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A homodecoupled diffusion experiment for the analysis of complex mixtures by NMR

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Abstract

Diffusion ordered spectroscopy (DOSY) relies on differences in translation diffusion as a means to separate components in a solution mixture. However, the analysis of spectra of mixtures can be problematic because spectral overlap. It is the aim of this article to propose a pulse sequence and processing method that leads to a complete 2D homodecoupled-DOSY experiment. This experiment offers several advantages that could extend the range of applications to more complex mixtures by achieving important improvements in both signal dispersion and sensitivity.

Keywords: NMR; Diffusion; DOSY; *J*-Resolved; Mixture analysis

1. Introduction

Diffusion ordered spectroscopy (DOSY) has introduced the translation diffusion of molecules into a bouquet of solution NMR experiments that provide innovative tools for the researcher [1]. When combined with appropriate data processing schemes, the technique becomes an exceptionally valuable tool for mixture analysis, the separation of which is based on the molecular size [2].

Every mixture analysis problem using pulsed gradient spin echo (PGSE) NMR involves differentiation of the resonances based upon their diffusion coefficient. This can be challenging in the analysis of complex mixtures with significant spectral overlap. To analyse overlapped components, different processing methods have been implemented such as the direct exponential curve resolution algorithm (DECRA) [3], component resolved (CORE) [4], multivariate curve resolution (MCR) [5], and maximum entropy [6]. In addition to these processing schemes, modified DOSY pulse sequences have been implemented to improve spectral selectivity.

Most of these enhanced pulse sequences include the bipolar-gradient pulse pairs and stimulated echo (BPP-STE) experiment [7] with or without an additional delay time for longitudinal eddy current compensation (LED) as the basic building block. One possibility that has found interest in drug screening [8] is to employ the difference in diffusion upon binding of a ligand to a receptor to distinguish between binding and non-binding ligands in a complex mixture [9–11]. The BPP-STE scheme can also be incorporated as a diffusion filter in standard liquid NMR sequence so as to obtain a diffusion weighted NMR experiment that simplifies the original spectrum by filtering out all the larger diffusing signals of the small components. An application that makes use of this idea is the diffusion filtered-NOESY experiment that allows the detection of inter-molecular NOEs between a small ligand and a large receptor alleviating cases of severe signal overlap [11]. Recently a gradient modified spin echo scheme

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(GOSE) in combination with the BPPSTE scheme, the 1D GOSE-DOSY [12], can effectively eliminates the signals arising from coupled spins in the homonuclear spectrum providing a much simplified spectrum in which only singlets are present making easier the quantification of the diffusion coefficients. Other possibilities combine the BPPSTE scheme with standard liquid sequences to add an extra dimension (diffusion dimension) that provides further editing of the signals alleviating the problem of signal congestion and its undesirable effects in the determination of diffusion coefficients (e.g., signal overlap leads to the formation of peaks that are between two components and actually represent the weighted average of the two resonances). Some of the sequences proposed are the 3D DOSY-TOCSY [13], 3D DOSY-COSY [14] or 3D DOSY-HMBC [15].

Following this latter strategy, a *J*-resolved experiment has recently been incorporated into the LED-BPPSTE scheme to obtain a 3D *J*-resolved-DOSY experiment that provides editing of the proton signals by means of the homonuclear scalar couplings spreading the spectroscopic information into the second (*J*) dimension to improve resolution [16]. In this experiment, the diffusion coefficients are estimated by means of the integration of peak volumes. It is the aim of this article to propose an alternative pulse sequence and processing method for this experiment that leads to a 2D homodecoupled-DOSY experiment. The proposed experiment offers several advantages that could extend the range of applications to more complex mixtures by achieving important improvements in both signal dispersion and sensitivity. In addition, the former 3D-*J*-resolved DOSY required volume integration of the 2D-*J*-resolved peaks, in which the inherent positive noise threshold can contribute to a systematic error in the measured diffusion coefficients, especially for the less intense signals. As will be seen this problem can be overcome by this new experiment in which the diffusion coefficients are determined by a simpler 1D peak area integration.

2. Results and discussion

The homodecoupled-DOSY pulse sequence proposed in Fig. 1 is a combination of the original LED-BPPSTE DOSY [7] and the two-dimensional spin echo *J*-resolved schemes. This sequence differs from the previously described *J*-resolved-DOSY experiment [16] in the order in which the spin-echo and DOSY elements have been incorporated in the sequence. For our purpose of obtaining the homodecoupled-DOSY spectrum, we placed the spin echo at the end of the sequence to preserve the sign information of the multiplet components, avoiding the possibility that they could be mixed by relaxation or other events in the sequence prior to the detection of the signal. Considering, for example, a doublet, after the DOSY part the *J*-resolved module generates a signal with two components f_+ and f_- which in the time domain are represented by Eqs. (1) and (2) [17]:

$$f_+(t_1,t_2) = (a/2)\exp(-t_1/T_2)\exp(\mathrm{i}\pi J t_1) \times \exp(-t_2/T_2^*)\exp[2\mathrm{i}\pi(v_a + J/2)t_2], \quad (1)$$

$$f_-(t_1,t_2) = (a/2)\exp(-t_1/T_2)\exp(-\mathrm{i}\pi J t_1) \times \exp(-t_2/T_2^*)\exp[2\mathrm{i}\pi(v_a - J/2)t_2], \quad (2)$$

where a is the signal amplitude that remains after the DOSY period, v_a is the frequency of resonance, and T_2 and T_2^* are the effective transverse relaxation times in t_1 and t_2 time domains, respectively. The two terms f_+ and f_- after Fourier transformation give rise to a doublet along the 45°-tilted *J*-resolved acquisition dimension.

From the different methods described in the literature to obtain a homodecoupled spectrum from *J*-resolved experiments ([17] and references therein [18]), a particularly simple and robust method is that described by Guenneau et al. [17]. In this method, the 2D *J*-resolved spectrum is processed with a Fourier transform along both dimensions, then the spectrum is tilted by 45° along the acquisition dimension, and displayed in power mode to remove the phase-twist problem. Finally, the projec-

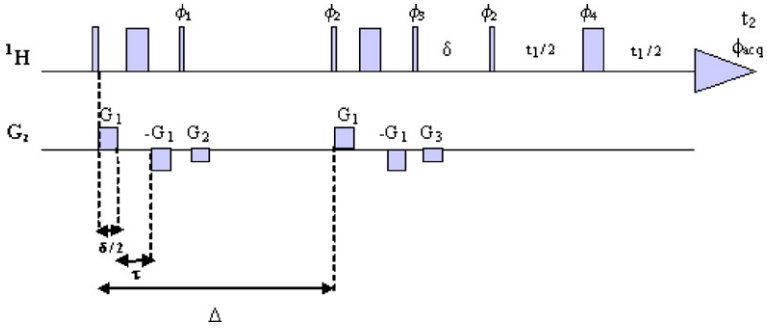

Fig. 1. Pulse scheme for the homodecoupled-DOSY experiment. Narrow and wide pulses correspond to 90° and 180° flip angles. Phase cycling is $\phi_1 = x, x, -x, -x$; $\phi_2 = (x)_4, (-x)_4$; $\phi_3 = \{x, -x, x, -x, -x, x, -x, x\}$; $\phi_4 = (x)_4, (-x)_4, (-x)_4, (x)_4, (y)_4, (-y)_4, (-y)_4, (y)_4$; $\phi_{acq} = (x, -x, -x, x, -x, x, x, -x)_4, (-x, x, x, -x, x, -x, -x, x)_4$. Phases if not indicated otherwise are on the x axis.

tion along the *J*-resolved dimension results in the 1D homodecoupled spectrum.

At low magnetic fields, one may consider the effect of proton–proton strong coupling which may cause the appearance of extra peaks in the 2D *J*-resolved spectrum [19] and consequently they will also result in extra peaks in the 1D homodecoupled spectrum projection. For each pair of strong coupled protons an extra singlet peak is obtained in the 1D homodecoupled spectrum at a resonance position located at the middle-point of the two protons involved [19]. Strong coupling effects are difficult to avoid with phase cycling or even with pulse field gradients. Recently, a new experiment has been presented at ENC [20] that suppress strong-coupling peaks. Nevertheless, although these extra peaks may cause confusion for the signal assignment, they do not cause much trouble with the diffusion analysis, since they will diffuse at the same rate as any other peak of the same molecule.

For the quantification of the diffusion with the experiment proposed in Fig. 1, it is worth mentioning that a single trace of the 1D homodecoupled spectrum has meaningless relative intensities, as opposed to the corresponding proton spectrum. However, it is still possible to obtain quantitative information when the method is applied to determine the change in intensity of any individual signal along a series of traces which are intensity modulated, as it has been originally described for T_1 relaxation measurements [17] and as we will shown for the proposed homodecoupled-DOSY experiment of Fig. 1.

Guenneau et al. [17] have described the analytical function for the lineshape in the homodecoupled power spectrum obtained (see Eq. [13] in [17]). Using that formalism, the relationship between the maximum of the peak in the homodecoupled power spectrum, $p^{\max}$, and the maximum intensity, $a^{\max}$, in the original (non-power mode) coupled modulated spectrum is given by Eq. (3)

$$p_i^{\max} = 2\left(\frac{a_i^{\max}}{2}\right)^2 \frac{T_2 \cdot T_2^*}{T_2 + T_2^*}, \tag{3}$$

where subscript i refers to the trace number in the modulated experiment.

From Eq. (3), the relationship for the intensity change of a signal in two traces i and j in the intensity modulated experiment can easily be deduced. The ratio of the intensities at the maximum in the (non-power) coupled spectrum, $a_i^{\max}$ and $a_j^{\max}$, can be obtained by simply taking the square root of the ratio of the intensities at the maximum in the homodecoupled power mode spectrum, $p_i^{\max}$ and $p_j^{\max}$ (Eq. (4)).

$$\frac{a_i}{a_j} = \sqrt{\frac{p_i^{\max}}{p_j^{\max}}}. \tag{4}$$

By using Eq. (4) it is possible to determine quantitative information in a homodecoupled power spectrum along

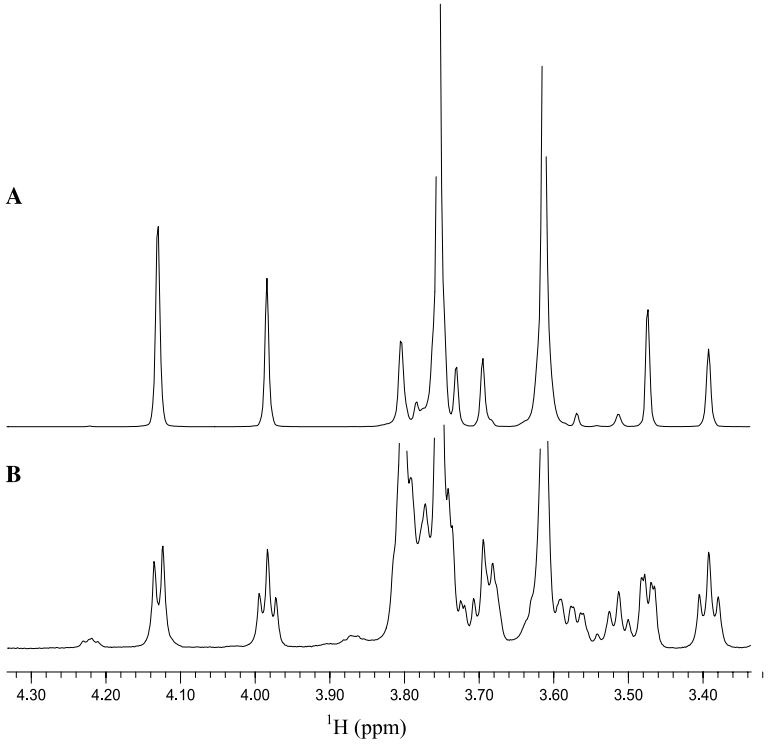

Fig. 2. Region of the spectrum of the mixture of compounds corresponding to the first 1D DOSY trace (acquired with $G_1 \sim 2$ G cm^{-}) at 35 °C in (A) homodecoupled-DOSY and (B) the standard DOSY experiment.

the extra intensity modulated dimension, as is the case with the diffusion dimension in the homodecoupled-DOSY experiment of Fig. 1.

We have experimentally tested the homodecoupled-DOSY experiment of Fig. 1 which is based in the LED-BPPSTE sequence to a sample consisting of a mixture of sucrose, β-cyclodextrin (β-CD), and 7,8-diol, 2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine (SKF) in D_2O. The diffusion coefficients obtained were compared with those derived from the standard LED-BPPSTE DOSY sequence [7] which was performed under virtually identical experimental conditions.

It can be seen in Fig. 2 that the homodecoupled-DOSY experiment clearly simplifies the analysis of the DOSY experiment since it all the coupled signals collapse into singlets with a significant reduction in the total signal width.

The determination of the diffusion coefficients in both DOSY experiments was done by performing a non-linear fit of the intensities to the well known Stejskal–Tanner equation. During this study all the analyzed signals in both experiments showed an excellent mono-exponential behaviour. An example of the curves obtained can be seen in Fig. 3. The mean diffusion coefficients

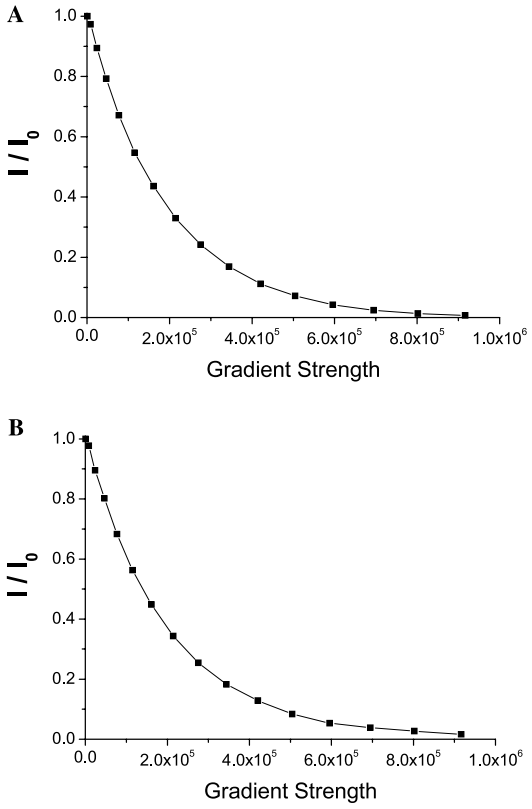

Fig. 3. Plot of the normalized signal intensity vs. the gradient strength corresponding to the signal H-3 Glc of sucrose in the mixture at 35 °C in experiments (A) 2D homodecoupled-DOSY ($D = 5.24 \times 10^{-6}$ cm^2 s^{-1}) and (B) standard DOSY experiment ($D = 4.99 \times 10^{-6}$ cm^2 s^{-1}).

Table 1
Mean diffusion results (D in cm^2 s^{-1}) obtained for different signals of the mixture of compounds obtained with homodecoupled-DOSY and DOSY experiments at 35 °C

	Homodecoupled-DOSY		DOSY	
	Average D	Standard error	Average D	Standard error
SKF	5.41E−6	± 7.2E−8	5.57E−6	± 7.7E−8
Sucrose	4.93E−6	± 12.1E−8	5.16E−6	± 7.4E−8
β-CD	3.94E−6	± 5.0E−8	3.88E−6	± 7.5 E−8

determined by the analysis of the different signals in the mixture for the two experiments are given in Table 1 and shows comparable diffusion coefficients.

3. Conclusions

The results shown in this paper validate the use of the homodecoupled-DOSY experiment for the determination of diffusion coefficients. The experiment proposed can be useful to avoid the spectral overlapping that complicates the determination of diffusion coefficients. Some of the advantages of this experiment are the simplicity in the processing scheme and the straightforward analysis required to determine the diffusion coefficients. In addition, this experiment is also an attractive alternative to other three-dimensional DOSY schemes described such as 3D DOSY-TOCSY [13], 3D DOSY-COSY [14] or 3D DOSY-HMQC [15], since the acquisition time for the homodecoupled-DOSY can be considerably shortened, because the reduced spectral width in its indirect J-resolved dimension can be recorded with a relative smaller number of transients.

4. Experimental

NMR experiments were acquired on a 750 MHz spectrometer Varian INOVA equipped with a triple gradient shielded probe and processed with MestReC software [21]. A mixture of three compounds was prepared by mixing 5 mg sucrose, 10 mg β-cyclodextrin (β-CD) and 10 mg of 7,8-diol, 2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine (SKF) in D_2O.

The homodecoupled DOSY experiment of Fig. 1 was acquired for the mixture of compounds at 35 °C. This 3D experiment was acquired as 16 independent 2D J-resolved experiments, each of them with a particular value of the DOSY encoding gradient G_1. The gradient G_1 was varied linearly along these 16 experiments from ~2 to 65 G cm^{-1} to obtain the diffusion dimension. Each J-resolved experiment was acquired with 16 scans and a total of 32 increments in the J-resolved dimension. The spectral width in the acquisition (proton) and J-resolved dimensions was 5600 and 40 Hz, respectively. The diffusion time Δ was set to 80 ms and the duration of all the

gradients in the sequence was set to 1 ms followed by a stabilization delay period of 0.2 ms. For the two purge gradients G_2 and G_3 a value of -8.5 and $-7.4\ \mathrm{G\ cm^{-1}}$ was used. The number of points in the acquisition dimension was 2900 and the total acquisition time for the whole experiment was about 6 h 30 min.

The processing of a 2D *J*-resolved experiment starts with the Fourier transformation in the acquisition (proton) and *J*-resolved dimensions with zero filling to 4096 and 64 data points without any type of digital filtering (e.g., apodization). The spectrum was then tilted by 45° along the acquisition dimension, and then a derivative function was applied along this dimension. The 2D spectrum obtained was then converted to the power mode from which the 1D homodecoupled spectrum was obtained from the projection obtained by summing all the rows along the *J*-resolved dimension. The 16 2D *J*-resolved experiments were processed in the same way and the sixteen 1D homodecoupled spectrum projections obtained were combined into a single 2D spectrum. This spectrum corresponds to the 2D homodecoupled DOSY in which the different rows in the diffusion dimension are ordered according to the strength of the gradient G_1 used (Fig. 1) and the horizontal dimension is the homodecoupled proton spectrum.

The determination of the diffusion coefficients in the 2D homodecoupled DOSY was done for each signal in the spectrum by measuring the raw intensity of the peak at its maximum (using parabolic interpolation) along the diffusion dimension. A square root of the values obtained gives the corresponding DOSY intensities (as showed in Eq. (4)) which were analyzed to determine the diffusion coefficients using the usual methodology [7].

For comparison purposes, a standard DOSY with the LED-BPPSTE sequence [7] was acquired for the same sample under virtually identical conditions to those used for the homodecoupled DOSY from which the diffusion coefficients were also determined.

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