictly focuses on the 3D DOSY-TOCSY experiment, the outlined methodology could in principle be applied to other 3D homonuclear (NOESY, COSY) experiments. Investigations along these lines are currently in progress in order to analyse complex mixtures.

## Notes and references

‡ Note that, although some of the features in Fig. 1 can be found in the STEP-DOSY experiment by Bradley *et al.*,<sup>27</sup> these authors did not intend to generate diffusion-encoded TOCSY spectra.

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