Journal of Magnetic Resonance 196 (2009) 78–83

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

Communication

Two-dimensional DOSY experiment with Excitation Sculpting water suppression for the analysis of natural and biological media

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ARTICLE INFO

Article history:
Received 24 July 2008
Revised 17 September 2008
Available online 30 September 2008

Keywords:
BPPSTE
Excitation sculpting
Water suppression signal
2D DOSY
Complex mixtures

ABSTRACT

The Bipolar Pulse Pair Stimulated Echo NMR pulse sequence was modified to blend the original Excitation Sculpting water signal suppression. The sequence is a powerful tool to generate rapidly, with a good spectrum quality, bidimensional DOSY experiments without solvent signal, thus allowing the analysis of complex mixtures such as plant extracts or biofluids. The sequence has also been successfully implemented for a protein at very-low concentration in interaction with a small ligand, namely the salivary IB5 protein binding the polyphenol epigallocatechine gallate. The artifacts created by this sequence can be observed, checked and removed thanks to NPK and NMRnotebook softwares to give a perfect bidimensional DOSY spectrum.

1. Introduction

The ^{1}H monodimensional NMR experiment with solvent suppression signal is an established protocol to analyze mixtures in non-deuterated water. A large number of NMR pulse sequences eliminating the strong water resonance has already been described in the literature. The most commonly-used water suppression technique is based on saturation of water magnetization by a presaturation pulse (CW) applied during the relaxation delay. Other methods include the WATERGATE scheme (WATER suppression by GrAdient Tailored Excitation) [1,2], ES (Excitation Sculpting) [3] or, more recently, the SOGGY sequence (Solvent-Optimized Gradient-Gradient spectroscopY) developed by Nguyen et al. [4].

Among all the techniques available in NMR, the analysis of a complex mixture can be simplified by the use of Diffusion-Ordered SpectroscopY (DOSY), in which the introduction of a second dimension allows a diffusion coefficient-based separation of the components [5,6].

The insertion of specific water suppression pulse sequences in a DOSY experiment is critical. The constant CW amplitude presaturation pulse is generally insufficient to completely suppress the signal of the protonated solvent. Alternatively, the WATERGATE or the ES pulse sequences inserted at the end of the diffusion measurement sequence produce some artifacts due to unavoidable accidental echoes between water suppressing Pulsed-Field Gradients (PFGs) and the incremented diffusion PFGs.

Another approach consists in combining two experiments, for example the Pulsed-field Gradient Spin Echo (PGSE) with the WATERGATE scheme [7] or with an ES water suppression sequence [8]. However, the dependence of the PGSE sequence on the transversal relaxation (T_2) precludes its application to systems with broad line widths such as polymers, proteins, lipids or macromolecules studied at low temperature. Recently, Zheng et al. [9] proposed an interesting diffusion measurement sequence, the Pulsed Gradient STimulated Echo (PGSTE) WATERGATE for protein and polymer analysis, which leads to an excellent solvent suppression. To work properly, this sequence relies on a diffusion gradient participating to water suppression, limiting the range of available gradient values. In consequence it can hardly be applied to the analysis of mixtures containing small and large compounds or of protein ligand interactions. PGSE-ES [8] and PGSTE-WATERGATE [9] are efficient sequences for 1D diffusion coefficient measurements but they lead to imperfect solvent suppression for weak gradient values, thus giving artifacts while acquiring and processing two dimensional diffusion spectra.

We propose a new two dimensional DOSY experiment based on a Bipolar Pulse Pair STimulated Echo (BPPSTE) sequence including an ES water signal suppression sequence. This experiment can be used for (i) diffusion measurements, (ii) analysis of complex mixtures such as biofluids (plasma...), plant extracts, proteins, or polymers, and (iii) protein–ligand interaction studies. We also show that the accidental echoes generated for some diffusion-encoding

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doi:10.1016/j.jmr.2008.09.022

gradient values and observed on 1D experiments can be theoretically explained by calculation of the coherence transfer pathways and eliminated.

2. Results and discussion

2.1. NMR sequence

Basic pulse sequences PGSE and PGSTE are two different approaches for diffusion measurement by NMR based on magnetization coherence during diffusion time. A variant of PGSTE, using a bipolar gradient pulse pair, reduced the effect of inhomogeneous background gradients [10] and the insertion of a supplementary delay at the end attenuated the longitudinal eddy current effects (LED) [11].

The BPPSTE-ES NMR pulse sequence is shown in Fig. 1. The stimulated echo with bipolar pulse pair was used to dephase the nuclear magnetization and rephase it after the diffusion-encoding delay (Δ) [10,12]. The $\pi/2$ pulses after the bipolar gradients transferred magnetization on the z-axis, thus reducing T_2 relaxation, and allowing spoiler gradients (G_1 and G_2) to be applied. A LED delay was incorporated between the BPPSTE and the final ES pulse sequence [3]. 2D spectra were obtained by incrementing the gradient strengths on a series of 1D experiments and by fitting the experimental signal attenuation to the Stejskal–Tanner equation

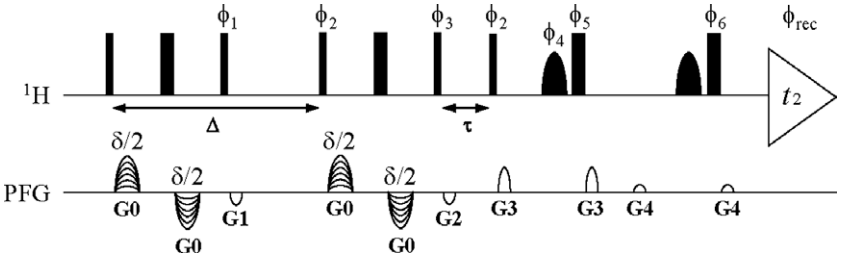

Fig. 1. BPPSTE-ES pulse sequence. Narrow and wide bars represent $\pi/2$ and π pulses, respectively. To excite the narrowest solvent spectral region, selective 180° square-shaped pulses of 1000 μs length were applied and centered at 4.7 ppm. The Δ and δ values depend on the mixture under study. A gradient recovery delay of 3 ms and a LED (τ) of 10 ms were used. The spoiler gradients G_1 and G_2 were 1 ms long, with a field strength of −7.92 and −6.09 G cm^{-1}, respectively. G_3 and G_4 for the ES sequence were 1 ms long, with a field strength of 14.33 and 5.08 G cm^{-1}, respectively. The diffusion-encoding gradient G_0 was used from 5 to 95%, with the gradient system previously calibrated to 46.25 G cm^{-1} at maximum intensity. Phases were x unless indicated. Phase cycles were $\phi_1 = x, x, -x, -x$; $\phi_2 = 4x, 4(-x), 4(y), 4(-y)$; $\phi_3 = x, -x, x, -x, -x, x, -x, x, y, -y, y, -y, -y, y, -y, y$; $\phi_4 = 16x, 16(y)$; $\phi_5 = 16(-x), 16(-y)$; $\phi_6 = -x$ and $\phi_{rec} = x, -x, -x, x, -x, x, x, -x, -y, y, y, -y, y, -y, -y, y, -x, x, x, -x, x, -x, -x, x, y, -y, -y, y, -y, y, y, -y$.

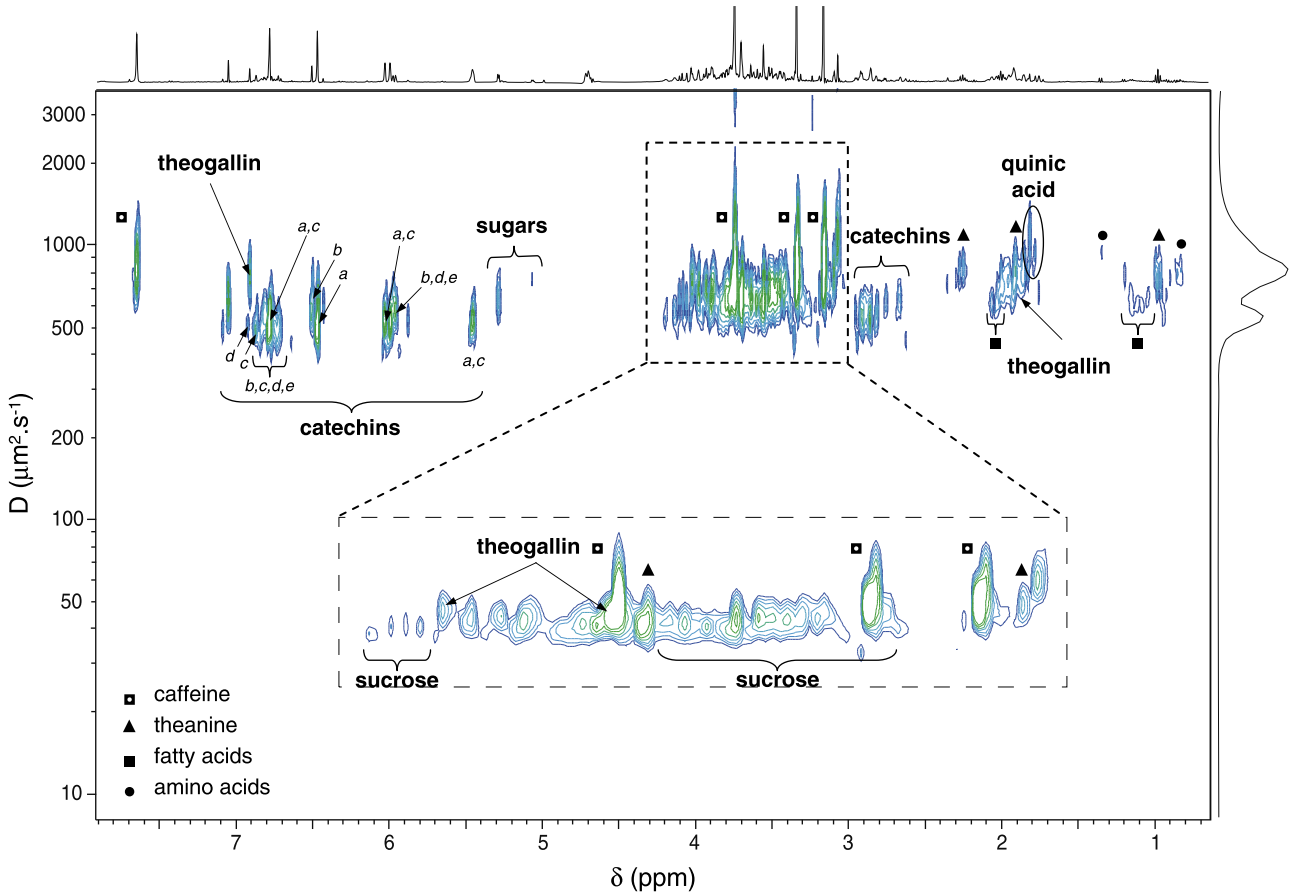

Fig. 2. ^{1}H DOSY-ES NMR spectrum of black tea infusion recorded at 298 K (10% D_2O, pH 5.2). [a]epigallocatechin gallate, [b]epigallocatechin, [c]epicatechin gallate, [d]epicatechin, [e]catechin. The blue and green colors represent weak and strong signal intensities, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

$$I = I_0 e^{-D\gamma^2\delta^2 g^2 \Delta'}$$

in which I and I_0 are the signal amplitude with and without gradient pulse, D the diffusion coefficient, γ the gyromagnetic ratio, δ the duration of the bipolar gradient pulse pair, g the gradient amplitude and Δ' the diffusion time including the correction delay due to the gradient pulse width [13].

This sequence has several advantages when compared to PGSE-ES or PGSTE-WATERGATE. The solvent suppression and the diffusion measure steps are performed at different periods of the sequence. This allows an optimum solvent suppression even with very small diffusion gradients (G_0 in Fig. 1), and the easy measure of light as well as heavy diffusing species. The ES technique presents superior water suppression when compared to the WATERGATE technique, but four spin-echoes are required in the sequence that may introduce J-modulation distortions and T_2 attenuation. However since each spin echo can be optimized independently, the total echo time can be minimized. The total echo time used in the examples presented here is of the order of 10 ms, which is comparable or smaller than in previous examples [8,9].

2.2. Application to natural or biological medium

1D ^{1}H and 2D DOSY-ES experiments were acquired on a 11.7 T Bruker AVANCE 500 spectrometer operating at 500.13 MHz, equipped with a 5 mm proton cryo-cooled probe. Experiments were performed at 298 K and TMPS (trimethylsilylpropane sulfonic acid) was used as an internal reference. All data were processed using the NPK software [14] with the inverse Laplace Transform method using the Maximum Entropy algorithm (MaxEnt). The NMRnotebook software [15] was used for spectra analysis.

The BPPSTE-ES sequence was successfully applied to analyze a natural medium, black tea infusion (Fig. 2), and human plasma (Fig. 3). Thirty-two scans and 64 or 80 increments (≈2 h) were sufficient to obtain a spectrum with satisfactory resolution in the second dimension and a good signal-to-noise ratio.

Fig. 2 depicts the black tea infusion 2D DOSY-ES spectrum. The assignment of ^{1}H signals has already been described in the literature [16]. The diffusion coefficient-based separation in the second dimension allows the localization of the proton peaks corresponding to the same organic component (e.g. caffeine, theogallin, theanine, or catechins), and to assign the main black tea constituents. The DOSY experiment also allows trace detection in complex mixtures [17]. In this case, some small signals in the 2D DOSY-ES spectrum corresponding to fatty acid or anomeric protons of sugars could be observed and attributed thanks to excellent water suppression.

The main interest of the 2D DOSY experiment is the molecular weight-based separation of the compounds. Combined with water signal suppression, the BPPSTE-ES sequence provides a powerful means of analyzing human plasma. The large difference between the molecular weights of "small" metabolites and macromolecules (proteins, lipids) leads to excellent separation on the 2D DOSY-ES

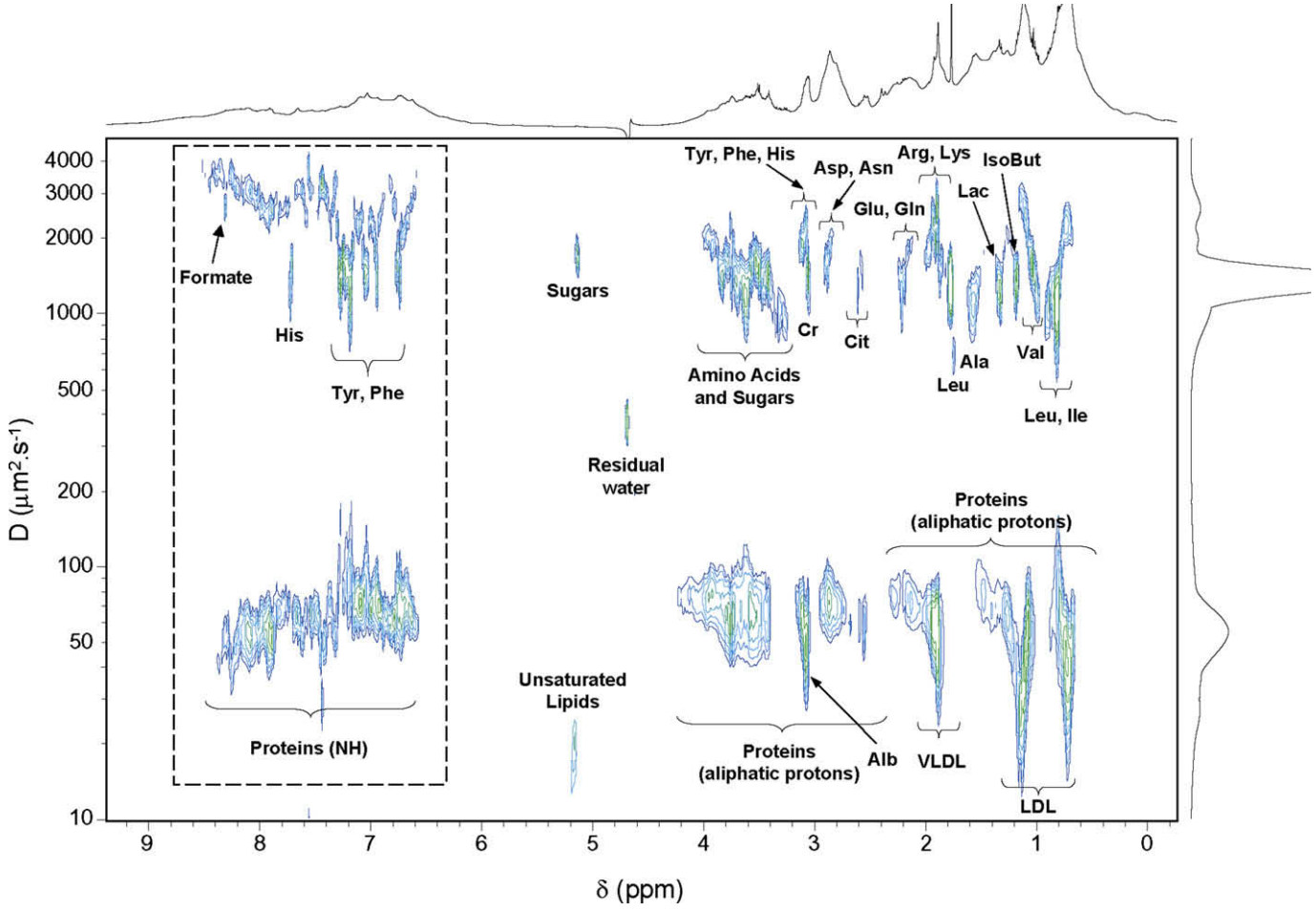

Fig. 3. ^{1}H DOSY-ES NMR spectrum of human plasma recorded at 298 K (pH 7.3). The spectrum was acquired with a 12 ppm spectral window and a recycling delay of 2 s. Partial assignments are reported on the spectrum according to the literature [20]. Isobutyrate (IsoBut), Creatine (Cr), Citrate (Cit), Albumine (Alb), low and very-low density lipoproteins (LDL and VLDL).

spectrum (Fig. 3). The plasma metabolite assignment generally needs at least two 1D NMR experiments [18]: a pulse-and-acquire sequence, in which large signals of macromolecules preclude small molecules analysis, and a transverse relaxation-edited sequence (also called a Carr-Purcell-Meiboom-Gill (CPMG)) in which small molecules are observed due to the suppression of signals from protons with short T_2 relaxation times. The signal of both types of components can be detected in only one step with the DOSY-ES experiment.

Low molecular weight metabolites (amino acids, lactate, creatine...) and proteins or lipids are localized at the top and bottom parts of the spectrum, respectively, due to high (>1000 $\mu m^2 s^{-1}$) and low (<100 $\mu m^2 s^{-1}$) diffusion coefficients (Fig. 3).

2.3. Application to protein–ligand interaction

The BPPSTE-ES sequence was also used for a protein–ligand interaction study. The experiment was performed on a Bruker AVANCE 600 spectrometer, equipped with a 5 mm z-gradient reverse triple resonance cryoprobe.

Fig. 4 shows the DOSY spectrum of salivary protein IB5 in interaction with polyphenol epigallocatechin gallate. It can be observed that the polydispersity of the DOSY peaks of the IB5 protein increased during the interaction (broad peak around 2 ppm). The hydrodynamic radius of IB5 increased at the same time and was measured, by using ethanol as a reference: it ranged from 13–27 Å, while it was in the range 13–15 Å for IB5 alone [19].

The excellent water signal suppression shown in Fig. 4 demonstrates that this sequence is a remarkable tool for protein ligand binding analysis; it has a good sensitivity and relatively weak acquisition time (2.5 h) for a very-low protein concentration (70 μM).

2.4. Resolution of artifacts

The presence of ES in the BPPSTE sequence allows the increase of the receiver gain in order to obtain a better signal-to-noise ratio compared with a conventional CW presaturation, and it provides a spectrum with more information on organic compounds at low concentrations. However, the gradients used in the ES sequence (G_3 and G_4) may produce accidental echoes for some diffusion-encoding gradient values.

In the black tea experiment, the 1D spectra for different G_0 values show that the fortuitous echoes between ES and BPPSTE gradients eliminate the water signal suppression effect (Fig. 5). The direct consequence is a saturation of the receiver and thus a non-exploitable 1D spectrum (Fig. 5C).

After applying a pulse sequence such as in Fig. 1, the magnetization intensity can be computed by evaluating the dephasing applied to magnetization, for a chosen magnetization coherence pathway and given a set of gradient intensities.

This procedure was tested with a home written python program, which was used to model the remnant water signal intensity, for all the gradient values used during the DOSY experiment. This program works on single isolated spins only, and does not try to model multiple quanta signals or depolarization field effects either.

With this modeling and in the experimental conditions of Fig. 5, it was found that the experiment with a diffusion gradient of

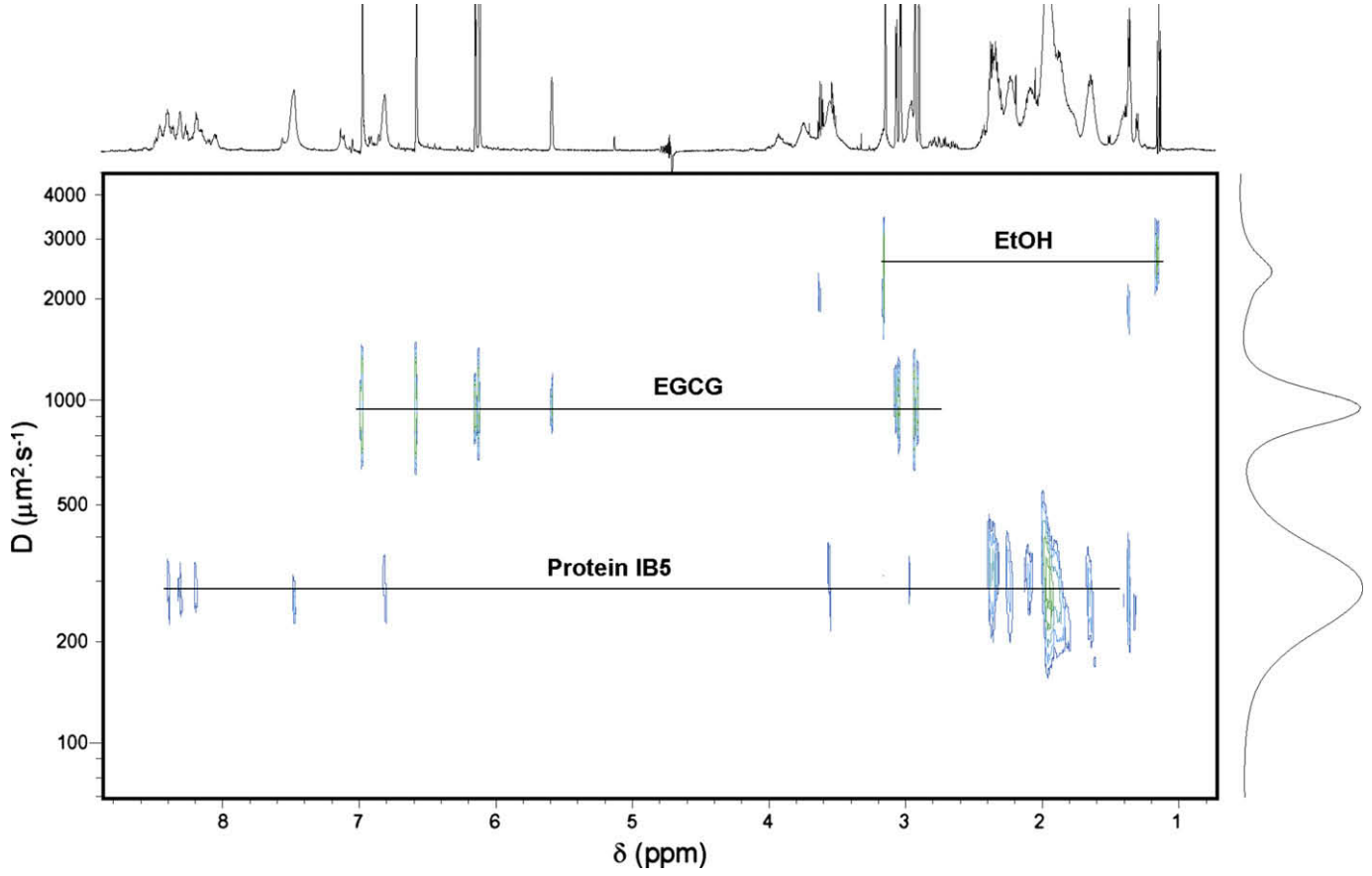

Fig. 4. ^{1}H DOSY-ES NMR spectrum, recorded at 297 K, for human salivary protein IB5 at 70 μM in H_2O/D_2O (90/10) with 12 equivalents of epigallocatechin gallate (EGCG) and 100 mM of NaCl (pH 3.5). Forty gradient increments were acquired in 128 scans, with a diffusion time of 150 ms and bipolar pulse field gradient total duration of 2.6 ms for a total of 2.5 h. Ethanol (EtOH) was used as an internal diffusion calibration compound [21,22].

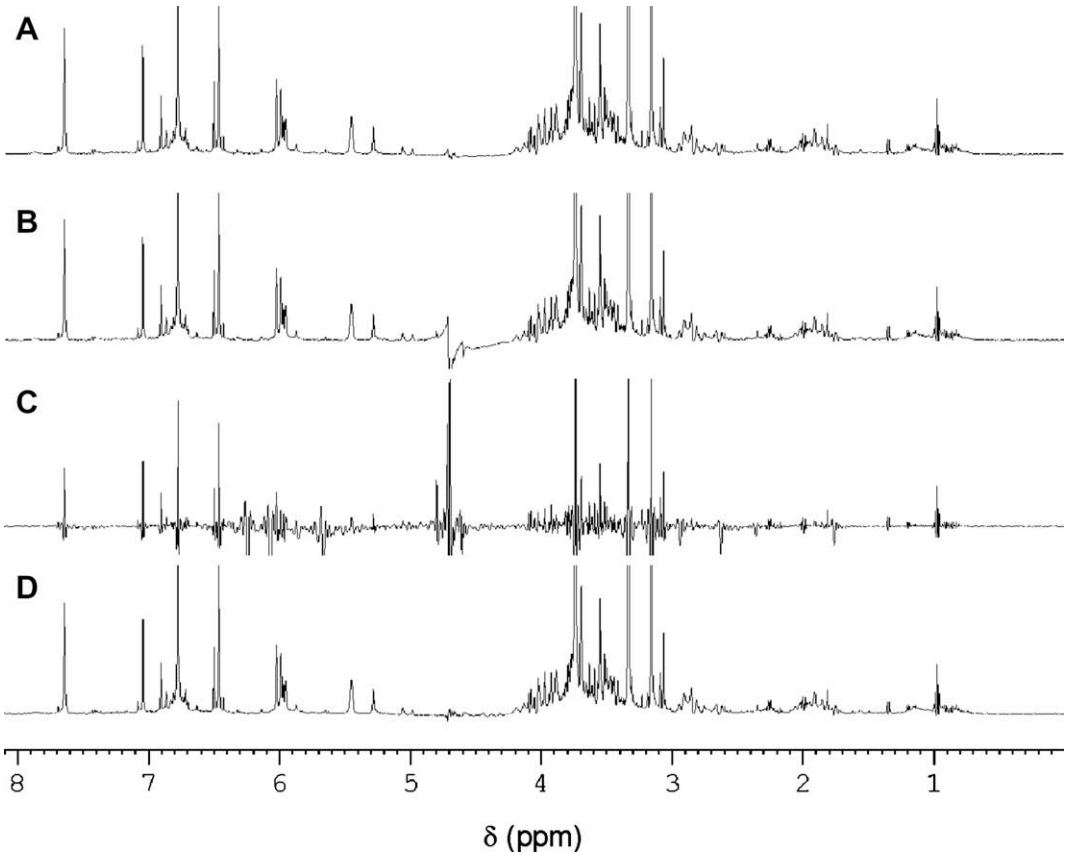

Fig. 5. ^{1}H 1D projections of the black tea infusion 2D DOSY-ES spectrum for different increments (12 (A), 13 (B), 14 (C), and 15 (D)) corresponding to gradient field strengths of 9.6, 10.2, 10.9, and 11.6 G cm^{-1}, respectively. Bipolar pulse field gradient duration is 1.5 ms with a sineshaped format. The 1D spectrum (C) corresponds to the distorted FID. This FID suffered from ADC overload due to the intense unsuppressed solvent signal. This distorted all resonances in the spectrum and made them appear less intense that they would otherwise be.

Table 1
Principal magnetization coherence transfer pathways for the BPPSTE-ES pulse sequence for 1° and 10° flip angle errors for different increments corresponding to ^{1}H 1D projections shown in Fig. 5

Grad	1° Error		10° Error	
	Maxt I	Pathway	Maxt I	Pathway
9.6	0.52	(1,−1,0,1,−1,0,−1,−1,−1)	0.78	(0,0,0,1,−1,0,−1,0,−1)
			0.42	(1,−1,0,1,−1,0,−1,−1,−1)
10.2	—	—	3.10	(1,−1,0,1,0,1,0,−1,−1)
10.9	1.04	(1,−1,0,1,0,−1,−1,−1,−1)	1.06	(0,−1,0,1,−1,−1,−1,−1,−1)
			10.11	(1,−1,0,1,0,−1,−1,−1,−1)
11.6	—	—	0.23	(0,0,0,1,0,1,0,0,−1)

Grad, gradient field strengths in G cm^{-1}.
Maxt I, maximum estimated intensity of the water artifact signal.

10.9 G cm^{-1} was the only one showing a strong spurious water signal, which comes from a coherence transfer pathway where magnetization goes to the *z*-axis after the second 180° pulse and is excited to −1 quanta by the fourth 90° pulse. This pathway is significantly populated for a 10° flip angle error (10.11 in Table 1), and probably associated with the imperfect B_1 homogeneity. In this case, limited spatial extension can reduce some artifacts, by using a Shigemi NMR tube, for example.

To obtain an exploitable spectrum, the solution is to delete the distorted FID and to correct accordingly the gradient list used for the processing. The NPK software [14] integrates a specific command to ignore one or several FID(s) and the corresponding gradient value(s) while processing the DOSY spectrum; it can thus generate a spectrum without artifacts as presented in this study. Alternatively, the magnetization coherence pathway model can be used in the DOSY-ES set-up procedure to build a diffusion gradient list devoid of gradients producing unwanted echoes.

3. Conclusions

We have demonstrated that BPPSTE-ES is a powerful diffusion measurement NMR sequence with a remarkable suppression of water signal leading to very good quality spectra of complex and diluted mixtures. Moreover, the protein–ligand interaction example showed the potential of the sequence for the analysis of very-low concentrations. The accidental echoes generated by this sequence can be easily detected, verified and eliminated to obtain a clean bidimensional DOSY spectrum, or they can be avoided by using a specific gradient list.

Acknowledgments

We thank Christine Pascal and Véronique Cheynier from INRA Montpellier for the salivary protein sample. Pr. Philippe Behra and Dr. Aurélie Mossion from INP Toulouse are acknowledged for sample preparation. S.B. thanks the Association pour la Recherche sur le Cancer (France) for a postdoctoral fellowship.

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