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A pure shift and spin echo based approach for high-resolution diffusion-ordered NMR spectroscopy

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ABSTRACT

Diffusion-ordered NMR spectroscopy (DOSY) can be used for separating mixture components according to their individual diffusion behaviors, thus offering a powerful tool for the analysis of compound mixtures. However, conventional DOSY experiments generally encounter the problem of limited resolution in the spectral domain, particularly for applications to complex mixtures that contains crowed resonances in 1D NMR. In addition, chemical exchange effects, bringing about spurious component signals, pose another limitation for interpreting DOSY measurements. Here, a general DOSY method is proposed based on pure shift extraction and spin echo evolution to obtain high-resolution 2D DOSY spectra, along with the suppression on effects of chemical exchange and *J* coupling. Both theoretical analyses and experimental results suggest that the proposed method is useful for high-resolution DOSY measurements on complex mixtures that contains crowded or even overlapped NMR resonances and exchanging spin systems.

1. Introduction

Diffusion-ordered NMR spectroscopy (DOSY) serves as a powerful tool for the analysis of mixtures by introducing information of diffusion coefficients [1–4]. Based on differences in diffusion coefficients among mixture components, DOSY can separate acquired NMR signals and achieve a "virtual" separation of mixture components, similar to the physical separation by HPLC NMR [5]. In general DOSY experiments, diffusion dependent signal decays, caused by a series of incremental gradient pulses, are acquired to be fitted into a theoretical expression for estimating diffusion coefficients and reconstructing the desired 2D spectra which correlate chemical shifts with diffusion coefficients. Benefitting from the minimum of *J* coupling modulation and T_2 relaxation effects during the diffusion encoding period, the stimulated echo scheme provides a significant mechanism of signal evolutions for DOSY experiments, albeit at the cost of half of acquired signal intensities [6,7]. From the original PFGSTE (Pulse Field Gradient STimulated Echo) to improved versions of BPPSTE (Bipolar Pulse Pair STimulated Echo) [8] and one-shot DOSY techniques [9], most DOSY pulse sequences used in common applications are designed based on the stimulated echo scheme. The successful implementation of high-resolution DOSY relies on a basic requirement that diffusion dependent 1D NMR spectra are presented in high-resolution manner, that is all peaks are well-resolved and different peaks are assigned to individual constituents [10]. After the exponential-curve fitting on the attenuation of these resolved peaks, accurate diffusion coefficients and corresponding errors are calculated to generate high-resolution diffusion domain with the resolution of the order of 1% differences [9].

However, due to the limited range of proton chemical shifts and the complexity of *J* coupling multiplet structures, 1D proton NMR spectra, commonly used in DOSY measurements because of its high natural abundance, generally suffer from spectral congestion and peak overlap in complex mixtures, even in relatively simple mixtures that contain extensive multiplet structures. In this case, diffusion coefficients may be calculated by the superimposed contributions from different diffusion decays, inevitably leading to inaccurate diffusion measurements and incorrect DOSY analyses. Multivariate analysis methods, such as DECRA [11] and MCR [12], have been designed from the aspect of data processing to improve the calculation accuracy of diffusion coefficients from complicated DOSY data sets. However, these data processing methods are generally suitable for the case when the diffusion differences among mixture components are larger than 30% [13], thus they cannot fit all DOSY measurements on complex mixtures. The fundamental solution to the problem of spectral congestion

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in DOSY experiments is proceed from experimental techniques which maximally improve resolution in the spectral domain. Intuitively, the solution for enhancing resolution in DOSY experiments is to extend the spectral domain from 1D to 2D manners by externally concatenating or internally integrating the diffusion encoding element to existing 2D NMR pulse sequences, thus introducing 3D DOSY variants, such as DOSY-COSY [14] by external diffusion encoding and 2D*J*-iDOSY [15,16] by internal diffusion encoding. It is inevitable for 3D DOSY-based experiments to require increased experimental time and complicated data processing [17]. Besides the spectral domain extension, the "pure shift" NMR technique by collapsing *J* coupling multiplets and preserving only chemical shifts provides an alternative to improve spectral resolution [18], intuitively benefitting to DOSY experiments. A pure shift based DOSY, ZS-DOSY [19], designed by prepending the stimulated echo diffusion encoding to the Zangger-Sterk (ZS) pure shift sequence [20], is capable of circumventing the challenge of peak overlaps in the spectral dimension and achieving high-resolution DOSY measurements on complex mixtures. Intrinsic signal penalty is generally encountered in pure shift NMR, and this may impact calculation accuracy of diffusion coefficients in pure shift based DOSY experiments. Morris and coworkers proposed a general method, PSYCHE-iDOSY [21], based on the combination of the PSYCHE module [22] (a pure shift technique with the enhanced signal intensity superior to other counterparts) and the embedded stimulated echo diffusion encoding element (holding higher signal intensity than the external diffusion encoding manner), to enhance signal intensity in pure shift based DOSY applications to complex mixtures.

Existing pure shift based DOSY experiments, e.g., ZS-DOSY and PSYCHE-iDOSY, are implemented based on the stimulated echo scheme, which minimizes *J* coupling modulation and T_2 relaxation by retaining the excited magnetizations along longitudinal z-axis during the diffusion period [23]. Additionally, chemical shifts are encoded as the position dependent phase information during the first diffusion encoding interval of stimulated echo based DOSY. The encoded chemical shifts can be dephased by diffusion gradients in general measurements. However, in some cases when chemical exchange effects occur with chemical shifts in the storage period of stimulated echo based DOSY experiments [24], the encoded chemical shifts would influence diffusion dependent signal decays and result in incorrect measurements on diffusion coefficients [25,26]. Alternatively, the spin echo scheme provides an effective way to suppress chemical exchange effects by refocusing chemical shifts during the echo period [27,28]. Because of the evolution of transverse magnetizations in the diffusion encoding period, the implementation of spin echo in DOSY experiments generally suffers from severe *J* coupling modulation. The Carr-Purcell-Meiboom-Gill (CPMG) method [29,30], containing a train of spin echo elements, is capable of suppressing *J* coupling modulation by adopting extremely short echo time 2τ (i.e. $\tau << 1/J$), but at the cost of massive π radiofrequency (RF) pulses along with the risk of sample heating and convection. The PROJECT (Periodic Refocusing of *J* Evolution by Coherence Transfer) scheme [31,32], implanting an extra $\pi/2$ pulse in the original CPMG element, offers the favorable performance on *J* coupling modulation suppression without the strict requirement on short echo time, thus benefitting to DOSY experiments. For example, a PROJECT-based DOSY experiment, PROJECTED, is designed to suppress chemical exchange effects on apparent diffusion coefficients [27]. Similar to conventional DOSY experiments, the PROJECTED also suffers from spectral congestion and peak overlap in measurements on complex mixtures.

Herein, a general DOSY method, dubbed as PSDE-DOSY (PSYCHE Pure Shift and PROJECT Diffusion Encoding DOSY), that holds the comprehensive advantages of pure shift based spectra in high-resolution manner and spin echo based evolution with exchange effect suppression, is presented for the analysis of complex mixtures with crowded resonances and chemical exchanges. The PSDE-DOSY is designed based on the combination of the PROJECT diffusion encoding scheme and the PSYCHE pure shift element. The PROJECT diffusion encoding scheme achieves diffusion dependent signal decays for DOSY implementation, along with the suppression on effects of chemical exchange and *J* coupling modulation. Although T_2 relaxation effect still exists in the PROJECT diffusion encoding scheme, the T_2 relaxation effect is comparable to T_1 relaxation effect in the routine stimulated echo diffusion encoding scheme when DOSY experiments are performed on mixtures in liquid state. The PSYCHE element serves on recording pure shift NMR spectra containing well-resolved singlets for accurate calculation of diffusion coefficients. Benefitting from the external diffusion encoding by prepending the PROJECT diffusion encoding scheme to the parent PSYCHE sequence, the PSDE-DOSY avoids the timing requirement of an interval for inserting a diffusion encoding scheme into the parent sequence and then it can preserve the integrality of the PSYCHE element, convenient for the extension of PSYCHE-like experiments to DOSY applications.

2. Theories and methods

Fig. 1 shows the pulse sequence diagram for the PSDE-DOSY method. The PSDE-DOSY sequence is composed of the PROJECT diffusion encoding module and the 1D PSYCHE module. The PSDE-DOSY starts with a non-selective $\pi/2$ pulse to excite the initial magnetization. Then the PROJECT diffusion encoding module, containing *N* repeating PROJECT diffusion units, introduces the diffusion dependent decay process. In each PROJECT diffusion units, two pairs of diffusion gradients G_D with incremental amplitudes are embedded into the double spin echo elements separated by a $\pi/2$ pulse, thus the unit diffusion time Δ of a pair of diffusion gradients should be less than a single spin echo evolution period 2τ, where τ is the interpulse interval. Because of the intrinsic feature of PROJECT evolution, τ can be adopted in a relatively long duration without the influence of *J* modulation effects, meeting the requirement of unit diffusion time Δ and avoiding the risk of sample heating and convection by RF pulses. In addition, chemical exchange effects are also suppressed by spin echo evolution in the PROJECT diffusion encoding module, because chemical shifts are refocused during the echo period. After the diffusion decay by the diffusion encoding module, the 1D PSYCHE module, containing a non-selective π pulse, a pair of coherence selection gradients G_1, a pair of separated indirect evolution period $t_1/2$, two double frequency swept chirp pulses α matching with weak pulsed gradient G_3 and a pair of coherence selection gradients G_2, and acquisition period t_2, is performed to remove *J* coupling effects and extract pure shift spectra, in which each singlet peaks are separated and related to individual constituents, for high-resolution DOSY calculations.

Since the PSDE-DOSY sequence is composed of two independent parts, namely the PROJECT diffusion encoding module for diffusion dependent decays and 1D PSYCHE module for pure shift spectra, we can understand the signal evolutions of the PSDE-DOSY sequentially. Without loss of generality, we adopt an AX spin-1/2 system that contains *I* and *S* spins coupled by a J_{IS} coupling, and deduce the theoretical expression of the PSDE-DOSY signals using the raising and lowering operator formalism. It is assumed that Ω_I and Ω_S are chemical shifts of *I* and *S* spins, respectively, that *D* is the diffusion coefficient of the AX molecule. Effects of radiation damping and relaxation are ignored to simplify theoretical descriptions in the following. With the high-temperature approximation, the reduced density operator at initial thermal equilibrium state is given as

$$\sigma_{eq} = I_z + S_z \quad (1)$$

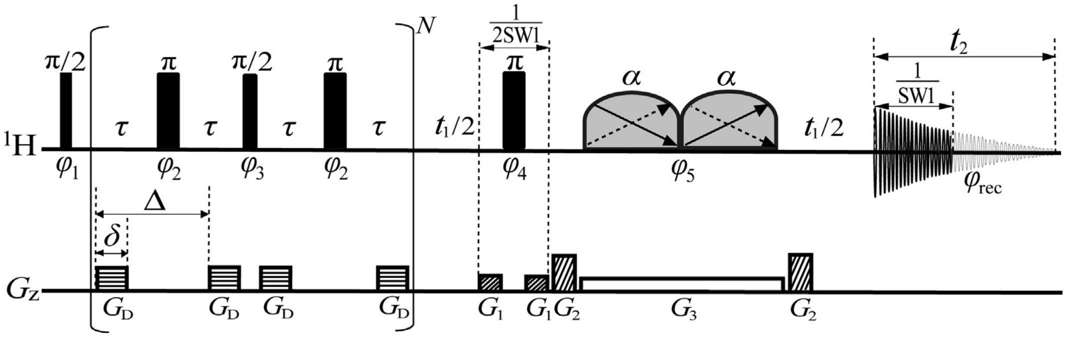

Fig. 1. Pulse sequence for the PSDE-DOSY experiment aiming for measurements on complex mixtures. The pulse sequence is composed of PROJECT diffusion encoding module and 1D PSYCHE module. Narrow and broad vertical bars indicate non-selective $\pi/2$ and π pulses, respectively. In the PROJECT diffusion module, G_D denotes diffusion gradients with incremental amplitudes, δ is the duration of diffusion gradients, Δ is the unit diffusion time, τ is the interpulse interval, N is the number of repeating PROJECT diffusion units. In the 1D PSYCHE module, two semicircle shaped pulses with two opposite diagonal arrows denote frequency-swept chirp pulses along with small flip angle α, applied matching with the week gradient G_3. Gradients G_1 and G_2 are used to select the desired coherence transfer pathway. In the acquisition period t_2, the highlighted part with the 1/SW1 interval indicates the chunk data for pure shift reconstruction. Phase cycling: $\varphi_1 = x, -x$, $\varphi_2 = y$, $\varphi_3 = y, y, y, y, -y, -y, -y, -y$, $\varphi_4 = x$, $\varphi_5 = x, x, y, y, x, x, -y, -y$, and $\varphi_{rec} = x, -x, -x, x$.

where I_z and S_z represent longitudinal magnetization components of spins I and S, respectively. After the first non-selective $\pi/2$ pulse, the density operator becomes

$$\sigma(0) = -\frac{1}{2i}\left(I^+ + S^+ - I^- - S^-\right) \tag{2}$$

From this point on, the PROJECT diffusion encoding module takes effect on the density operator evolves for introducing the diffusion dependent decay process. Because spin evolutions originated from the PROJECT scheme and diffusion gradients are independent, to clearly show the overall effect of the PROJECT diffusion encoding module, we first deduce spin evolution of the density operator, $I^+ + S^+ - I^- - S^-$, caused by a single PROJECT element of $\tau - \pi - \tau - \pi/2 - \tau - \pi - \tau$, then we consider the diffusion weighting evolution by diffusion gradients G_D. According to spin evolution of the PROJECT echo, the spin term of $I^+ + S^+ - I^- - S^-$ evolves into $-I^- - S^-$ with chemical shift refocusing and J modulation suppression. According to theoretical analyses of spin echo based diffusion element and rectangular shape gradient pattern given by Stejskal-Tanner equation [33], a diffusion weighting factor of $\exp\left[-2DG_D^2\gamma^2\delta^2(\Delta-\delta/3)\right]$ is taken into to the spin term of $-I^- - S^-$ after a PROJECT diffusion unit. N repeating PROJECT diffusion units are contained in the whole PROJECT diffusion encoding module to guarantee sufficient diffusion dependent decays. Similar to varied increments related to the indirect dimension of conventional 2D NMR experiments, diffusion gradients G_D are the incremental variable related to diffusion dimension and it can be set as an experimental variable to record the diffusion dependent decay process. Thus, after the evolution of the whole PROJECT diffusion encoding module [27], the density operator becomes

$$\sigma(G_D) = \frac{1}{2i}\left(I^- + S^-\right)e^{-2ND\gamma^2G_D^2\delta^2(\Delta-\delta/3)} \tag{3}$$

Subsequently, the density operator evolves under the 1D PSYCHE module for collapsing J coupling and recording pure shift spectra. The evolution of chemical shifts and J couplings are included into the density operator during the first indirect evolution period $t_1/2$. The gradients G_1 and G_2 are applied to select the desired coherence transfer pathway of $-1 \rightarrow +1 \rightarrow 0 \rightarrow -1$ for spin evolution. Two frequency-swept chirp pulses with the weak gradient G_3 can suppress zero-quantum coherence signals (ZQCs) and preserve desired pure shift signals with amplitudes proportional to $\sin^2\alpha$ and spurious signals with amplitudes proportional to $\sin^4\alpha$. In general PSYCHE experiments, the small flip angle is adopted in the frequency-swept chirp pulses α for suppressing spurious signals. Therefore, only desired pure shift signals are considered for the evolution in the second period $t_1/2$, and the reduced density operator evolves into

$$\sigma(G_D,t_1)=\frac{1}{4i}e^{-2ND\gamma^2G_D^2\delta^2(\Delta-\delta/3)}e^{i\Omega_I t_1}\sin^2\alpha\left[I^-\cos\left(\frac{\pi J_{IS}}{2SW1}\right)-2iI^-S_z\sin\left(\frac{\pi J_{IS}}{2SW1}\right)\right] \tag{4}$$

For simplification, we only consider the evolution of I spin in the deduction shown as Eq. (4) because of the identical evolution of I and S spins in AX spin-1/2 system. Since then, signal acquisition starts in the acquisition period t_2, in which the observable spin term I^- in Eq. (4) are acquired along with the evolution of chemical shifts and J couplings. The reduced density operator of the acquired spin term I^- gives

$$\sigma(G_D,t_1,t_2)=\frac{1}{4i}I^-e^{-2ND\gamma^2G_D^2\delta^2(\Delta-\delta/3)}e^{i\Omega_I t_1}\sin^2\alpha\cos\pi J_{IS}\left(t_2-\frac{1}{2SW1}\right) \tag{5}$$

Accordingly, the total complex transverse magnetization for I spin is given as

$$\begin{aligned}M_+^I(G_D,\ t_1,\ t_2) &= M_0^I Tr\left\{I^+\sigma(G_D,\ t_1,\ t_2)\right\}\\ &= \frac{M_0^I}{4i}\sin^2\alpha e^{-2ND\gamma^2G_D^2\delta^2(\Delta-\delta/3)}e^{i\Omega_I(t_1+t_2)}\cos\pi J_{IS}\left(t_2-\tfrac{1}{2SW1}\right)\end{aligned} \tag{6}$$

Theoretical signal expression of I spin evolved by the PSDE-DOSY sequence is presented in Eq. (6). It is suggested that the resulting signal by the PSDE-DOSY is a 3D signal varied with incremental diffusion gradients G_D, incremental variables t_1 and t_2. The incremental variable G_D corresponds to the diffusion dependent decay process caused by the PROJECT diffusion encoding module, and related data processing can be performed with respect to the incremental variable G_D to obtain diffusion information. Incremental variables t_1 and t_2 are related to pure shift evolution by the 1D PSYCHE module. Similar to the pseudo-2D acquisition and pure shift reconstruction in 1D PSYCHE experiments, a chunk of acquired data of 1/SW1 duration are combined together to generate an integrated free induced decay (FID) for pure shift spectrum. In this reconstruction procedure, t_2 is the running time variable during the chunk interval 1/SW1 (i.e. $0 \le t_2 \le 1/\mathrm{SW1}$), and the chunk of data during 1/SW1 is arranged according to the t_1 increment to form pure shift evolution as a new evolution time variable t_2', where t_2' is equal to $t_1 + t_2$, namely $t_2' = t_1 + t_2$. After the pure shift reconstruction, the chemical shift term $e^{i\Omega_I(t_1+t_2)}$ is converted into $e^{i\Omega_I t'_2}$ in Eq. (6). In addition, J couplings are refocused in the middle of each piece of chunked data in the data concatenation, thus variable t_2 can be set as 1/(2SW1). Then the theoretical expression after pure shift reconstruction is given as

$$M_+^I(G_D,\ t'_2) = \frac{M_0^I}{4i}\sin^2\alpha e^{-2ND\gamma^2G_D^2\delta^2(\Delta-\delta/3)}\mathrm{e}^{i\Omega_I t'_2} \tag{7}$$

Eq. (7) gives the signal expression of the reconstructed 2D dataset for high-resolution pure shift DOSY by the PSDE-DOSY. In this reconstructed 2D dataset, chemical shift evolution is displayed as the exponential term of $\mathrm{e}^{i\Omega_I t'_2}$ and 1D Fourier transformation is performed with respect to the variable t'_2 to yield the direct dimension containing only chemical shift information. Diffusion weighted evolution is given by the exponential decay term of $e^{-2ND\gamma^2G_D^2\delta^2(\Delta-\delta/3)}$. Due to the sufficient separation along the pure shift based spectral domain, in which each peak corresponds to individual constituents, the simple data process of monoexponential-curve fitting can be performed on the diffusion dependent decays of resolved peaks to obtain the diffusion information containing accurate diffusion coefficients and corresponding errors. Finally, a high-resolution 2D DOSY spectrum is generated by matching Gaussian pattern signals including diffusion coefficients and errors along the diffusion domain and pure shift peaks along the spectral domain [34,35]. In general, different molecular groups from the same molecule holds the identical diffusion coefficient, thus the DOSY is useful for component separation on mixtures.

3. Experiments

All experiments were performed at 295 K using a Varian NMR System (Varian 500 MHz spectrometer, Agilent Technologies, Santa Clara, CA, USA), equipped with a 5 mm XYZ indirect detection probe. A series of mixture samples are selected for designed experiments. A simple mixture of dichloromethane (80 mM), n-propanol (100 mM) and 2,3-dihydrofuran (55 mM) in DMSO-d_6 was used to illustrate the implementation of the PSDE-DOSY for high-resolution DOSY measurements. A complex two-component sample of quinine (200 mM) and geraniol (180 mM) in DMSO-d_6, which contains crowded or even overlapped NMR resonances, was used to show the ability of the PSDE-DOSY for diffusion coefficient measurements and component separations on complex mixtures. Then a complex three-component mixture of estradiol (200 mM), geraniol (200 mM) and 2,3-dihydrofuran (180 mM) in DMSO d_6 was used to verify the applicability of the PSDE-DOSY on practical samples with different components. Conventional BPPSTE DOSY and PSDE-DOSY experiments were performed on these three mixtures for comparison. To further show the actual utility of the PSDE-DOSY in practical samples that contain crowded NMR resonances and exchanging spin systems, we performed comparative DOSY experiments on a sample of maltotriose (96 mM) in DMSO-d_6 by using BPPSTE DOSY, PROJECTED, PSYCHE-iDOSY, and PSDE-DOSY methods. In addition, to demonstrate the performance of the PSDE-DOSY on suppressing effects of chemical exchange and *J* coupling compared to other existing DOSY approaches (i.e. BPPSTE DOSY, PROJECTED, PSYCHE-iDOSY, and Oneshot PSYCHE-DOSY), we performed experiments on a mixture sample of catechin (94 mM) and flavone (95 mM) in DMSO-d_6. Another comparative experiments by using BPPSTE DOSY, PSYCHE-iDOSY, Oneshot PSYCHE-DOSY and PSDE-DOSY are performed on a mixture of 200 mM quinine in DMSO-d_6 to show their performance on signal intensity. Detailed experimental settings and parameters for all aforementioned experiments are given in Supplementary Information.

4. Results and discussion

4.1. Simple mixture

Experimental results on the simple mixture of dichloromethane, n-propanol and 2,3-dihydrofuran are presented in Fig. 2. Molecular structures of three components are given in Fig. 2(a), and the standard 1D NMR spectrum accordingly assigned with molecular proton groups is shown in Fig. 2(b). Due to the simple molecular structures, all peaks are well-resolved in the 1D NMR spectrum in spite of *J* coupling splittings. Because of resolved peaks in the spectral domain, it is intuitive to obtain high-resolution DOSY spectrum with all individual constituents separated by the conventional BPPSTE DOSY method. Unfortunately, the conventional BPPSTE DOSY spectrum cannot perform well to separate components of the simple mixture (see Fig. 2(d)). Because of *J* coupling splittings, coupled protons yield multiplet peaks in the 1D spectrum, such as multiplets derived from protons 2 to 8 in Fig. 2(b). Splitting peaks of a given multiplet should share the same diffusion behavior since they are originated from the identical proton. However, due to *J* coupling evolution, splitting peaks of the multiplet generally differ in signal intensity variations during the diffusion decay process, and this leads to variations in calculated diffusion coefficients for splitting peaks of a given multiplet. For example, in the BPPSTE DOSY spectrum (Fig. 2(d)), a triplet from proton 4 at 0.92 ppm has three different calculated diffusion coefficients, corresponding to three single splitting peaks. Although this type of diffusion difference does not influence DOSY analyses on the simple sample, it may become a serious problem for measurements on complex mixtures in which extensive multiplets may introduce various diffusion coefficients and make DOSY analyses challenging. The singlet resonances from uncoupled protons intrinsically avoid the problem of diffusion coefficient variations, see the singlet peak from proton 1 at 5.65 ppm of dichloromethane in Fig. 2(d). Second, except for the influence of *J* coupling effects, the BPPSTE DOSY spectrum is also influenced by chemical exchange effects. In Fig. 2(d), it is available to distinguish peaks from dichloromethane and 2,3-dihydrofuran while it is confused to recognize signals from n-propanol. The peak from proton 2 at 3.46 ppm (marked by red dotted circle) belong to the n-propanol component, but it appear to be a spurious signal with an increased diffusion coefficient, different from other two peak signals at 0.92 ppm and 1.08 ppm. This is because the n-propanol contains a hydroxyl group connecting to proton 2 and chemical exchange effect between the hydroxyl group and the residual water in the sample increase the apparent diffusion coefficient of proton 2.

Accordingly, to overcome the influence of *J* coupling and chemical exchange effects, we propose the PSDE-DOSY method which avoids *J* coupling splitting and evolution by the PSYCHE pure shift module and suppresses chemical exchange effects by the PROJECT diffusion module. In the PSDE-DOSY experiment, a 3D raw dataset are obtained after data acquisition. Related data processing is performed to convert the original 3D raw data to a pure shift based 2D DOSY spectrum. Similar to the 1D pure shift reconstruction data processing for pseudo-2D based PSYCHE experiments, we first process the original 3D raw data into a reconstructed 2D data, which contains pure chemical shift varied with the variable t'_2 and diffusion weighted evolution varied with incremental diffusion gradients G_D. After 1D Fourier transformation on the reconstructed 2D data the variable t'_2, an array of 1D pure shift spectra with their peak intensities decayed with diffusion gradients G_D is obtained (Fig. 2(c)). From the expanded 1D pure shift spectrum in Fig. 2(c), it can be seen that all multiplet peaks are simplified into singlets, exhibiting high-resolution spectral domain for diffusion coefficient calculation. Subsequently, the standard monoexponential-curve

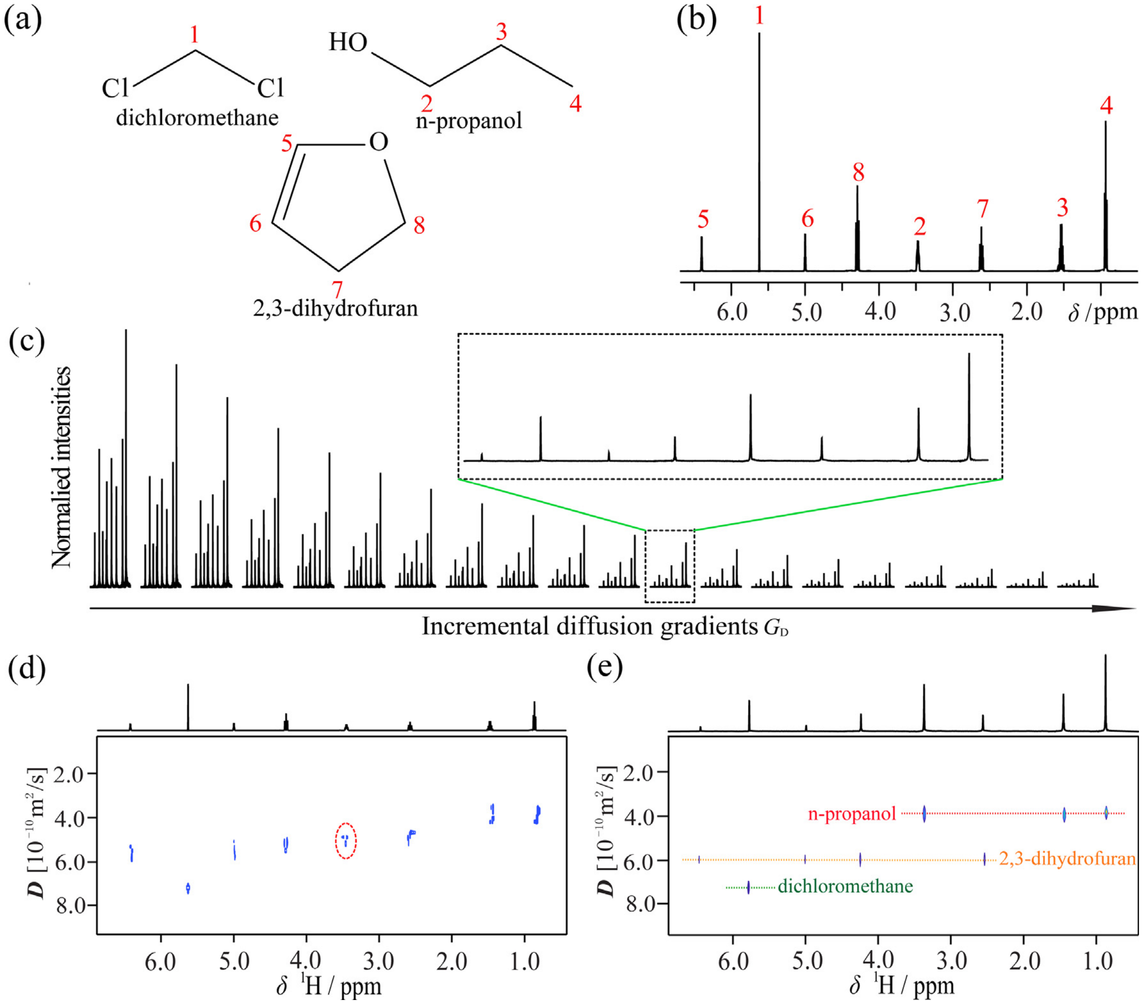

Fig. 2. Experimental results for a simple mixture of dichloromethane, n-propanol and 2,3-dihydrofuran. (a) Molecular structures of three components assigned with observed protons from 1 to 8. (b) Standard 1D NMR spectrum of the mixture. (c) An array of 1D pure shift spectra with peak intensities decayed with incremental diffusion gradients G_D obtained by the PSDE-DOSY. (d) Conventional 2D DOSY spectrum by the BPPSTE. (e) Pure shift based 2D DOSY spectrum by the PSDE-DOSY.

fitting is directly performed based on diffusion dependent peak intensities in the 1D array spectra to obtain diffusion coefficients for each peak and generate the pure shift based high-resolution 2D DOSY spectrum (Fig. 2(e)). At the first sight, all signals are displayed as singlet peaks in the PSDE-DOSY spectrum (Fig. 2(e)) and they are aligned into three group according to different diffusion coefficients along the diffusion domain, suggesting that three individual components are separated. All singlet peaks, assigned to individual protons of the sample, give the defined diffusion coefficients in the diffusion domain and avoid the diffusion coefficient variations of multiplet peaks in BPPSTE DOSY spectrum. In addition, benefitting from the suppression of chemical exchange effects by the PROJECT module, the singlet peak from proton 2 connecting a hydroxyl group of the n-propanol component shows the correct diffusion coefficient, consistent with that of other two protons 3 and 4. Therefore, compared to the conventional BPPSTE DOSY experiment, the PSDE-DOSY experiment is more applicable for accurate diffusion measurements and DOSY analyses on mixtures that contains *J* coupled molecular structures or exchanging spin systems.

To further demonstrate the performance of the proposed PSDE-DOSY on suppressing effects of chemical exchange and *J* coupling compared to other existing DOSY approaches, we performed experiments on a mixture of catechin and flavone in DMSO-d_6. Comparative 2D DOSY results are given in Fig. S1 of Supplementary Information. The BPPSTE DOSY spectrum (Fig. S1(b)) suffers from diffusion coefficient variations caused by *J* couplings and spurious component signals caused by chemical exchange effects from the catechin, and it is difficult to achieve well component separation for this mixture. The PROJECTED spectrum (Fig. S1(c)) can suppress chemical exchange effects and achieve component separation. However, it remain unable to eliminate *J* coupling splittings, and this introduces extensive muliplets peaks and leads to diffusion coefficient variations, thus influencing the resolution in the diffusion domain. Both the PSYCHE-iDOSY spectrum (Fig. S1(d)) and the Oneshot PSYCHE-DOSY spectrum (Fig. S1(e)) can eliminate *J* coupling splittings and avoid diffusion coefficient variations, thus giving accurate diffusion calculation and higher resolution in the diffusion domain. However, these two PSYCHE based DOSY approaches remain sensitive to chemical exchange effects and cannot separate catechin and flavone components due to the influence of spurious component signals. In contrast, the PSDE-DOSY spectrum (Fig. S1(f)) simultaneously suppresses effects of chemical exchange and *J* coupling. Thus it performs well for component assignments and separations high resolution along both spectral and diffusion domain in the resulting 2D DOSY spectrum. Therefore, compared to existing DOSY methods, the PSDE-DOSY may be more applicable for high-resolution DOSY measurements on mixtures that contains *J* coupled molecular structures or exchanging spin systems.

4.2. Complex two-component mixture

The PSDE-DOSY aims for high-resolution DOSY measurements on complex mixtures that suffer from crowded or even overlapped peaks in 1D NMR. The comparative experiments on the complex two-component mixture of quinine and geraniol were performed to show the ability of the PSDE-DOSY for high-resolution measurements (Fig. 3). Molecular structures of quinine and geraniol marked with observable protons are shown in Fig. 3(a). According to the acquired 1D NMR spectrum in Fig. 3(b), twenty peaks are detected and assigned to protons 1–20 of the quinine component and eight peaks are assigned to protons 21–28 of the geraniol component. Because of numerous resonances and extensive *J* coupling splittings, the phenomenon of spectral congestion is observed the 1D NMR spectrum, e.g. the region from 1.5 ppm to 2.5 ppm. In particular, multiplets from protons 12 to 14 of the quinine, and multiplets from proton 10 of the quinine as well as proton 22 of the geraniol are overlapped. Well-resolved peaks in the 1D spectral domain are the basic requirement for high-resolution DOSY calculation. Based on the calculation from 1D diffusion dependent decayed spectra, such as the 1D in Fig. 2(b), conventional 2D DOSY spectrum (Fig. 3(c)) obtained by the BPPSTE cannot give accurate diffusion coefficients for crowded and overlapped multiplet peaks. From the congested regions marked by red dotted circle in Fig. 3(c), it can been seen that signals tangle together and yield inaccurate diffusion coefficients in the diffusion domain. Therefore, it is difficult to separate mixture components from the conventional 2D DOSY spectrum.

In contrast, the PSDE-DOSY experiment can bypass spectral congestion and resolve peaks in the spectral domain by suppressing *J* coupling splittings, thus generating the desired high-resolution 2D DOSY spectrum (Fig. 3(d)). In this PSDE-DOSY spectrum, each signal appears as well-resolved singlet peak in the chemical shift dimension. The corresponding relationship between each singlet peak and individual components are observed in the PSDE-DOSY spectrum (Fig. 3(d)), by tracking different sites in the spectral domain and related diffusion coefficients in the diffusion domain. The tangled signals observed in the conventional BPPSTE DOSY spectrum (see signals in red dotted circle in Fig. 3(c)) are well separated and classified to different components in the PSDE-DOSY spectrum (see signals in blue dotted circle in Fig. 3(d)). Even for overlapped signals in the conventional BPPSTE DOSY spectrum, e.g. multiplets at 5.28 ppm from two components (proton 10 of the quinine and proton 22 of the geraniol), are distinguished and associated with the respective components in the PSDE-DOSY spectrum. Consequently, it is intuitive to separate individual components of quinine and geraniol by tracking different diffusion coefficients along the diffusion domain. There exist some slight variations on diffusion coefficients for observed signals from the same component in Fig. 3(d). This may be caused by the influence of gradients used in the PSYCHE module and the simple data fitting of diffusion dependent decays. However, these slight variations do not influence identification and separation of two mixed components. Besides enhanced resolution in the pure shift based spectral domain, the PSDE-DOSY spectrum also presents higher resolution in the diffusion domain in contrast with conventional

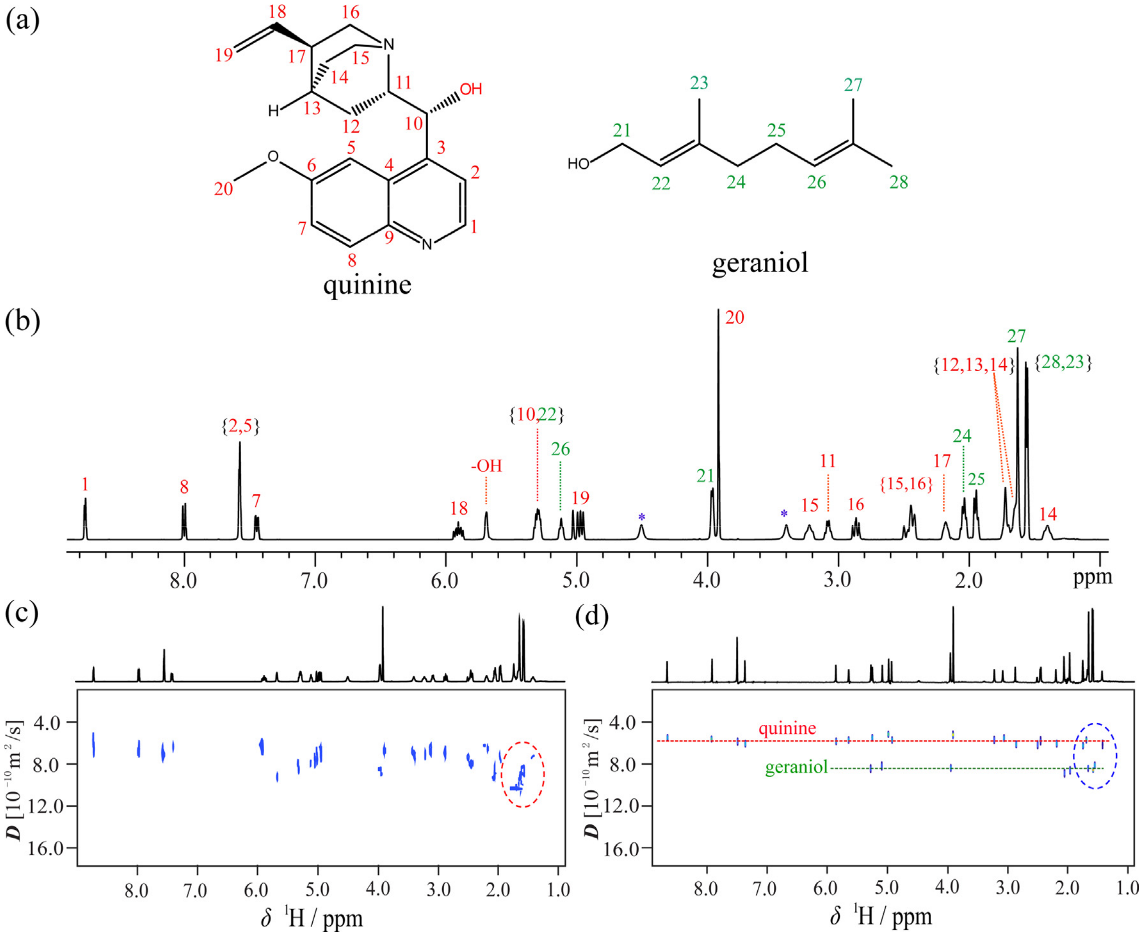

Fig. 3. Experimental results for a complex two-component mixture of quinine and geraniol. (a) Molecular structures of quinine and geraniol assigned with observed protons from 1 to 28. (b) Standard 1D NMR spectrum of the two-component mixture, and all peaks are assigned to corresponding protons. (c) Conventional 2D DOSY spectrum by the BPPSTE. (d) Pure shift based 2D DOSY spectrum by the PSDE-DOSY.

DOSY spectrum. Therefore, the PSDE-DOSY is more suitable for accurate diffusion measurements and component separations on complex mixtures.

4.3. Complex three-component mixture

In this section, the PSDE-DOSY is applied to a three-component mixture of estradiol, geraniol and 2,3-dihydrofuran to further show its applicability of component separation on complex mixtures, and experimental results are shown in Fig. 4. Molecular structures of three components are given in Fig. 4(a). The BPPSTE sequence is applied for conventional 2D DOSY spectrum (Fig. 4(b)), which contains *J* coupling splittings in the spectral domain. A wide chemical shift distribution (0.5–9.0 ppm) is shown in the spectral dimension of the conventional DOSY spectrum. However, there exist spectral regions where observed signals from different components are crowded and even overlapped. For example, in the spectral region between 0.90 and 2.40 ppm, numerous peaks derived from estradiol and geraniol components are included, and they give rise to severe spectral congestion and overlap by additional *J* coupling splittings. It is clear that the conventional DOSY spectrum confronts the problem of distinguishing individual resonances in the congested region of the spectral domain. This subsequently leads to inaccurate diffusion coefficients, which may be contributed from different decays of overlapped resonance, and gives tangled signals in the congested region of the conventional DOSY spectrum (Fig. 4(b)). Although the 2,3-dihydrofuran component is partly separated due to its resolved peaks and large diffusion coefficients, it remains difficult to completely separate three components by the conventional BPPSTE DOSY. Contrastively, the pure shift based 2D DOSY spectrum by the PSDE-DOSY performs well to resolve peaks even overlapped ones in the spectral domain (Fig. 4(c)). After the mono-exponential fitting on the diffusion decay process, high-resolution diffusion information is generated in the diffusion domain in Fig. 4(c). Then signals from estradiol, geraniol and 2,3-dihydrofuran components are separated according to their diffusion coefficients. Although signals from the same component still have slight variations on diffusion coefficients, these variations are smaller than the diffusion coefficient differences among different components, thus the separation for the three-component mixture is not influenced.

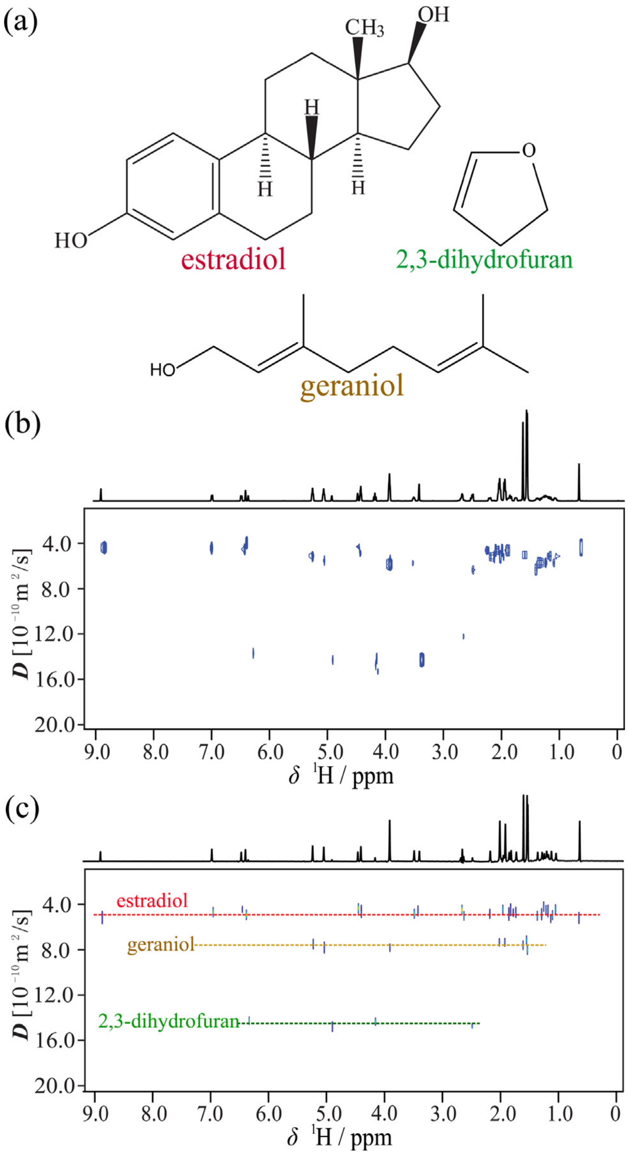

Fig. 4. Experimental results for a complex three-component mixture of estradiol, geraniol and 2,3-dihydrofuran. (a) Molecular structures of three components. (b) Conventional 2D DOSY spectrum by the BPPSTE. (c) Pure shift based 2D DOSY spectrum by the PSDE-DOSY, along with separated components.

4.4. Practical sample

We further performed experiments on a sample of maltotriose in DMSO d_6 to show the utility of the PSDE-DOSY in practical samples that contain crowded NMR resonances and exchanging spin systems (Fig. 5). Fig. 5a shows the molecular structure of maltotriose along with hydroxyl groups marked by red fonts. This molecular structure contains carboatomic rings, generally yielding crowded *J* coupling multiplet resonances, and eleven hydroxyl groups, responsible for chemical exchange effects. Because of these two influence factors, it is difficult to obtain accurate diffusion measurements and correct component assignments for the maltotriose in the conventional BPPSTE DOSY spectrum (Fig. 5(b)). In this spectrum, crowded multiplet peaks (particularly in the region between 3.0 and 4.0 ppm) are observed in the spectral domain, which leads to obvious diffusion coefficient variations and gives inaccurate measurements in the diffusion domain. Additionally, chemical exchange effects increase apparent diffusion coefficients of protons from hydroxyl groups, and generate spurious component signals in the BPPSTE DOSY spectrum (see signals marked by red dotted circles in Fig. 5(b)). These spurious signals belong to the maltotriose and they deviate from the desired diffusion coefficient around 1.0×10^{-10} m^2/s, thus hindering correct component assignments for the maltotriose. The PROJECTED based on PROJECT echo evolution can effectively suppress chemical exchange effects and yield correct diffusion coefficients for protons from hydroxyl groups. From the PROJECTED spectrum in Fig. 5(c), it is clear that all signals align correctly near to the desired diffusion coefficient in the diffusion domain. However, similar to the BPPSTE DOSY, the PROJECTED also suffers from crowded *J* coupling multiplet peaks in the spectral dimension, and this yields variations on diffusion coefficients and influences resolution in the diffusion domain. The PSYCHE-iDOSY presents an effect manner to generate pure shift spectra for diffusion calculations. Compared to BPPSTE DOSY and PROJECTED, the PSYCHE-iDOSY provides the pure shift based 2D DOSY spectrum with simplified singlet peaks in the spectral domain and with higher resolution in the diffusion domain, as shown in Fig. 5(d). The PSYCHE-iDOSY is intrinsically designed based on the stimulated echo diffusion encoding, therefore it is difficult to avoid chemical exchange effects from hydroxyl groups. Similar to the BPPSTE DOSY spectrum (Fig. 5(b)), spurious component signals with increased apparent diffusion coefficients are also observed in the PSYCHE-iDOSY spectrum (see signals marked by

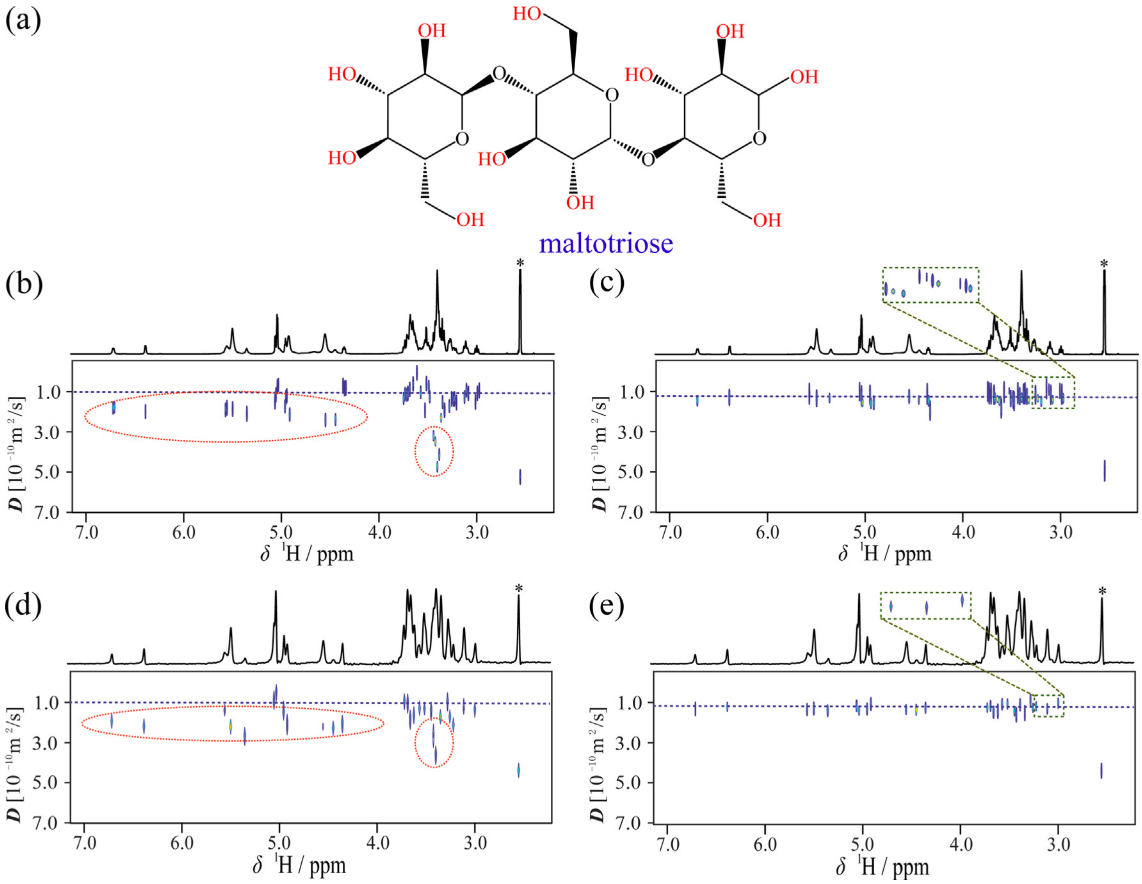

Fig. 5. Experimental results for a sample of maltotriose in DMSO-d_6. (a) Molecular structure of the maltotriose, along with hydroxyl groups marked by red fonts. 2D DOSY spectra obtained by BPPSTE DOSY (b), PROJECTED (c), PSYCHE-iDOSY (d), and PSDE-DOSY (e). Spurious component signals are marked by red dotted circles in (b) and (d). Expanded regions are marked by green dotted boxes in (c) and (e). Residual solvent signals are marked by *. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

red dotted circles in Fig. 5(d)). Combining the advantages of pure shift extraction and PROJECT echo evolution, the PSDE-DOSY can simultaneously suppress chemical exchange effects and simplify *J* coupling multiplets in the spectral domain, thus yielding higher resolution in both spectral and diffusion domain. From the PSDE-DOSY spectrum in Fig. 5(e), it is clear that all signals present as singlet peaks in the spectral domain, which is benefit to the reduction of diffusion coefficient variations in the diffusion domain. From the comparison of expanded regions marked by green dotted boxes in PROJECTED and PSDE-DOSY spectra, it can be seen that the PSDE-DOSY can convert multipelt signals into singlets in the spectral domain and generate high resolution along the diffusion domain. Additionally, benefitting from the suppression on chemical exchange effects, spurious signals are removed and all desired component signals align correctly along the diffusion domain of the PSDE-DOSY spectrum, useful for the component assignments and separations.

The performance of the PSDE-DOSY for high-resolution DOSY measurements on complex mixtures are demonstrated in aforementioned experiments. The PSDE-DOSY method is designed based on pure shift based elements. Similar to other pure shift DOSY experiments, e.g. ZS-DOSY and PSYCHE-iDOSY, the PSDE-DOSY intrinsically inherits the disadvantage of low signal intensity in contrast with conventional DOSY experiments. The low signal intensity in DOSY experiments may influence the calculation accuracy of diffusion coefficients. Despite that, significant enhancement on spectral resolution by pure shift based DOSY experiments can relieve the influence of low signal intensity for accurate calculation on diffusion coefficients and achieves satisfactory component separation for complex mixtures that contains crowded and even overlapped signals. It is proved that internal diffusion encoding DOSY experiments (i.e. iDOSY) have double signal intensity compared to external diffusion encoding DOSY experiments. Different from iDOSY based experiments (e.g. PSYCHE-iDOSY), the PSDE-DOSY, based on the design of diffusion gradients externally concatenated with the pure shift element, is unable to gain this signal benefit. However, the PSDE-DOSY gains the signal compensation from the spin echo evolution, which holds twice signal intensity higher than that from the stimulated echo evolution. The comparative experiments by using BPPSTE DOSY, PSYCHE-iDOSY, Oneshot PSYCHE-DOSY and the proposed PSDE-DOSY are performed on a sample of 200 mM quinine in DMSO d_6 to demonstrate their performance on signal intensity (see Fig. S2 in Supplementary Information). Because of the signal loss caused by the PSYCHE pure shift evolution, SNR of 1D pure shift spectra by three PSYCHE based DOSY are an order of magnitude lower than that of standard 1D NMR spectrum by BPPSTE DOSY. Then, we focus on the SNR comparison among three PSYCHE based DOSY experiments. Benefitting from internal diffusion encoding mode, the SNR of the PSYCHE-iDOSY (Fig. S2(b)) is around twice than that of Oneshot PSYCHE-DOSY (Fig. S2(c)). The PSDE-DOSY adopts the same external diffusion encoding mode as the Oneshot PSYCHE-DOSY, but the SNR of PSDE-DOSY (Fig. S2(d)) remains 1.57 times higher than that of Oneshot PSYCHE-DOSY, resulting from the spin echo evolution. Although the PSDE-DOSY in current version remains inferior to the PSYCHE-iDOSY on the performance of signal intensity, the PSDE-DOSY may be convenient for the extension of PSYCHE-like experiments to DOSY measurements since it preserves the integrality of the PSYCHE element and avoids the timing requirement for inserting a diffusion encoding element. In addition, the PSDE-DOSY also holds the ability of suppressing chemical exchange effects due to the spin echo evolution. Therefore, the PSDE-DOSY can solve the problem of chemical exchange effects encountered in PSYCHE-iDOSY as well as Oneshot PSYCHE-DOSY, and provides an effect way for high-resolution DOSY measurements on complex mixtures with crowded NMR resonances and exchanging spin systems.

Compared to conventional DOSY experiments, the PSDE-DOSY yields higher diffusion resolution along the diffusion domain, thus providing accurate diffusion coefficient values and achieving decent component separation for investigation mixtures, particularly for complex mixtures. Due to the influence of gradients used in the PSYCHE module and the simple data fitting of diffusion decays (i.e. monoexponential-curve fitting), there exist slight variations on diffusion coefficient values for signals from the identical component in resulting spectra by the PSDE-DOSY, particularly for applications to complex mixture samples. This phenomenon is also observed in previous pure shift based DOSY experiments [21], and it does not influence the identified separation for most mixture samples, even for which diffusion coefficients of individual components differ slightly. For our experiments on complex mixtures shown as Fig. 3(c) and Fig. 4(c), the PSDE-DOSY performs well to distinguish mixture components. Additionally, in the data processing, we also consider diffusion effects of gradients G_1, G_2 and G_3 used in the PSYCHE module to further correct the diffusion scale of resulting DOSY spectra and improve the calculated accuracy of diffusion coefficients. However, the phenomenon of diffusion coefficient variations still exists, and some other advanced DOSY processing approaches, such as inverse Laplace transform [36] or PALMA [37], may be introduced to the PSDE-DOSY for further enhancements on diffusion coefficient accuracy.

5. Conclusion

In summary, we present a DOSY method, PSDE-DOSY, to achieve high-resolution 2D DOSY measurements on complex mixtures by presenting resolved pure shift peaks in the spectral domain and generating accurate diffusion coefficients in the diffusion domain. Benefitting from the combination of the PSYCHE element collapsing multipelts into singlets and the PROJECT diffusion encoding scheme suppressing effects of chemical exchange and *J* coupling modulation, the PSDE-DOSY is suitable for DOSY applications to complex mixtures that contains crowded or even overlapped resonances and exchanging spin systems. Experiments from simple chemical mixture to complex mixtures are performed to demonstrate the ability of the PSDE-DOSY for high-resolution DOSY measurements on diffusion coefficient measurements and component separations. With simple data processing and convenient pulse sequence design, the PSDE-DOSY may be an interesting choice for pure shift DOSY applications to complex mixtures. Similar to other pure shift based experiments, the PSDE-DOSY also suffers the lower signal intensity than conventional DOSY, and improvement of the PSDE-DOSY for wide applications may be achieved by combining with other pulse sequence techniques and data processing approaches.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmr.2019.07.021.

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