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Improving Pulse Sequences for 3D Diffusion-Ordered NMR Spectroscopy: 2DJ-IDOSY

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An improved pulse sequence for the 3D DOSY experiment 2DJ-DOSY, using diffusion encoding internal to the parent 2DJ spectroscopy sequence (2DJ-IDOSY), is presented. The diffusion-encoding pulses are used to enforce the desired coherence transfer pathway, reducing the minimum experimental time by at least a factor of 4, as compared to existing techniques, and approximately doubling the signal-to-noise ratio for small molecules. The new sequence is demonstrated on a simple mixture and on a complex sample with a high dynamic range (port wine). The principle of internal diffusion encoding can be applied with profit to a range of other 3D DOSY experiments.

Diffusion-ordered spectroscopy (DOSY) is a powerful tool for the study of mixtures, involving the addition of an extra dimension-displaying signal as a function of diffusion coefficient to a conventional 1D or 2D NMR spectrum. The extra information is provided by adding diffusion encoding to the normal *n*D pulse sequence, typically by appending or prepending a pulsed field gradient stimulated echo sequence (although a different approach is advocated here). Diffusion coefficients are calculated by fitting the observed signal decay as a function of pulsed field gradient to the appropriate theoretical expression, and the diffusion dimension of the spectrum is then calculated by extending the *n*D spectrum into an $(n + 1)$th dimension in which the line shape is a Gaussian centered on the apparent diffusion coefficient and with a width determined by the standard error estimated in the fitting process. The extra spectral dimension that results may be regarded as a pseudoseparation by apparent molecular size (i.e., hydrodynamic radius). DOSY has been extensively reviewed elsewhere.[1−3]

DOSY suffers from a fundamental difficulty. Where signals from species of similar size overlap, it is not in general possible to determine the separate diffusion coefficients with high accuracy. Here, two approaches are valuable. The first is to use techniques such as further spectral dimensions or signal filtering to improve the separation of individual signals, the route used here; the second is to employ sophisticated data analysis methods to optimize the extraction of diffusion data. Such methods divide roughly into single channel[4−7] and multichannel[8−12] methods. The former focus on resolving the superimposed decays by some approximation to the inverse Laplace transform, a particularly intractable numerical problem requiring severe constraints to be placed on solutions and yielding low resolution in the diffusion dimension. In multichannel processing, the full band shape is decomposed into component spectra using assumptions about the number of distinct diffusion coefficients present, but it is not generally possible to resolve overlapping signals with similar diffusion coefficients.

In the absence of signal overlap, monoexponential fitting of the individual signal decays allows diffusion coefficient differences of the order of 1% to be resolved, an approach sometimes known as high-resolution DOSY (HR-DOSY).[5] Where signal overlap in a 1D spectrum is not too severe, fully resolved spectral signals can often be obtained by using familiar 2D techniques such as COSY or HMQC, leading to the 3D DOSY−COSY and DOSY−HMQC experiments.[13−21] These have mostly relied on the concatenation

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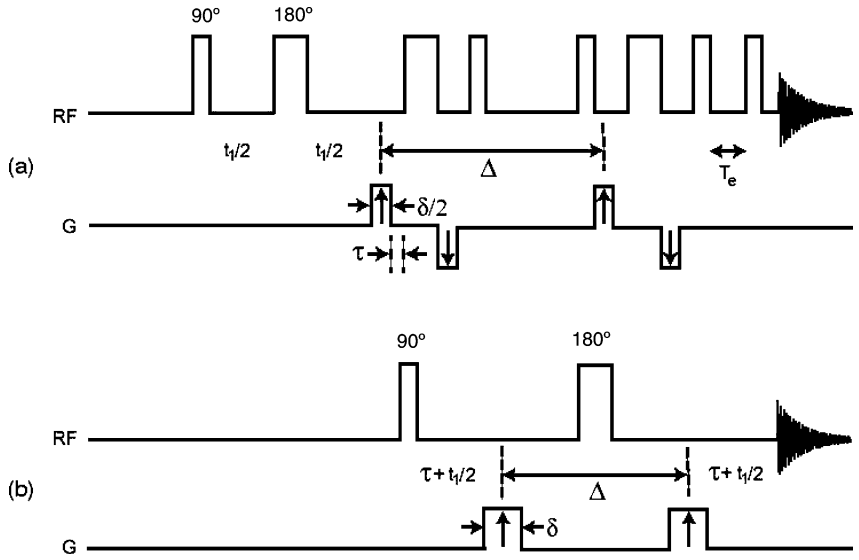

Figure 1. Pulse sequences for 3D DOSY derived from 2DJ spectroscopy. (a) The 2DJ-BPPSTE pulse sequence of Lucas et al.[17] and (b) the proposed 2DJ-IDOSY experiment. The pictures show the radio frequency (RF) and gradient (G) pulses, with vertical arrows indicating the gradient levels changed to vary the diffusion encoding; a delay, τ, allowed for gradient stabilization after each gradient pulse.

of existing pulse sequence elements, typically pre- or appending a pulsed field gradient stimulated echo to a familiar 2D sequence. This is often an effective solution, but it carries a significant penalty in signal-to-noise ratio. Where the timing of the sequence permits, it is sometimes possible to incorporate diffusion weighting of a spin or gradient echo within the parent pulse sequence. This improves sensitivity by avoiding the 50% signal loss associated with the stimulated echo and by reducing the sequence duration and number of pulses. The basic requirement for internal diffusion encoding is that the parent pulse sequence allow the inclusion of a diffusion delay, Δ, typically of the order of tens of milliseconds. Thus, in the case of TOCSY [14] or NOESY, the existing mixing period can also do duty as a diffusion delay. Long-range HMQC, as for example in the case of ^{1}H−^{29}Si HMQC of siloxanes,[18] allows the incorporation of two separate diffusion weighting segments, but unfortunately, the coherence transfer delays are too short in one-bond ^{13}C or ^{15}N HMQC. We also suggest here a minor change to current nomenclature, using DOSY-X to indicate a 3D experiment in which a diffusion-encoding step precedes a 2D pulse sequence for experiment X, X-DOSY where the diffusion-encoding follows the evolution period, and X-IDOSY where diffusion encoding is incorporated internally. Clearly, related distinctions can be drawn for experiments of higher dimensionality.

This paper illustrates the principle of internal diffusion encoding with an archetypically simple IDOSY experiment, 2DJ-IDOSY, which uses only two radio frequency pulses and is considerably faster and more sensitive than competitive techniques. At least one 3D DOSY experiment using internal diffusion encoding implicitly (a TOCSY−IDOSY) has been described previously.[14] Very recently, a convection-compensated HMQC−IDOSY experiment has also been described.[18] The most appropriate choice of 3D DOSY experiment depends on the nature of the mixture under study. DOSY−HMQC and related techniques offer the highest spectral resolution available, but they suffer from low sensitivity and are best suited to complex mixtures of high concentration.[13] Where signals from chemically disparate species overlap in the normal spectrum, cross-peaks in homonuclear correlation (e.g. COSY) spectra are more likely to be well-separated, making experiments such as DOSY−COSY attractive (COSY−IDOSY is even more so). Where multiplets from similar chemical species overlap, however, COSY is much less likely to provide resolved cross-peaks, because the chemical shifts of coupled partners in similar species are likely to be correlated. Here, an effective solution is to introduce a diffusion dimension into a 2DJ[17] spectrum, with the added advantage of a much smaller minimum experimental duration. Although historically important and still occasionally exhumed,[22] 2DJ spectroscopy[23] has been almost completely overshadowed by homonuclear and heteronuclear correlation methods, such as COSY, NOESY, HMQC, and HMBC, which have lower resolution at the multiplet level but much higher information contents overall. Where interpenetrating multiplets in small molecules are to be resolved, however, *J* spectroscopy remains a very useful tool. In favorable cases it offers nearly an order of magnitude improvement in the ability to resolve single peaks, despite the penalty incurred by the need for absolute value mode display; the utility of the basic experiment in the study of complex mixtures has been noted previously.[24]

A 2DJ-DOSY (in the nomenclature introduced above) experiment using the normal 2DJ sequence followed by a bipolar pulse pair stimulated echo (BPPSTE) sequence (Figure 1a) has been shown to separate the signals of a mixture of sugars very effectively.[17] The converse DOSY-2DJ experiment, in which a spin−echo evolution period is appended to a BPPSTE sequence, would also be possible; this would have the potential advantage of leaving longer for field perturbations to die down before the onset of data acquisition. Here, it is shown that similar results may be obtained much more economically by incorporating the diffusion weighting into the spin−echo, as in the 2DJ-IDOSY

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sequence of Figure 1b. The penalty for introducing the diffusion weighting into the parent sequence is that the minimum evolution time, t_1, is increased from near 0 to Δ, the diffusion time. For mixtures of small molecules, the principal likely application of 2DJ-IDOSY, this has little effect on the 2DJ spectrum: the extra delay is short compared to typical T_2 values, and the effects of F_1 phase modulation are masked by the absolute value mode display. The brevity and simplicity of the pulse sequence give several advantages over and above the factor of ~2 in sensitivity stemming from the elimination of the stimulated echo sequence. No phase cycling is needed for the diffusion encoding, while the diffusion-encoding pulses themselves serve to enforce the spin−echo coherence transfer pathway, meaning that the sequence as a whole can if appropriate be run in single transient mode, reducing to a matter of minutes the minimum time needed to acquire a 3D DOSY spectrum. If desired, the new pulse sequence can very simply be compensated for the effects of convection using the same logic as in HMQC−IDOSY,[18] but this would then require the usual phase cycling. Here, the speed and efficacy of the new 2DJ-IDOSY sequence are demonstrated in practical applications to a mixture of medium-chain alcohols and a complex liquid food sample.

EXPERIMENTAL SECTION

All spectra were recorded on a narrow-bore Varian Unity 500 MHz spectrometer fitted with a $^1H/^{13}C/^{15}N$ triple probe equipped with a 50 G/cm gradient coil. Three samples were used: the first consisted of sucrose (4.2% w/w), ethanol (0.8% w/w), and TSP (sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4) (1% w/w) in D_2O; the second was a 1% (v/v) solution in D_2O of an equimolar mixture of 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methyl-1-propanol, and 1-propanol; and the third was a port wine (type "Ruby", 2002 harvest) mixed with 10% D_2O. Sample 2 used 0.2%, and sample 3, 0.05% TSP as an internal chemical shift reference. Because the port wine sample contained high concentrations of both H_2O and ethanol, the water, methyl, and methylene proton signals were presaturated for all experiments on this sample. During the 3.3-s presaturation delay the water signal was irradiated at low level using the transmitter, and the ethanol methyl and methylene multiplets were irradiated using the decoupler. The decoupler frequency was switched back and forth between the two multiplets at intervals of 48.7 ms. All experiments were carried out at 30 °C without spinning.

The first sample was used to assess the relative sensitivities of sequences 1a and 1b. 2DJ spectra were recorded for a single value of gradient strength of ~5 G/cm, with 16 transients, each of 10 432 complex points, and a recycle delay of 15 s. After sinebell weighting, Fourier transformation, and baseline correction, the signal strengths produced by the two sequences for each compound were determined by summing the heights of all relevant peaks (peaks overlapping between compounds were excluded). 2DJ-IDOSY spectra of samples 2 and 3 were acquired using the sequence of Figure 1b with gradient strengths chosen to give equally spaced steps of gradient squared using six levels between 5 and 25 G cm^{-1} for the alcohol sample and 12 for the port sample. The diffusion delay Δ was 0.05 s, and the width δ of the diffusion encoding gradient pulses was 3 ms. Sixteen t_1 increments of 10 368 complex points with spectral widths in F_2 and F_1 of 3500 and 55 Hz, respectively, were acquired for the alcohol mixture, and 32 t_1 increments of 16384 complex points with spectral widths of 5500 and 43 Hz, respectively, for the port wine. 2D Fourier transformation was carried out following sinebell time-domain weighting, with one zero-filling in each dimension. 3D DOSY spectra were calculated as described previously[13] by monoexponential fitting of cross-peak volumes. Cross-peaks were selected manually using the same size and shape of integration region for each peak; cross-peak volumes were corrected for baseplane noise by subtraction of the appropriate proportion of the integrated volume of a large sample of baseplane from an empty region of the spectrum. In cases of partial overlap of cross-peaks, integration areas were offset slightly to minimize cross-contamination. Depending on the signal-to-noise ratio of the experimental data, diffusion coefficients can be determined either individually for all peaks or collectively for a given chemical shift using the sums of the peak volumes. The former approach is preferable when all peaks of interest have good signal-to-noise ratios, allowing peaks from multiplets with the same chemical shift but different peak positions to be separated in the diffusion domain; the latter is appropriate with the poorer signal-to-noise ratios generally encountered, maximizing the interpretable information content at the expense of ambiguity where the J spectroscopy fails to achieve complete resolution of multiplets and is the method used here. 2D projections of 3D DOSY spectra were reconstructed by integrating the 2D line shapes between defined limits in the diffusion domain, with peak regions larger than those used for the volume integration.

RESULTS AND DISCUSSION

The relative sensitivities of the 2DJ-IDOSY and 2DJ-DOSY pulse sequences of Figure 1a and b were compared using the sucrose/ethanol/TSP test sample described earlier. The 2DJ-IDOSY sequence showed an improvement in signal strength for all three species, with a factor of 2.0 for sucrose, 2.3 for ethanol, and 2.5 for TSP. These ratios reflect a balance between the advantage of omitting the stimulated echo sequence (which recovers less than one-half of the original signal) and the disadvantage of having the magnetization transverse during Δ (where relaxation losses are governed by T_2 rather than T_1 and unresolved J couplings can cause signal dephasing).

The 2DJ-IDOSY data of Figure 2 for the mixture of four medium chain alcohols in D_2O were recorded in 3.5 min using a single transient for each increment and illustrate some of the strengths and weaknesses of 3D DOSY methods based on J spectroscopy. At the top of Figure 2 is the normal proton spectrum; below it, three 2D projections of the 3D 2DJ-IDOSY spectrum are shown for different diffusion ranges. The 2D projections factorize the full 2DJ spectrum into the contributions from (bottom, fastest diffusing) 1-propanol, (middle) 2-methyl-1-propanol, and (top, most slowly diffusing) the pentanols 2-methyl-1-butanol and 3-methyl-1-butanol. The three projections are almost identical to the 2DJ spectra of the individual alcohols. The separation is not perfect; the peaks originating from 1-propanol around 1.55 ppm in the bottom slice also contain contributions from 2-methyl-1-butanol. These are near-degenerate and could not be resolved but have little effect on the apparent diffusion

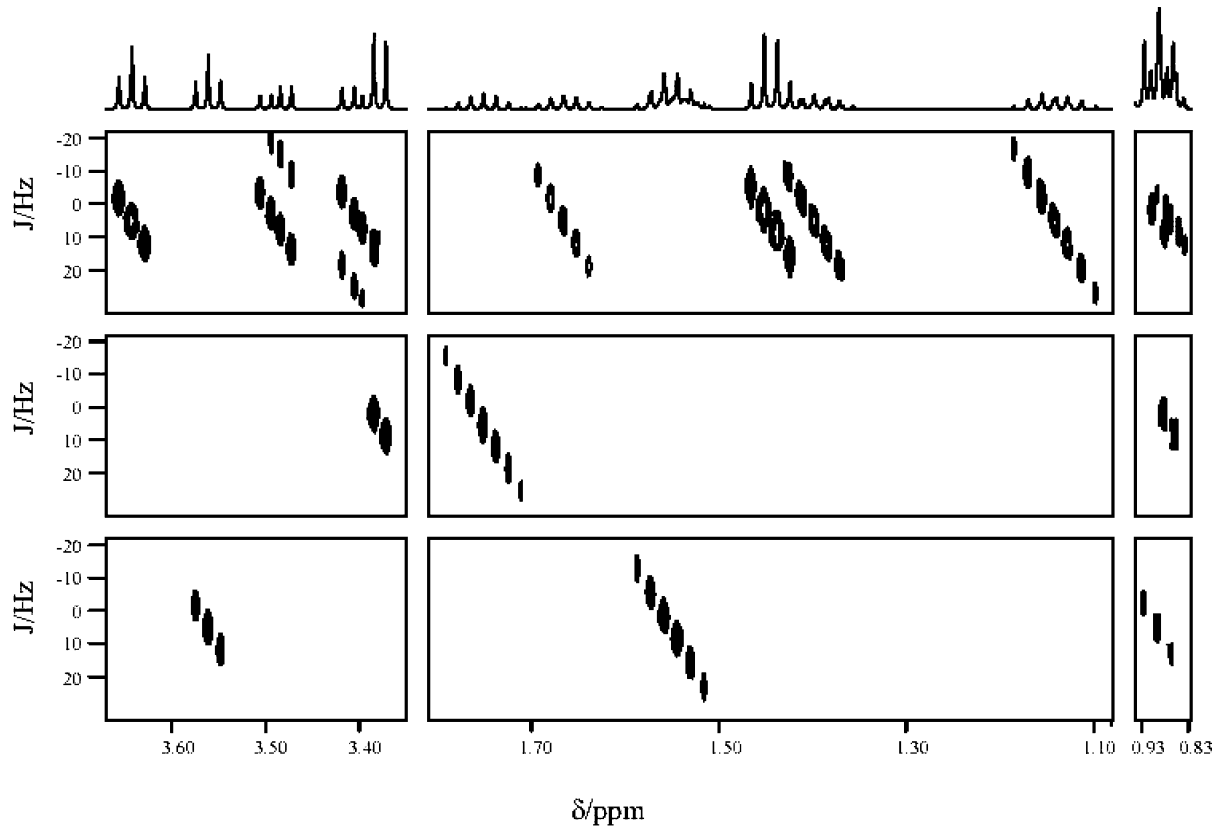

Figure 2. Projections of the diffusion dimension of a 3D 2DJ-IDOSY spectrum of the mixture of alcohols described in the text onto the 2DJ plane. As a reference, a ^{1}H spectrum of the mixture is plotted at the top. The top plane projects the diffusion region between 7.0 and 7.4 $\times$ 10^{-10} m^2 s^{-1}; the middle, between 7.5 and 7.9 $\times$ 10^{-10} m^2 s^{-1}; and the bottom, between 8.4 and 9.0 $\times$ 10^{-10} m^2 s^{-1}. The three projections are almost identical to the 2DJ spectra of the individual alcohols (see text).

coefficient of the stronger 1-propanol peaks. In addition, spurious peaks originating from the strong coupling of the signals at 3.36 and 3.46 ppm are present in the top slice as the two triplets at 3.38 and 3.48 ppm.

In a previous study on port wine, the signals originating from the alcohols in the test sample were seen to be poorly resolved in the diffusion dimension, especially for the methyl peaks at around 0.9 ppm and for peaks in the range 3.35−3.75 ppm.[25] As a practical test, a 3D 2DJ-IDOSY spectrum of the port wine sample was acquired. The strength of the 2DJ-IDOSY experiment is illustrated by the clean diffusional separation of the corresponding signals in the three 2DJ projections of the 2DJ-IDOSY 3D data matrix shown in Figure 3. Substantial time-averaging had to be used (32 transients for each of 12 gradient levels), so in this case, the experimental time was just over 15 h. The severe overlap in the region 0.84−0.95 ppm is adequately resolved. The diffusion projections show the same separation as in the simple alcohol mixture, with the exception of the highest chemical shift signals, which have been omitted because they were impossible to resolve from the strong sugar signals, and the signals around 1.38 and 1.11 ppm, which were swamped by the strong ^{13}C satellite signals of ethanol. The differences in diffusion coefficient between the alcohols in the pure alcohol mixture and in the port wine sample are likely to reflect the different viscosities of the two samples.

2DJ-IDOSY clearly augments the resolving power in the spectral dimensions, making high-resolution DOSY data available for a wider range of mixtures; for concentrated samples, this can be achieved very rapidly. The pulse sequence used can be run in single transient mode and is both simpler and more sensitive than the existing sequence. The very short minimum experiment times offered by 2DJ-IDOSY could be reduced still further by appropriate use of linear prediction to extend data in t_1.[26,27] Two generic problems with 2DJ-DOSY are the occurrence of strong coupling artifacts, which can confuse very crowded spectra, and the difficulty of achieving the accurate baseplane correction necessitated by absolute value mode display. In a previous study on 2DJ-DOSY,[17] an added complication to the latter was identified in an extra noise contribution at 0 Hz in F_1. In our data, the only 0-Hz interference seen was the expected contribution from the absolute value tails of strong F_1 peaks, which was minimized by appropriate choice of weighting function.

CONCLUSION

The principle of integrating diffusion encoding into multidimensional pulse sequences has been illustrated by the new 2DJ-IDOSY pulse sequence, which improves the signal-to-noise ratio by approximately a factor of 2 and reduces minimum experimental

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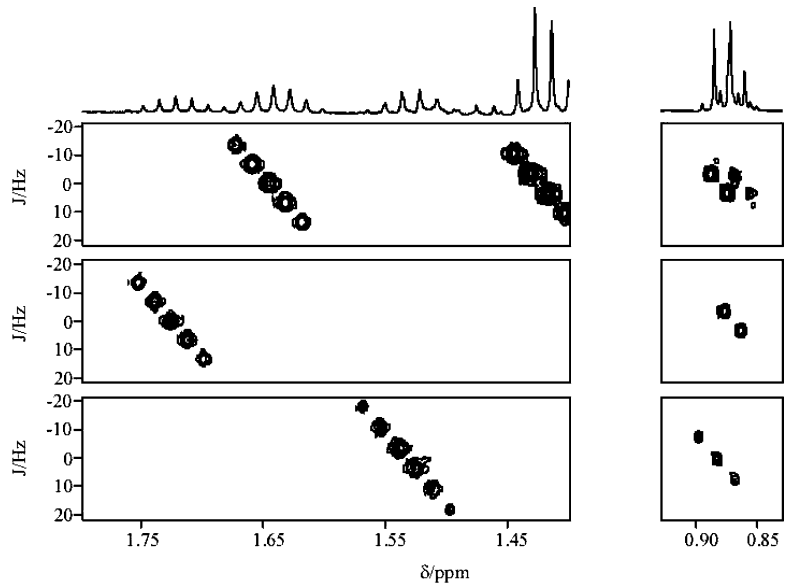

Figure 3. Projections of the diffusion dimension of a 3D 2DJ-IDOSY spectrum of port wine onto the 2DJ plane. The normal proton spectrum is plotted at the top. The top plane projects the diffusion region between 4.80 and 5.15 $\times 10^{-10}$ m^2 s^{-1}; the middle, between 5.2 and 5.6 $\times$ 10^{-10} m^2 s^{-1}; and the bottom, between 5.65 and 6.3 $\times$ 10^{-10} m^2 s^{-1}.

time at least 4-fold compared to the best previous method. 2DJ-IDOSY has been shown to be an efficient means of improving the resolution of DOSY experiments, as evidenced by its application to a highly complex mixture with a large dynamic range (port wine). The integration of diffusion encoding into existing multidimensional NMR pulse sequences is possible in many, although by no means all, cases and is particularly straightforward when data will be processed in absolute value mode. Other potential examples of integrated DOSY experiments, including HMQC−IDOSY[18] and COSY−IDOSY, are under investigation.

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