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The 2D-*J*-DOSY Experiment: Resolving Diffusion Coefficients in Mixtures

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Many *d*iffusion-*o*rdered *s*pectroscop*y* (DOSY) NMR techniques have recently been developed to aid in the deconvolution of complex mixtures. Spectroscopic separation based on chemical and physical properties facilitates the identification of mixture components while eliminating time-consuming separation steps and preserving the chemical environment. One way to improve resolution in such experiments is to spread the spectroscopic information into two dimensions. The 2D-*J*-DOSY experiment has been designed to resolve mixture components in terms of a chemical shift and proton coupling constant as well as distinguishing them on the basis of translational diffusion. Acquiring a series of spectra as a function of gradient amplitude permits the determination of diffusion coefficients for components that cannot be resolved in the one-dimensional (1D) ^{1}H NMR spectrum. Comparison of the resulting values with those obtained through the traditional 1D diffusion experiment for a mixture of sugars validates The 2D-*J*-DOSY technique.

Key Words: **NMR; diffusion, 2D-*J*-resolved spectroscopy; mixture analysis.**

INTRODUCTION

The analysis of complex mixtures is a challenge commonly faced by scientists working in the chemical and pharmaceutical industries. Spectroscopic approaches for analyzing mixtures intact without effecting a separation can offer benefits of simplicity and speed of analysis. Nuclear magnetic resonance (NMR) spectroscopy has a great advantage over many other analytical techniques in that a vast amount of structural information can be gained in a single analysis without destroying the sample. Multidimensional NMR experiments allow a pseudo-separation and identification of mixture components without requiring physical separation, eliminating a time-consuming and nontrivial step in the overall analysis. Furthermore, preservation of the mixture's chemical environment often means component interactions such as aggregation and partitioning can be probed more realistically (*1, 2*). The spectra of mixtures can quickly become complicated, however, and the typical two-dimensional (2D) experiments that rely on spin–spin coupling become difficult to interpret, especially when the molecules of interest contain singlet resonances arising from isolated protons and methyl groups. In extreme cases, severe overlap results in seemingly broadened resonances that cannot be resolved, even in 2D experiments (*3*).

Noninvasive mixture analysis by NMR is possible using *d*iffusion-*o*rdered *s*pectroscop*y* (DOSY)(*4*), where differential translational diffusion permits the identification of mixture components (*2, 5*). Since diffusion coefficients are inversely proportional to hydrodynamic radii, DOSY is particularly useful for mixture analysis where the components encompass a wide range of molecular sizes or for studying intermolecular interactions between components, as in ligand–receptor binding (*6, 7*).

Diffusion coefficients are measured using a pulse sequence incorporating pulsed-field gradients such as the *b*ipolar *p*ulse *p*air *s*timulated *e*cho (BPPSTE) pulse sequence shown in Fig. 1A (*8*). Diffusion coefficients are obtained from the BPPSTE spectra by monitoring signal attenuation as a function of the applied magnetic field gradient amplitude and fitting Eq. [1] to the experimental results

$$I = I_o \exp[-D(\gamma\delta G)^2(\Delta - \delta/2 - \tau/3)], \qquad [1]$$

where I is the resonance intensity measured for a given gradient amplitude, G, I_o is the intensity in the absence of the gradient pulse, γ is the gyromagnetic ratio, δ is the duration of the bipolar gradient pulse pair, Δ is the diffusion delay time, and τ is a short gradient recovery delay time during which relaxation and spin–spin coupling evolution are not significant. Many recent applications of this pseudo-2D technique have been developed under the broad term "affinity NMR" (*9*) and applied as screening methodologies in the pharmaceutical industry (*6*).

A primary limitation of this approach is that reliable diffusion coefficients can only be determined for well-resolved resonances. This can be problematic in the analysis of complex mixtures where spectral overlap or matrix background can complicate spectra, even at high magnetic field strengths. Computational programs and processing methods have been introduced to analyze spectral peaks containing multiple components and extract meaningful diffusion coefficients. If the overlapped components have diffusion coefficients that differ by at least a factor

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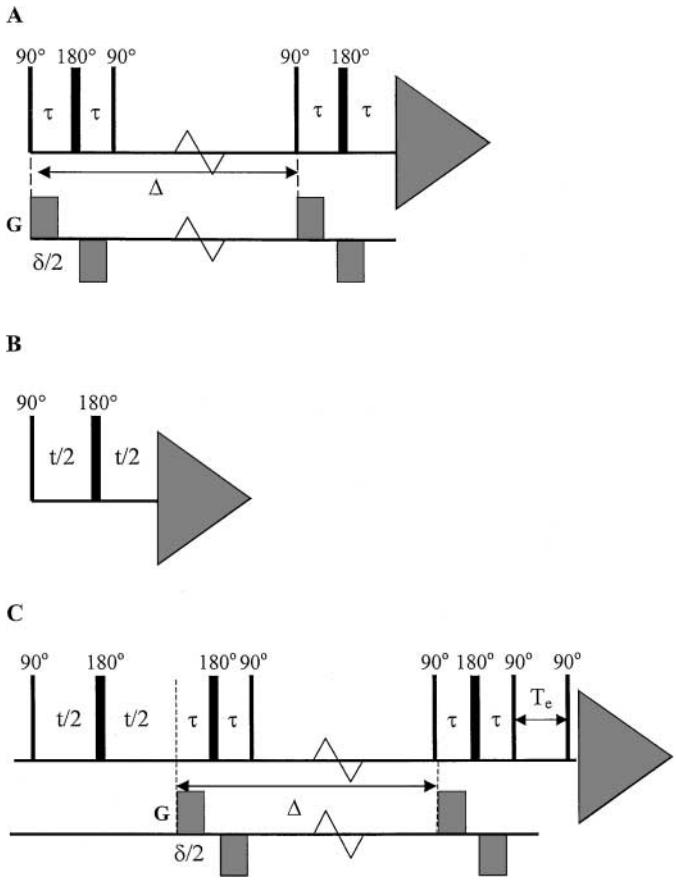

FIG. 1. Relevant pulse sequences. (A) Bipolar-pulse pair stimulated echo (BPPSTE) pulse sequence for diffusion measurements, where gradient amplitude (G) is successively increased. (B) Homonuclear 2D-J pulse sequence, where t is the incremented delay. (C) 2D-J-DOSY pulse sequence utilized in this work. T_e is an optional delay to permit the decay of residual eddy currents (resulting from the gradient pulses).

of two, SPLMOD can be used to calculate the diffusion coefficients of each species (*10*). The program CONTIN has been used to analyze DOSY data producing a distribution of diffusion coefficients that describe the diffusion of polydisperse samples (*10*). In this method, the user defines a finite baseline value (typically zero), which assumes the absence of a positive noise threshold. The CORE-NMR method complements CONTIN, utilizing all spectral information with a global least squares minimization that treats the noise randomly (*11*). The DECRA algorithm relies upon high quality exponential signal decay and requires maximum signal-to-noise ratios in order to resolve components with similar diffusion coefficients (*12*). With regard to uncharacterized complex mixtures, a drawback of these computational methods is their dependence upon user-defined parameters that generally require some *a priori* knowledge about the sample.

To address these issues, modified DOSY pulse sequences have been created to improve spectral selectivity. One approach is to modify the BPPSTE sequence itself, as in the *g*radient m*o*dified *s*pin–*e*cho (GOSE) experiment (*13*). This experiment incorporates a spin–echo period to dephase coupled spins followed by a gradient pulse, which removes residual magnetization of the coupled protons. Other spectral editing methods for complex mixture analysis have been reviewed by Dixon and Larive (*14*).

An alternate approach is to use the diffusion experiment as a building block for new pulse sequences where spectral information can truly be spread into two dimensions. For example, in the TOCSY-DECODES experiment, the magnetization is allowed to evolve during a TOCSY spin-lock added at the end of the diffusion pulse sequence (*15*). Collecting a series of spectra as a function of gradient amplitude permits the identification of mixture components and the measurement of cross peak intensities that can then be used to calculate diffusion coefficients. Other related examples include the homonuclear DOSY–NOESY (*16*), the heteronuclear DOSY–HMBC (*17*), and the so-called three-dimensional (3D) experiments: 3D DOSY–TOCSY (*18*) and COSY–DOSY (*19*).

The pulse sequence for the homonuclear 2D-J experiment is shown in Fig. 1B. One of the first 2D NMR experiments to be developed, the fundamental basis of this pulse sequence relies upon the well-known properties of spin–echo experiments to refocus chemical shift (*20*). For homonuclear spin-coupled systems, however, the echoes are modulated with respect to spin–spin coupling. In the 2D-J experiment the total incorporated delay time (t) is incremented according to the coupling constants of sample components to allow for J evolution in t_1, which complements the chemical shift information in t_2. Successive Fourier transformations generate a 2D spectrum that distinguishes components of identical or overlapped chemical shift on the basis of differential coupling constants without compromising chemical shift. The projection of the data along the coupling dimension reveals the homonuclear decoupled spectrum. Because of the advantages of both DOSY and homonuclear 2D-J spectroscopy for resolving mixture components based on differing physical and chemical molecular properties, the two pulse sequences have been combined to generate the 2D-J-DOSY experiment (Fig. 1C) which was successfully used to resolve a mixture of sugars.

RESULTS AND DISCUSSION

The one-dimensional (1D) ^{1}H NMR spectra of the D_2O solutions of sucrose (4.64 mM), glucose 6-phosphate (G6P, 17.5 mM), and the mixture of the two sugars (at the same concentrations) are shown in Fig. 2. These sugars were chosen due to their similar structural and diffusion properties. The anomeric protons of the sugars are well resolved in the mixture and observed downfield of the solvent peak (HOD, 4.78 ppm) to which all spectra were referenced. A sucrose doublet at 4.21 ppm and a G6P triplet at 3.27 ppm are also baseline resolved. Diffusion coefficients were not measured for the G6P doublet at 4.66 ppm just upfield of HOD due to its proximity to the solvent resonance. The HOD peak often suffers from spectral artifacts in diffusion experiments, which can influence the signal integrals

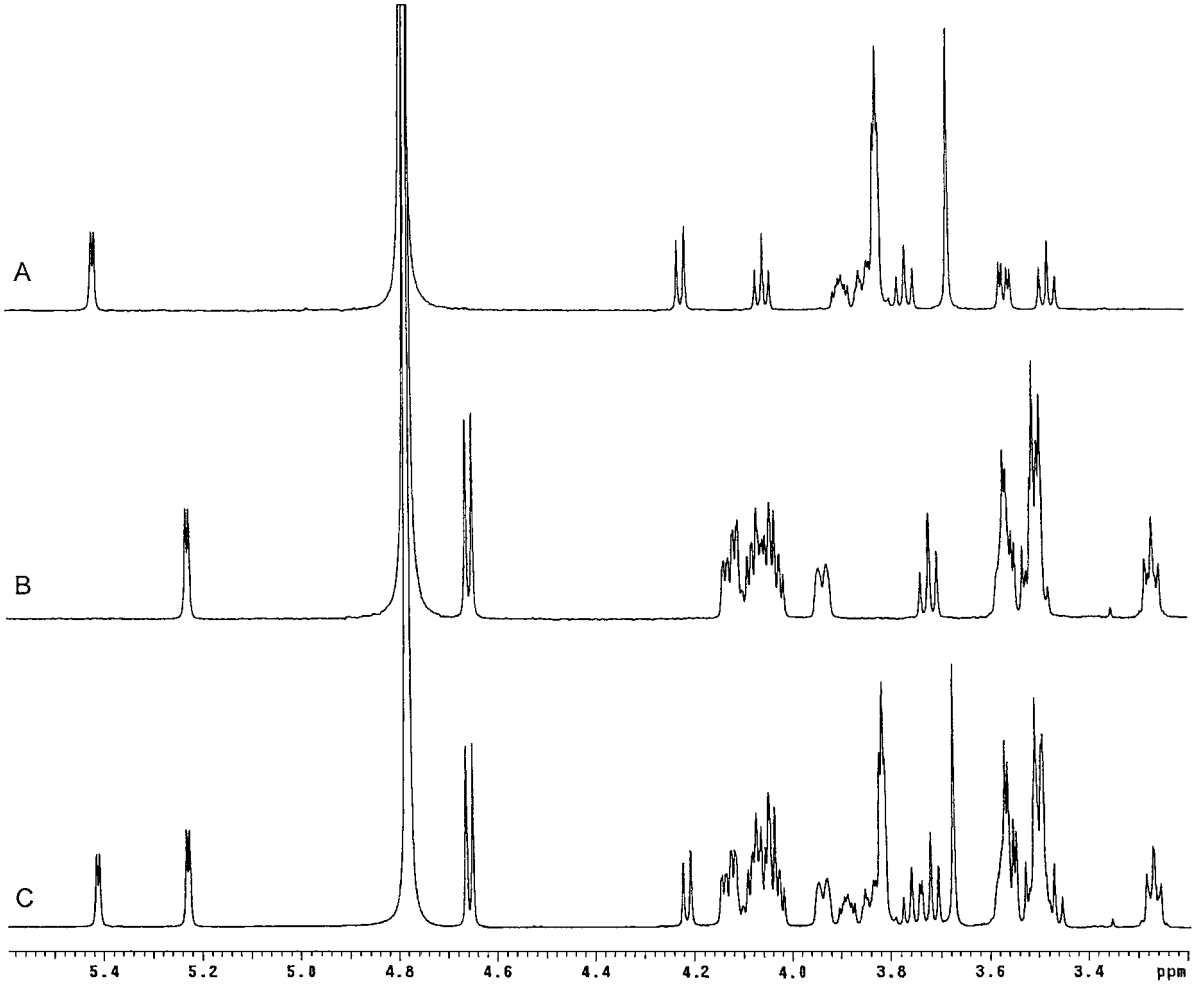

FIG. 2. ^{1}H 1D-NMR spectra for (A) 4.64 mM sucrose, (B) 17.5 mM glucose-6-phosphate (G6P), and (C) mixture of the two sugars. All spectra were referenced to the solvent peak (HOD, 4.78 ppm).

and/or intensities of nearby peaks. Figure 3 shows the corresponding 2D-*J*-DOSY spectrum for the sugar mixture, with its 1D spectrum included for reference. Figure 4 provides a close-up view of the overlapped resonances between 3.20 and 4.30 ppm.

The diffusion coefficients (D) measured for selected 2D-*J*-DOSY peaks are shown in Table 1 along with the reference values obtained for the mixture components using the standard BPPSTE experiment. Equation [1] was fit to each data set using a nonlinear least squares alogorithm. The errors reported for individual peaks in Table 1 are the fitting errors. The errors reported for average diffusion coefficients are calculated as either the standard deviation of the mean or the pooled fitting errors, whichever produced the greater error. Average diffusion coefficients were determined for each sugar in the mixture and have values of $5.08 \pm 0.10 \times 10^{-6}$ cm^2 s^{-1} (sucrose) and $5.50 \pm 0.10 \times 10^{-6}$ cm^2 s^{-1} (G6P). The obtained values are consistent with the known structural properties of the molecules: sucrose, a disaccharide, should diffuse more slowly in solution than the modified monosaccharide G6P. The overlapped region of the BPPSTE spectrum for the complex mixture between 3.43 and 3.53 ppm was also integrated and produced a diffusion coefficient of $5.53 \pm 0.03 \times 10^{-6}$ cm^2 s^{-1}. This value is indistinguishable from the value obtained for G6P alone due to its nearly 3.5-fold higher concentration relative to sucrose.

2D-*J*-DOSY integral regions were defined by setting the vertical scale of the spectrum to a level sufficient to visualize all peaks (except the broadened G6P doublet at 3.94 ppm. This resonance was not observed due to complicated coupling patterns and/or fast T_2 relaxation.) Specific integration regions just large enough to contain the peaks of interest were then selected. The diffusion coefficients calculated from the volume integrals of these regions are given in Table 1. The diffusion coefficient for the well-resolved G6P anomeric proton at 5.23 ppm measured with the 2D-*J*-DOSY experiment agrees within error with the average value measured by the traditional BPPSTE experiment. However, The 2D-*J*-DOSY diffusion coefficient for the G6P triplet at 3.27 ppm was significantly lower than the average value

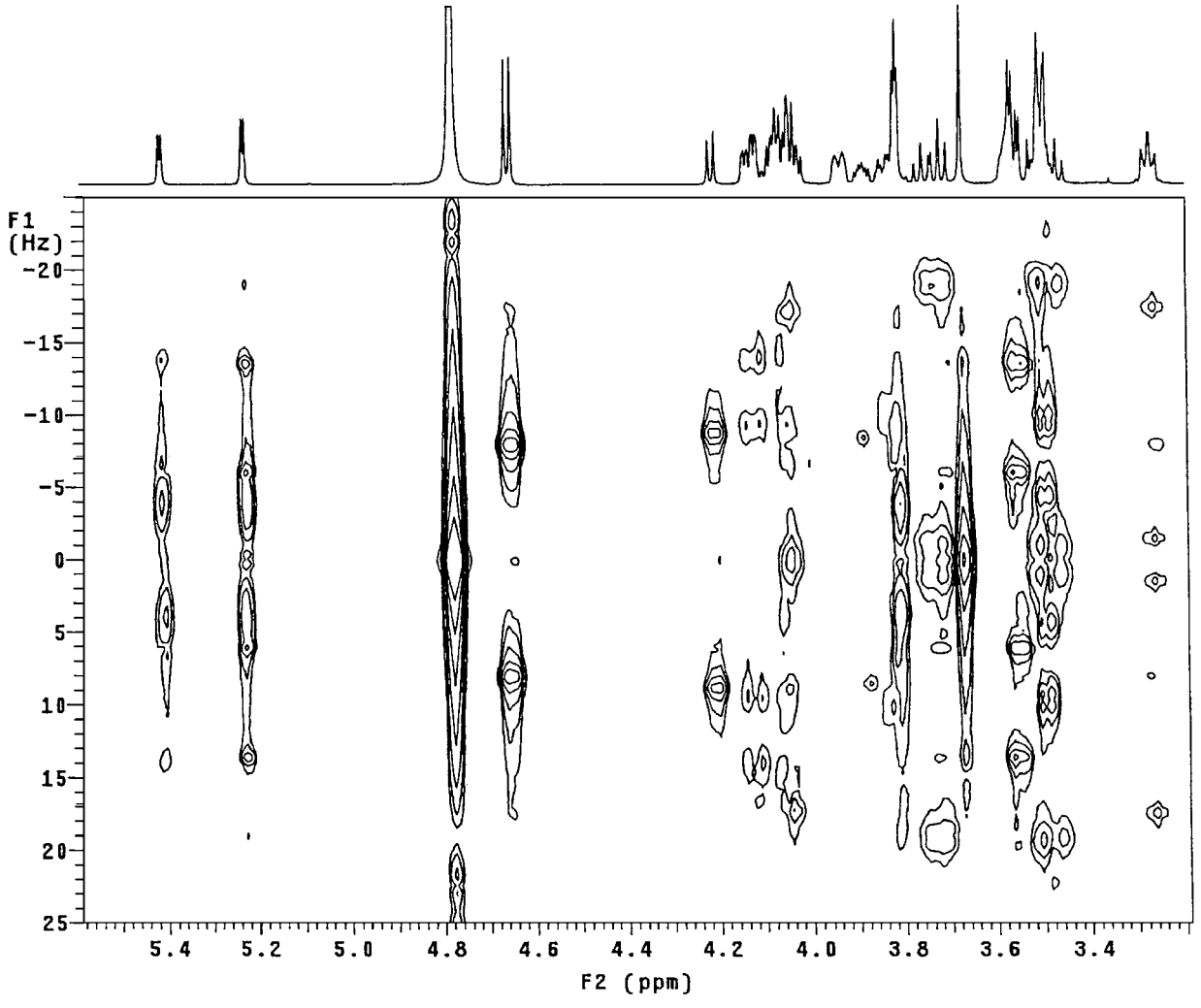

FIG. 3. Spectrum for the first increment of the 2D-*J*-DOSY experiment for the sugar mixture. The corresponding ^{1}H spectrum is shown for reference. Although the experiment is called 2D-*J*-DOSY, it is recognized that the data are not represented in a true DOSY format, since the spectrum is not displayed with a diffusion coefficient dimension.

obtained by BPPSTE and has a fitting error three times greater. Figure 3 reveals relatively weak 2D peaks for this resonance. By nature, the homonuclear 2D-*J* experiment is acquired in absorption mode, thus eliminating cancellation of baseline noise. The noise threshold will then represent a greater percentage of a volume integral determined for a less intense peak compared with a more intense signal. The plot of resonance intensity, calculated using integral volumes, versus gradient amplitude squared for this G6P resonance is shown in Fig. 5A, illustrating the nature of the baseline noise contribution as a positive offset especially obvious at larger gradient values.

Correction for the contribution of the spectral baseline noise to the volume integrals was attempted as shown in Fig. 5B. To determine the appropriate correction factor, ten spectral regions without peaks were selected and the average volume integral of the noise, normalized for the area of each region, was calculated. This value of the baseline noise was subtracted from each volume integral prior to the determination of the diffusion coefficients, which are given in Table 1. When this correction was applied, an improved fit to the experimental data for the G6P triplet was obtained as illustrated by Fig. 5B. The diffusion coefficient calculated using corrected integral volumes was $6.31 \pm 0.03 \times 10^{-6}$ cm^2 s^{-1}, a value that exceeds by 14.7% the average diffusion coefficient for G6P determined by BPPSTE. The correction is applied with the assumption that there is a positive noise threshold above which all peaks exist; however, this threshold may become indistinguishable for a weak signal where the signal-to-noise ratio is problematic, especially at high gradient amplitudes. When the correction factor is applied, any signal obscured by the noise is lost, and the data appear overall to decay more rapidly. The end result is a diffusion coefficient skewed to higher values than are experimentally reasonable. A similar result is observed for the sucrose multiplet at 3.89 ppm, reflected by the values listed in Table 1.

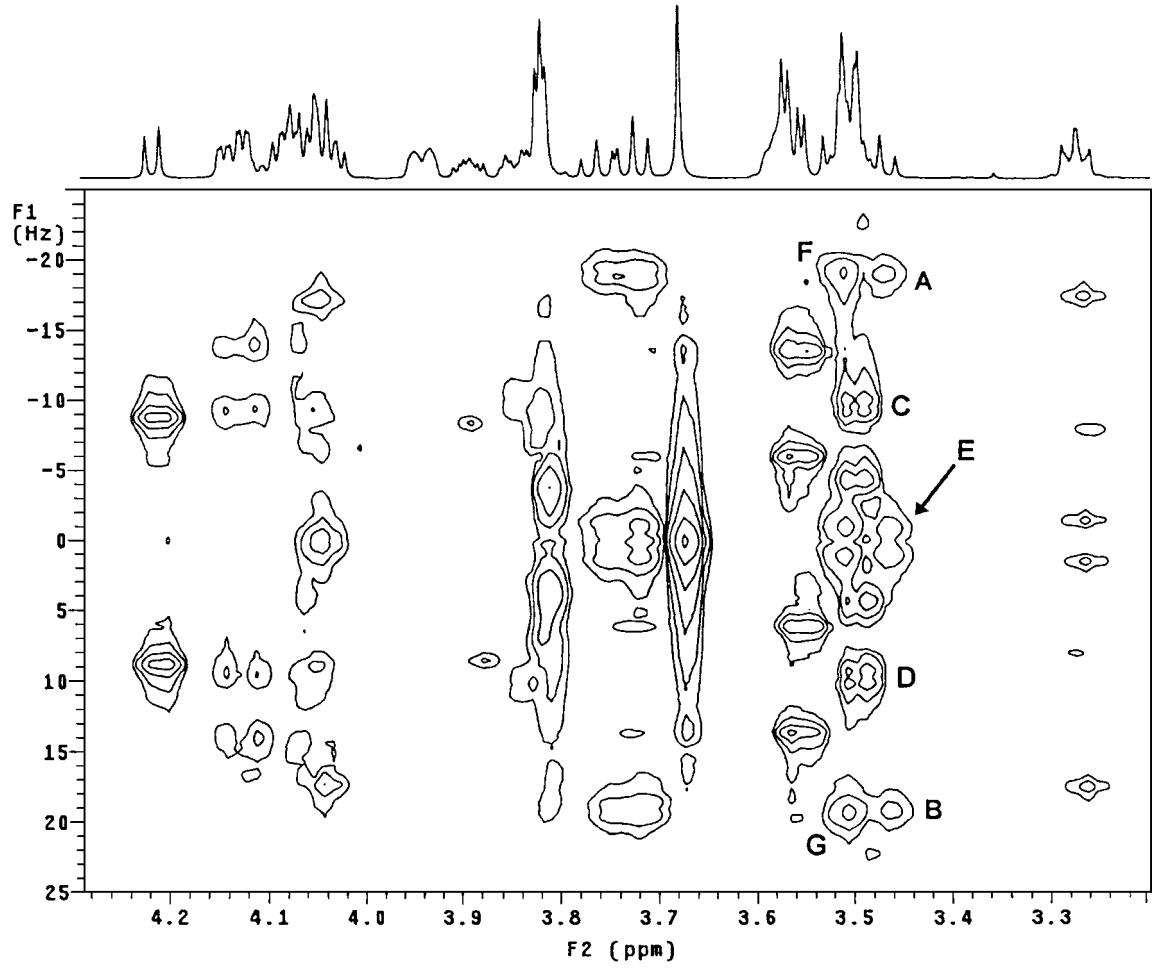

FIG. 4. Expanded region (3.20–4.30 ppm) of the 2D-*J*-DOSY spectrum shown in Fig. 3. The arrow indicates the overlapped peak, E, which contains components from both sucrose and G6P.

To overcome this problem, resonance intensities were determined from the maximum amplitude of the peaks contained within the integral regions and were then used to calculate diffusion coefficients, also given in Table 1. For comparison, the intensities measured for the G6P triplet are plotted against gradient amplitude squared in Figure 5C. The resulting diffusion coefficient, $5.58 \pm 0.30 \times 10^{-6}$ cm^2 s^{-1}, is in good agreement with the average value obtained by BPPSTE. The intensity fit for the sucrose anomeric peak, which Fig. 3 reveals as less intense relative to the G6P anomeric peak, also produced a diffusion coefficient in good agreement with the value obtained by BPPSTE. In both cases, though, the fitting error is still much greater than was obtained with the BPPSTE experiment. In general, the fitting errors for the diffusion data measured by the 2D-*J*-DOSY experiment are higher than those obtained with the BPPSTE pulse sequence because of the poorer digital resolution of the 2D spectra.

The positive noise offset is visible in the spectrum by adjusting the threshold of the spectrum and through data processing programs that display peak contours in a color series to illustrate intensity. The volume selected for integration is user-defined based on the boundaries selected in both dimensions. The threshold levels were adjusted to minimize the noise contribution to defined integral regions, although data analysis reveals that complete noise elimination is not possible, even with optimized processing parameters. A further complication of this experiment is additional t_2 noise at 0 Hz which impairs analysis of singlets, triplets, etc., as well as signals for which t_1 noise is apparent (such as the anomeric protons seen in Fig. 3). The 0-Hz noise ridge can be avoided for many multiplets by integrating multiplet components individually as discussed below.

One advantage of the 2D-*J* experiment is separation of multiplets in terms of chemical shift and coupling constant. In the traditional BPPSTE experiment, entire multiplets are usually integrated for the diffusion measurement. The sucrose doublet at 4.21 ppm is well resolved from other peaks in the spectrum and serves as an example of integration of the individual components of the multiplet in the 2D experiment for determining

TABLE 1
Diffusion Coefficients (D) Measured with the BPPSTE and 2D-*J*-DOSY Experiments

Component	^{1}H Chemical Shift (ppm)	BPPSTE D × 10^{-6} (cm^2 s^{-1}) (integral areas)	2D-*J*-DOSY D × 10^{-6} (cm^2 s^{-1}) (integral volumes)	2D-*J*-DOSY D × 10^{-6} (cm^2 s^{-1}) (corrected volumes)	2D-*J*-DOSY D × 10^{-6} (cm^2 s^{-1}) (peak intensities)
Sucrose	3.67[b]	5.29 ± 0.03	5.34 ± 0.06	5.51 ± 0.05	5.35 ± 0.04
	3.76	5.13 ± 0.03	NR	NR	NR
	3.79–3.87	5.15 ± 0.04	4.89 ± 0.09	5.26 ± 0.01	4.99 ± 0.06
	3.89	5.05 ± 0.05	5.00 ± 0.16	6.23 ± 0.10	5.13 ± 0.14
	4.21	4.98 ± 0.05	4.91 ± 0.08	5.13 ± 0.04	4.86 ± 0.07
			4.90 ± 0.08	5.15 ± 0.04	4.86 ± 0.08
	5.41	5.11 ± 0.03	4.89 ± 0.02	5.41 ± 0.03	5.07 ± 0.11
Average D		5.08 ± 0.10	4.92 ± 0.25	5.44 ± 0.46	4.98 ± 0.21
RSD		1.91%	5.04%	8.39%	4.27%
G6P	3.27	5.51 ± 0.03	5.00 ± 0.30	6.31 ± 0.03	5.58 ± 0.30
	3.72	5.51 ± 0.06	NR	NR	NR
	3.94	5.43 ± 0.05	ND	ND	ND
	4.13	5.43 ± 0.05	5.38 ± 0.13	5.94 ± 0.04	5.57 ± 0.12
			5.40 ± 0.12	5.98 ± 0.04	5.59 ± 0.12
	5.23	5.61 ± 0.03	5.58 ± 0.10	5.93 ± 0.04	5.83 ± 0.07
Average D		5.50 ± 0.10	5.34 ± 0.33	6.04 ± 0.18	5.64 ± 0.35
RSD		1.84%	6.26%	3.00%	6.21%
Overlapped	3.47 (A)[a]				5.16 ± 0.09
Sucrose + G6P	3.47 (B)				5.11 ± 0.09
	3.49 (C)				5.61 ± 0.07
	3.49 (D)				5.61 ± 0.07
	3.51 (E)				5.62 ± 0.09
	3.51 (F)				5.62 ± 0.05
	3.51 (G)				5.63 ± 0.05
Average D		5.53 ± 0.03			
RSD		0.62%			

Note. RSD, Relative standard deviation. NR, Not resolved in the 2D-*J*-DOSY spectrum. ND, Not detected in the 2D-*J*-DOSY spectrum.

[a] Letters correspond to the peaks labeled in Fig. 4.

[b] The diffusion coefficients for this singlet are consistently high relative to the other values obtained for sucrose. This is likely due to a small contaminant in the mixture. As such, the values for this peak are not included in the averages.

diffusion coefficients. Using volume integrals, the two peaks of the sucrose doublet in the 2D-*J*-DOSY spectra were integrated separately. The diffusion coefficients determined from each component of the doublet were 4.91 ± 0.08 × 10^{-6} cm^2 s^{-1} (4.20 ppm) and 4.90 ± 0.08 × 10^{-6} cm^2 s^{-1} (4.22 ppm), which agree with each other and, within error, with the average value obtained by BPPSTE (4.98 ± 0.05 × 10^{-6} cm^2 s^{-1}). The individual diffusion coefficients obtained using peak intensities for this doublet also agree within error with those obtained by volume integration. However, the average diffusion coefficient determined from the corrected volumes was again skewed high (5.14 ± 0.06 × 10^{-6} cm^2 s^{-1}) relative to the BPPSTE average value.

Examination of the NMR spectra of sucrose and G6P, shown in Figs. 2A and 2B, respectively, reveals a region around 3.50 ppm where the sucrose triplet is obscured by the complicated G6P multiplet pattern in the NMR spectrum of the mixture (Fig. 2C). As mentioned above, the diffusion coefficient measured by BPPSTE by integrating this spectral region was 5.53 ± 0.03 × 10^{-6} cm^2 s^{-1}. Since this value is within error of the average diffusion coefficient experimentally measured for G6P (5.50 ± 0.10 × 10^{-6} cm^2 s^{-1}), if no reference spectra for mixture components were available, as is often the case in uncharacterized complex mixtures, it would be easy to assume from the 1D spectrum that this region consisted solely of G6P resonances.

The 2D-*J*-DOSY spectrum in Fig. 4 reveals otherwise. Peaks labeled A and B appear to represent the outer edges of the sucrose triplet while the complicated G6P multiplet is composed of peaks C, D, F, and G. The homonuclear 2D-*J* experiment resolves only weak couplings and thus the *J* values for strongly coupled sugar peaks are distorted. The center peak of the sucrose triplet appears to be encompassed in peak E (shown with an arrow) but not resolved from G6P in terms of chemical shift or *J*.

Since it is difficult to accurately define volumes in overlapped regions of the 2D spectra, peaks A–G were evaluated individually using peak intensities. The results are included in Table 1. Indeed, peaks A and B (5.16 ± 0.09 × 10^{-6} cm^2 s^{-1} and 5.11 ± 0.09 × 10^{-6} cm^2 s^{-1}, respectively) have diffusion coefficients that much more closely resemble the values measured for the well-resolved sucrose peaks by 2D-*J*-DOSY and BPPSTE. Peaks C, D, F, and G similarly have diffusion

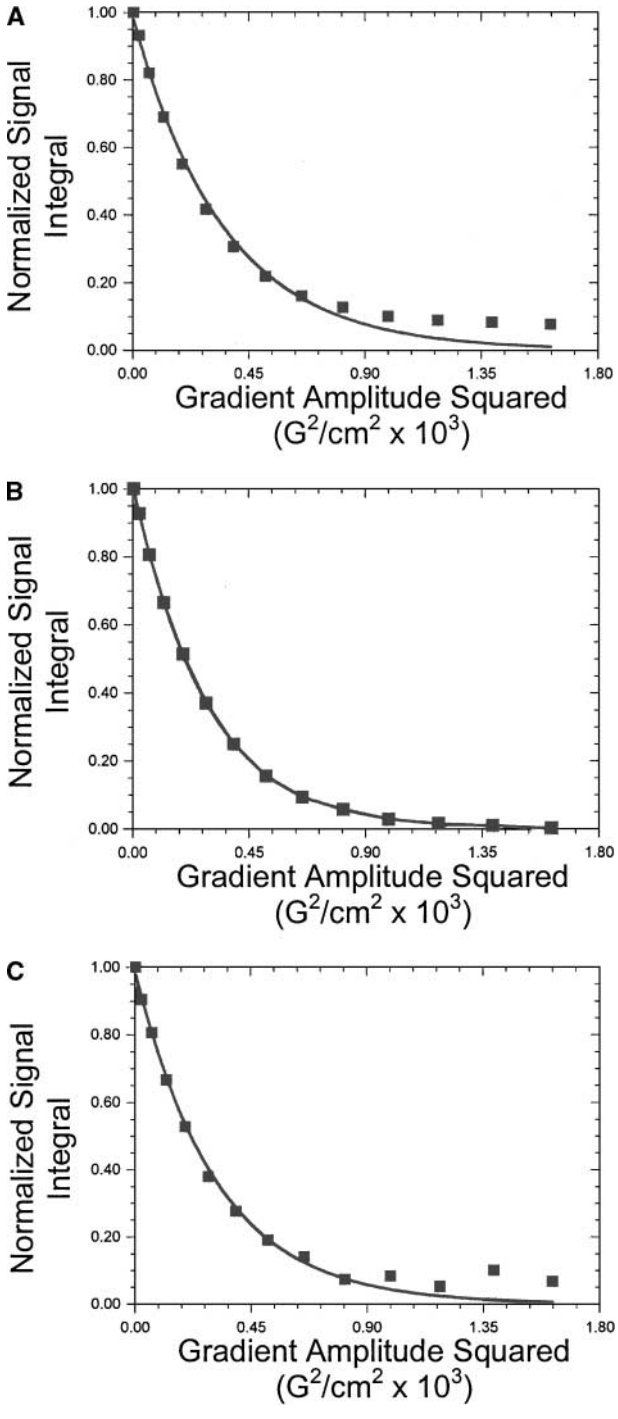

FIG. 5. Diffusion results for the G6P triplet at 3.27 ppm using volume integrals (A), noise-corrected volume integrals (B), and peak intensities (C). These graphs were obtained by plotting signal volume (A and B) or intensity (C) against gradient amplitude squared according to Eq. [1].

coefficients that agree with the average value for G6P by BPPSTE. The unresolved peak E has a diffusion coefficient of $5.62 \pm 0.09 \times 10^{-6}$ cm^2 s^{-1}, reflective of the average BPPSTE value for G6P. This is likely due to the large excess of G6P in the mixture. Weighted diffusion coefficients can be calculated to account for known concentration differences of mixture components, normalized for the number of protons contributing to the peak. However, because of the complicated coupling patterns evident in the spectrum, it is difficult to quantitatively analyze peak E in this way. Still, comparison of diffusion coefficients for resolved and unresolved peaks in the 2D-J-DOSY spectra can provide insight regarding the number of peak components and aid in mixture deconvolution.

CONCLUSION

The 2D-J-DOSY experiment provides reliable diffusion coefficients for mixture components for which the resonances are overlapped in the 1D ^{1}H NMR spectrum. Spreading the spectroscopic information into the second (J) dimension improves resolution such that individual peaks can be identified and integrated. When using volume integration of the 2D-J peaks, the inherent positive noise threshold can contribute to a systematic error in the measured diffusion coefficients, especially for the less intense signals. This problem is generally overcome by using peak intensities at their maximum amplitude instead of volume integrals in the calculations. The experimental value of 2D-J-DOSY is realized in terms of its ability to provide a great deal of relevant structural information that is potentially useful for characterizing complex mixtures.

EXPERIMENTAL

Reagents. D-Glucose-6-phosphate, monosodium salt (G6P, Sigma, St. Louis, MO), table sugar (sucrose, Sweet Harvest brand), and D_2O (Fluka, Milwaukee, WI) were used without further purification.

NMR spectroscopy. G6P (17.5 mM) and sucrose (4.64 mM) solutions in D_2O were analyzed by ^{1}H NMR at 298 K on a Varian INOVA 600-MHz spectrometer (Varian Instruments, Inc., Palo Alto, CA) using a 5-mm triple-axis gradient probe. The z-gradient coil constant was calibrated at 1.74×10^{-3} G/cm/DAC using a 10.0 mM solution of β-cyclodextrin (Sigma) in D_2O. The known diffusion coefficient of β-cyclodextrin was calculated according to the equation provided by Uedaira and Uedaira (*21*). The BPPSTE pulse sequence was used for the calculation of reference diffusion coefficients. Sixteen transients (48,000 points per transient) were collected for each of 14 increments, where the z-gradient amplitude (G) varied from 2.23 to 40.2 G/cm. The duration of each gradient in the pulse pair ($\delta/2$) was 1.0 ms. A 50-μs gradient recovery delay (τ) was used, and the diffusion delay time (Δ) was 200 ms. The FIDs were apodized by multiplication with an exponential decay equivalent to 1-Hz line broadening.

The 2D-J-DOSY experiment utilized the same diffusion parameters described above. In the F2 dimension, 2048 points were collected. Sixteen transients were co-added for each of 96 increments in F1. The data in F1 were zero-filled to 2048. Fourteen

experiments were collected, each at a different gradient amplitude (corresponding to the same array used in the BPPSTE experiment) to generate the complete 2D-*J*-DOSY data set. Pseudo-echo apodization was applied, and the data were corrected for DC offset. Diffusion coefficients were calculated by measuring signal areas (BPPSTE) and volumes or peak intensities at maximum amplitude (2D-*J*-DOSY) for the individual peaks in each spectrum in the array, followed by a nonlinear least squares fit of Eq. [1] to the data using Scientist® (MicroMath®, Inc., Salt Lake City, UT).

Error analysis. Each diffusion coefficient results from the fit of 14 data points (peak areas, volumes, or intensities), except the sucrose multiplet at 3.89 ppm, for which signal decay was sufficient after 11 increments. Errors reported with individual diffusion coefficients represent fitting errors calculated using Scientist®. Errors of average diffusion coefficients were calculated as the standard deviations of the means or the square root of the sums of the squares of the errors in the individual values, whichever was larger, to avoid underestimation of the errors.

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