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Relaxation-encoded NMR experiments for mixture analysis: REST and beer†

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A new family of NMR experiments for mixture analysis (Relaxation-Encoded Selective TOCSY, REST) allows the extraction of component subspectra from mixtures. It uses isotropic mixing to label whole spin systems with the relaxation times (*e.g.* T_1, T_2) of individual spins.

NMR is one of the most useful non-destructive spectroscopic tools for the characterization of organic compounds, and works particularly well for pure compounds. However, nature is often more complicated, and some of the most interesting challenges in characterization present themselves as complex mixtures. Methods are needed that can analyse intact mixtures when physical separation is costly, impractical, or impossible. Diffusion-ordered spectroscopy (DOSY) is one example, in which NMR signals are separated according to their diffusion behaviour.[1–3] In the absence of exchange, the diffusion coefficient is the same for every spin in a molecule, allowing the signals of different mixture components to be distinguished. In principle, spin relaxation can be used as a filter[4,5] or to distinguish between components of a sample, and several examples of DOSY-style analysis of relaxation experiments have been reported previously. These were variously termed ROSY (Relaxation-Ordered SpectroscopY),[6] TOSY (raTe of relaxation Ordered SpectroscopY)[7] and RAS (Relaxation Assisted Separation);[8] here we use the first of these names. One complication of using relaxation is that, in contrast to diffusion, different spins from the same spin system typically show different relaxation. Here we illustrate a new class of experiments that circumvents this limitation, and allows the use of spin relaxation for mixture analysis without the need for physical separation. In the REST (Relaxation-Encoded Selective TOCSY) class of experiments a combination of selective excitation and isotropic mixing[9] is used to label each spin in a given system with the same relaxation weighting, so that the experimental data obtained can be analysed in similar ways to DOSY data. The solid-state ROSY experiment[6] uses dipolar-driven spin diffusion to ensure that all spins of a given species share the same relaxation characteristics; REST experiments in liquids use isotropic mixing to achieve this.

An illustration of the power of the REST approach is given in Fig. 1. The proton spectrum of a German lager beer (Fig. 1c) reflects the complexity of beer chemistry, with almost wall-to-wall peaks. From this highly complex mixture, a REST experiment using T_2 weighting ($REST_2$), in combination with multivariate processing, *e.g.* OUTSCORE (Optimized Unmixing of True Spectra for COmponent REsolution),[10] extracts clean spectra of the α-glucose moiety of maltose (Fig. 1a) and the free α-glucose (Fig. 1b). It is not possible to isolate these spectra by TOCSY alone, because of the overlap between the anomeric signals of the two species.

DOSY works well in many cases, but cannot succeed when different species have very similar diffusion coefficients. This degeneracy can sometimes be lifted by manipulating the medium (or "matrix") in which the solutes diffuse,[13–18] but this complicates sample preparation and changes the sample composition. In such cases, REST can come to the rescue, allowing mixture analysis without alteration of sample composition. A simple illustration is afforded by a model mixture of two disaccharides, lactose and melibiose (both present in some beers, at a very low concentration), where simple DOSY fails completely (Fig. 2a) because of the virtually identical diffusion coefficients. The only structural difference between the two saccharides is in the connectivity between the glucose and galactose rings: melibiose has an α-1,6 linkage and lactose a β-1,4, leaving them with virtually identical hydrodynamic radii.

Relaxation, in contrast to diffusion, depends on the chemical environment of an individual nucleus. Thus different protons in a given species will in general have different relaxation times, at first sight ruling out the use of relaxation to distinguish between the signals of different species in the manner of DOSY. However, differences in relaxation between different compounds can be exploited to separate signals in experiments that combine selective excitation and isotropic mixing to ensure that all the signals measured for a given species originate from a single proton.[19] Constructing a 2D ROSY spectrum, in which the 1D proton

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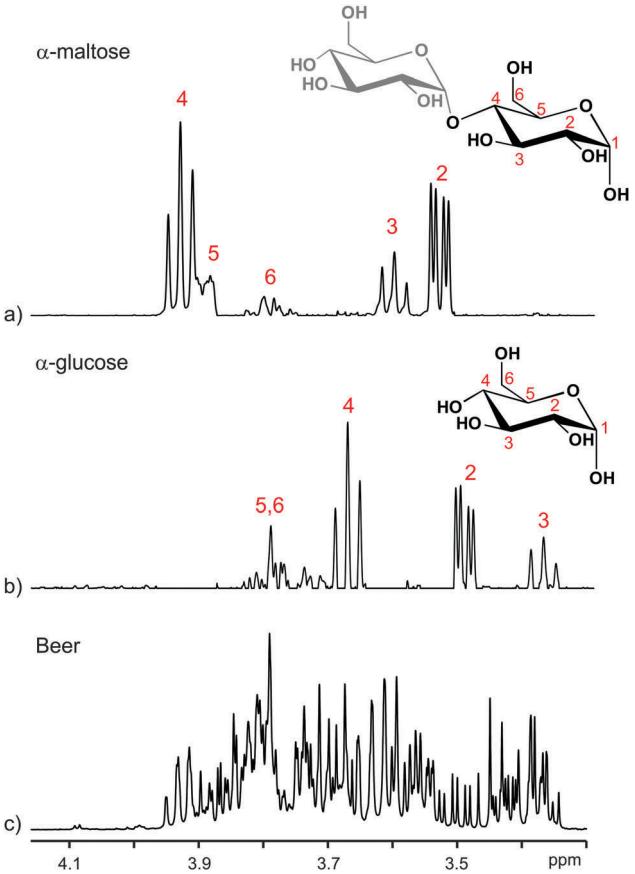

Fig. 1 $REST_2$ analysis (37 min 10 s) of beer (Clausthaler Classic premium low alcohol lager) with 10% added D_2O. (a) OUTSCORE[10] component for the α-glucose unit in α-maltose, (b) OUTSCORE component for free α-glucose, and (c) full ^1H spectrum of the beer. All processing, including OUTSCORE, was done in the DOSY Toolbox.[11] Data were acquired using the sequence in Fig. 3; full experimental details are given in the ESI.†

spectrum of a mixture is dispersed as a function of relaxation time, *e.g.* T_2, should then allow the signals of different species to be distinguished even if they have identical diffusion coefficients. Both DOSY and REST experiments suffer from spectral overlap. This problem can be addressed *e.g.* by increasing the magnetic field strength, by using more advanced (*e.g.* multivariate) processing,[10,20] by using nuclei with wider chemical shift ranges,[21–24] or by coupling with multidimensional[25–27] or pure shift NMR methods.[28–30]

Fig. 2 compares the Oneshot DOSY spectrum (Fig. 2a) of the disaccharide mixture, in which all the sugar signals show the same diffusion coefficient, with ROSY spectra (Fig. 2b) measured using the PROJECT sequence to weight each signal according to its T_2 while suppressing J modulation,[31] and using the $REST_2$ pulse sequence of Fig. 3 to weight each signal according to the T_2 of the associated anomeric signal (Fig. 2c). As expected, neither Fig. 2a nor Fig. 2b allows the signals from the two different species to be distinguished, in the case of Fig. 2a because they have almost identical diffusion coefficients and in the case of Fig. 2b because the differences in T_2 between different protons in a given sugar are far greater than any systematic difference in relaxation between the two sugars. In contrast, Fig. 2c shows clean resolution between the signals of α-glucose in the two disaccharides, exploiting the 10% difference in T_2 between the anomeric signals of the two species. Again, a simple TOCSY experiment is not readily interpretable because the anomeric signals both resonate close to the same chemical shift of 5.2 ppm. The sensitivity of T_2 to chemical environment is a great advantage here, the subtle changes in dynamics at the reducing terminus of the disaccharide caused by the distal linkage being sufficient to allow clean separation of signals and facilitating the use of multivariate methods, such as SCORE[20] or OUTSCORE.[10]

The basic structure of the pulse sequence of Fig. 3 consists of an initial preparation period prefaced by a spherical randomisation pulse to dephase any residual magnetization;[32] the generation

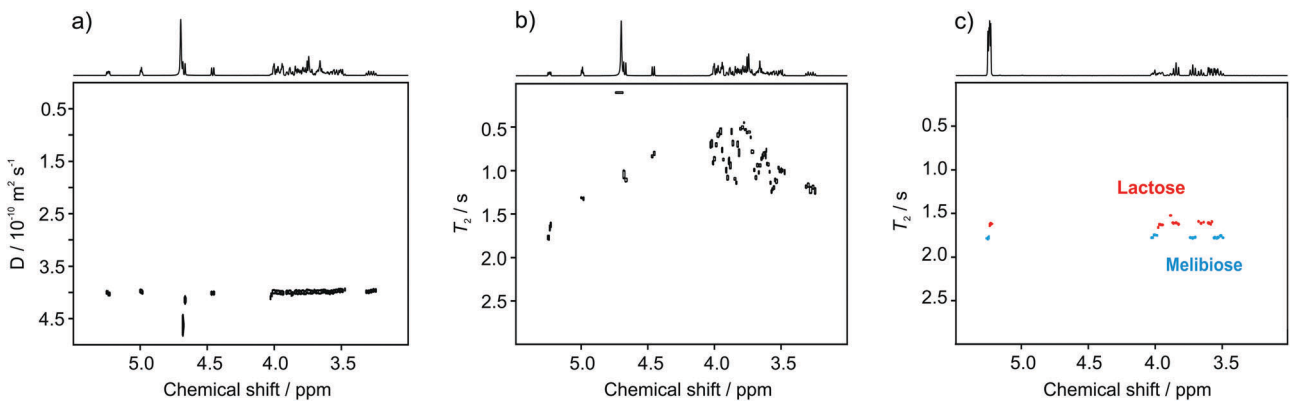

Fig. 2 (a) Oneshot DOSY (1 h 27 min), (b) PROJECT-ROSY (53 min) and (c) $REST_2$ ROSY (1 h 13 min) spectra of a sample of lactose and melibiose in D_2O. $REST_2$ used a 30 ms RSNOB pulse at 5.239 ppm and a mixing time of 120 ms. All spectra were processed using reference deconvolution with the TSP-d_4 signal.[12] The DOSY experiment (a) shows that the two disaccharides have very similar diffusion coefficients of about 4×10^{-10} m^2 s^{-1}. The PROJECT-ROSY experiment (b) shows that there is a variety of relaxation values that can be exploited. In the $REST_2$ experiment (c) the difference in relaxation between the anomeric signals of glucose at 5.2 ppm is used to separate the signals of the glucose spin systems in the two disaccharides (lactose signals in red and melibiose in blue). Full experimental details are given in the ESI.† ROSY spectra (like DOSY spectra) are statistical constructs: the positions of peaks in the relaxation (diffusion) domain show scatter because of the statistical uncertainty introduced into the fitting process by spectral noise. This is in contrast to conventional multidimensional NMR, which use Fourier transformation rather than least squares fitting and hence peak positions are well-defined.

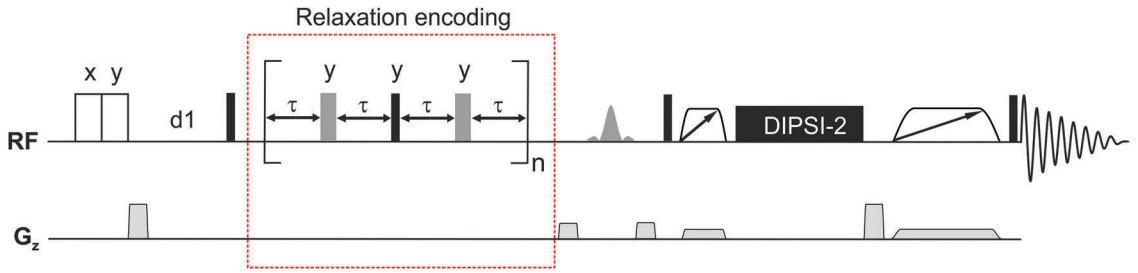

Fig. 3 Pulse sequence for a $REST_2$ experiment using PROJECT[31] for T_2 relaxation encoding. Light grey trapezoids represent field gradient pulses. White, black and dark grey rectangles represent spherical randomization pulses,[32] 90° hard pulses, and 180° hard pulses, respectively. White trapezoids with arrows represent chirp pulses used to suppress zero-quantum coherences,[33] and DIPSI-2[34] is used for isotropic mixing. The grey shaped pulse represents an 180° selective pulse. The initial 90° pulse and PROJECT element (outlined in red) can be replaced with other types of relaxation encoding, for example inversion recovery[35] for $REST_1$. Further information on the pulse sequence is given in the ESI.†‡

of relaxation-weighted single quantum coherence, in the example shown here using the PROJECT sequence, a CPMG[36,37] analogue that suppresses homonuclear J modulation; a selective 180° pulse to select signals at a single chemical shift; and an isotropic mixing sequence, here DIPSI-2[34] with zero-quantum coherence suppression,[33] to transfer coherence throughout each spin system. It is straightforward to adapt the pulse sequence to encode other types of relaxation information, including T_1, $T_{1\rho}$ and dynamic NOE; in the ESI† we show results for $REST_1$ using inversion recovery.[35] In principle, subtleties of multispin relaxation such as cross-correlation effects could complicate REST, but in the systems investigated here at least no such complications were observed. The basic sensitivity of REST methods is very similar to that of conventional 1D selective TOCSY. For the TSP-d_4 signal in the disaccharide sample the signal-to-noise ratios, with the same number of scans, were: 20200 (PROJECT), 14500 (selective TOCSY), and 14500 ($REST_2$), for the first increment of each experiment.

While relaxation is used purely qualitatively in the experiments of Fig. 1 and 2, to distinguish the signals of different species, it is of course possible to use REST methods quantitatively, to gain information on structure and dynamics. One possible application would be to use the strategy of Fig. 1 and 2 in reverse, applying the selective pulse to a region of a spectrum that is unresolved in order to measure relaxation indirectly through resolved signals.

The lager beer sample of Fig. 1 was also subjected to DOSY and ROSY analysis. Beers are complex mixtures, including saccharides such as glucose, maltose and maltotriose.[38] The DOSY spectrum (Fig. 4a) showed a range of diffusion coefficients, but due to the large number of different components and the limited resolution, little detailed insight can be gained. The anomeric doublet at 5.23 ppm was selected for further analysis with a $REST_2$ experiment (Fig. 4b). The 2D TOCSY spectrum and HSQC spectra (see ESI2 and 3,†) showed that this contains contributions from more than one species, but further analysis was not straightforward. Because of poor dispersion of the ^{13}C signals for these species, of just a few hertz, HSQC-TOCSY would also not be informative. Selective TOCSY, exciting at 5.23 ppm (see ESI4,†), did not provide much further information, even with the considerably simpler spectrum. In contrast the $REST_2$ experiment (Fig. 4b) clearly indicated that

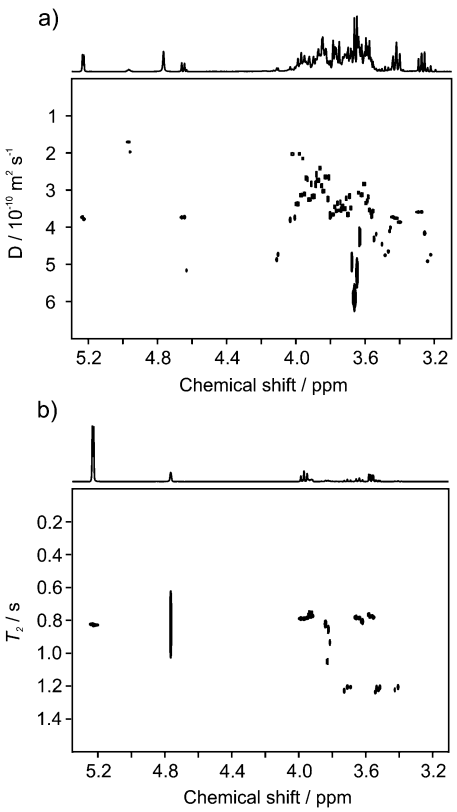

Fig. 4 (a) Oneshot DOSY (32 min 26 s) and (b) $REST_2$ ROSY (37 min 10 s) spectra for lager beer in D_2O. The difference in T_2 of the signals at 5.23 ppm (α-glucose H_1s) makes them suitable for REST analysis. REST was performed using a 50 ms RSNOB pulse at 5.23 ppm and a mixing time of 100 ms. The broad signal at 4.75 ppm in (b) arises from exchange between sugar OH signals and water. All spectra were processed using reference deconvolution with the TSP-d_4 signal.[12]

there were two separate species contributing to the anomeric doublet. The remaining ambiguity, due to spectral overlap around 3.85 ppm, was resolved by OUTSCORE processing (Fig. 1a and b), clearly identifying the individual spectra of the species α-glucose and the terminal α-glucose moiety of α-maltose. Similar information could be extracted using $REST_1$ (see ESI5b,†).

The REST family of experiments is a new tool for NMR mixture analysis, allowing the extraction of (sub)spectra of

contiguous spin systems from intact complex mixtures that are currently difficult or impossible to analyse. Some notable successes in the NMR analysis of mixtures of this level of complexity have been reported previously,[39,40] but these used heteronuclear multidimensional experiments that required both chemical derivatisation and very long acquisition times, in contrast with REST. We expect the new experiments presented here, alone and in combination with *e.g.* DOSY,[41,42] to be useful in many areas of chemistry, including metabolomics, natural products, and organic synthesis. When spectral overlap remains a problem, relaxation encoding can be combined with pure shift selective TOCSY experiments to provide further resolution.[43]

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‡ Related pulse sequence codes are available from http://nmr.chemistry.manchester.ac.uk/. Full experimental data and pulse sequences can be downloaded from DOI: 10.15127/1.307570.

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