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Spectral unraveling by space-selective Hadamard spectroscopy

Nagarajan Murali *, William Marcus Miller, Boban K. John, Daina A. Avizonis, Stephen H. Smallcombe

Varian Inc., 3120 Hansen Way, Palo Alto, CA 94304, USA

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Abstract

Spectral unraveling by space-selective Hadamard spectroscopy (SUSHY) enables recording of NMR spectra of multiple samples loaded in multiple sample tubes in a modified spinner turbine and a regular 5 mm liquids NMR probe equipped with a tri-axis pulsed field gradient coil. The individual spectrum from each sample is extracted by adding and subtracting data that are simultaneously obtained from all the tubes based on the principles of spatially resolved Hadamard spectroscopy. The well-known Hadamard spectroscopy has been applied for spatial selection and the method utilizes standard configuration of NMR instrument hardware. The SUSHY method can be easily incorporated in multi-dimensional multi-tube NMR experiments. This method combines the excitation multiplexing, natural advantage of FTNMR, and sample multiplexing and offers high-throughput by reducing the total experimental time by up to a factor of four in a 4-tube mode.

Keywords: SUSHY; Hadamard; Multi-tube NMR; Space selective encoding; Fast NMR

1. Introduction

Fourier transform NMR (FTNMR), by its inherent property of multiplexing in excitation that increase the signal to noise per unit time, has enabled complex studies of wide range of molecular systems. Further sensitivity gains, up to a factor of four or more, achieved by cryogenically cooled probes lead to substantial reduction in experimental times. In addition to the natural advantages of FTNMR and improved probe technologies, efficient sample handling techniques such as flow NMR and tube-based robotics have allowed study of multiple samples with even higher throughput. However, flow NMR is plagued with problems of sample recovery and cross contaminations, leading to spectral artifacts and requirement of extensive wash cycles that take up significant time from data acquisitions. The tube-based sample transport methods that avoid the problems of sample recovery and contamination, are not truly sample-multiplexing techniques as only one sample is used per NMR data acquisition. To improve high-throughput further, it is desirable to be able to study multiple samples in a given acquisition scheme and unravel the NMR spectra to improve the throughput of samples. A tube-based multiple sample acquisition is advantageous as it keeps the fidelity of the samples intact and ensures clean recovery. In recent years, methods have been developed that enable simultaneous data acquisition of multiple samples by utilizing multiple-coil probe designs to hold the samples and multiple receivers to receive signals from each sample [1–13]. Such methods increase high-throughput, but require special probes and spectrometer hardware modifications for implementation. The method described in this paper utilizes conventional spectrometer hardware and enables simultaneous data acquisition of multiple samples followed by extraction of NMR spectrum of individual samples.

It is quite possible to load multiple sample tubes of smaller diameter, instead of a single conventional 5 mm

* Corresponding author. Fax: +1 650 852 9688.
E-mail address: nagarajan.murali@varianinc.com (N. Murali).

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sample tube, in the observation window of a standard NMR probe and devise techniques to unravel the NMR spectrum from the individual sample tubes. A straightforward approach at this point is to use simple selective rf pulses in the presence of pulsed field gradients that excite either one or the other sample tube and subsequent detection in the absence of any gradients to yield NMR spectra of either sample separately. This would essentially be a sequential data collection approach leading to no net gain in signal to noise or increased speed in throughput. It is thus desirable to incorporate simultaneous excitation of all the spins in all the tubes and improve the signal to noise arising from sample multiplexing.

Combining spatial resolution through the application of pulsed field gradients (pfg) in a suitable direction and Hadamard type spin-state-encoding by radio frequency (rf) pulses in the presence of pfg pulses, one can separate high-resolution NMR spectrum from individual samples. This method, with an acronym SUSHY (Spectral Unraveling by Space-selective Hadamard spectroscopy), retains the sensitivity advantage of FTNMR as all the spins in all the tubes are excited for detection and suitable data addition and subtraction given by a Hadamard scheme yield NMR spectra from each individual sample. This method combines, elegantly, the principles of imaging and spectroscopy and uses standard spectrometer hardware and probe configuration to obtain the NMR data.

Spatial Hadamard encoding, described in this paper, is not an entirely a new idea espoused for the first time. Volume localization methods are well known in imaging and the ISIS technique [14] is the first that allowed multiple volume localization. The subsequent method designed for simultaneous volume localization that acquire as many volumes (voxels) as the number of scans based on a Hadamard excitation scheme, known by the acronym HSI [15,16], is an improvement of the ISIS method and the HSI method is similar to the SUSHY approach. When a single volume is selected the ISIS and Hadamard-based methods are similar but they diverge when multiple volumes are selected. In the HSI and SUSHY, the initial state is prepared according to a Hadamard matrix scheme that allows utilization of the signals from all the spins in every volume, thus increasing the speed of data acquisition. Hadamard schemes have also been utilized in high-resolution NMR to increase the speed of data collection [17,18] and belong in the family of fast NMR methods. The SUSHY technique combines the same principle used in the imaging application to provide high-resolution spectra from multiple samples in a standard probe, thus accelerating high-throughput.

2. Experimental methods and discussion

As an example, let us consider a sample arrangement in which two sample tubes each of approximately 1.7 mm outer diameter (~1.3 mm inner diameter) are positioned symmetrically across the axis of a turbine (Fig. 1). The samples used were tetracycline in one tube and glucose in the other. The samples were dissolved in deuterated water and the concentrations were approximately 10 mM. Such a sample arrangement was then inserted in to a probe equipped with a tri-axis pfg coil. A simple proton profile experiment based on gradient spin-echo method, in which an x- or y- (or a combination of both) defocusing/refocusing pfg-applied during the acquisition, spread the signals along that axis showing the alignment of the two NMR tubes in space with respect to the axis of the effective pulsed field gradient direction. Fig. 2 shows the dependence of the spatial profiles of the two tubes with respect to the effective pfg direction (shown by the arrows). The two broad peaks in Fig. 2B show the spatial distribution of spins in the two tubes when the effective pfg direction is aligned across the two tubes. The areas under the lobes are proportional to the number of spins in the tubes. One can, thence, conceive an experiment in which rf excitations in the presence of applied pfg pulses tailored to spatially encode the spins in the two tubes followed by a non-selective read pulse without any pfg pulses and detection of the NMR signals to extract the high-resolution NMR spectra.

A simple pulse scheme, that allows recording of one-dimensional spectra, is shown in Fig. 3. This sequence happens to be similar to the ISIS [14] pulse sequence

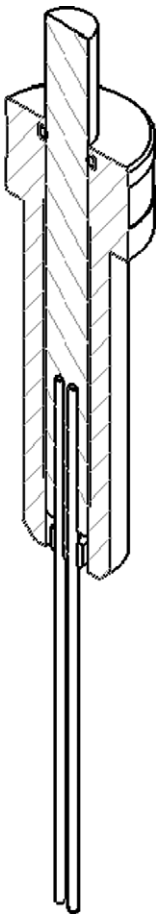

Fig. 1. Cross-section of a sample holder showing two tubes of each outer diameter of 1.7 mm positioned across the axis of the spinner.

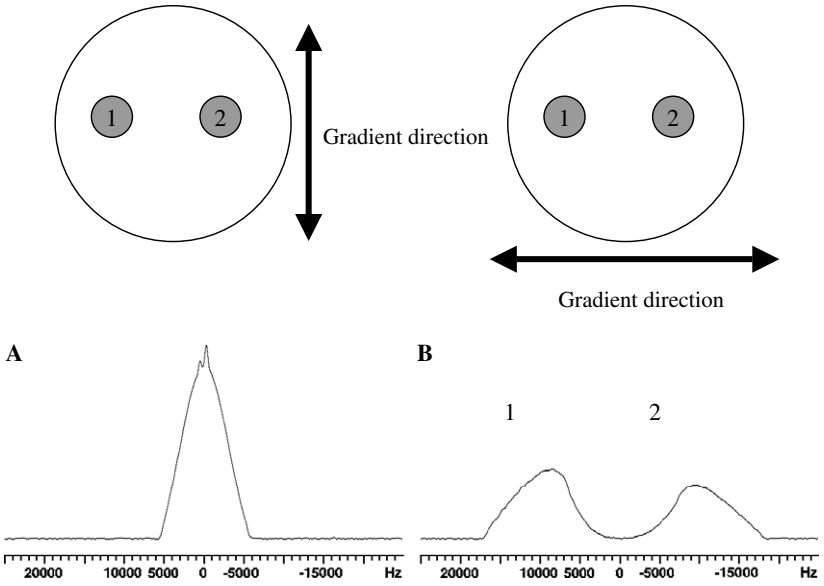

Fig. 2. Profile of the two tubes with respect to the orientation of the effective transverse gradient direction. The profile in (A) is obtained with the gradient along the perpendicular direction to the tube distribution axis and does not distinguish the two tubes. (B) The gradient direction is along the sample tubes distribution axis and distinguishes the spatial position of the two tubes. A 20 G/cm effective gradient strength was used in all the experiments. A single transient was collected for each gradient orientation.

and is illustrated here for completeness. The difference between the SUSHY sequence and ISIS is in the details of the excitation characteristics of the initial shaped-pulse. In the SUSHY experiment, the initial shaped-pulse in the presence of the pulsed field gradients prepare the necessary spatial encoding by manipulating the Z-magnetization of the spins in the tubes according to a Hadamard matrix (Table 1). The shaped-pulse in this case is chosen to be an inversion pulse of hyperbolic-secant type. In the presence of the gradient pulses, the shaped-pulse is tailored to invert the spins, either from the two tubes (both the lobes in Fig. 2B) or just one tube (one of the lobes in Fig. 2B). The non-selective 90° observe pulse in the absence of gradient pulses reads the state of the magnetization of all the spins in both the tubes. Two experiments are performed with the Z-magnetizations prepared according to the Hadamard scheme in Table 1, one experiment for each row (or column). A negative sign in the table implies that the spins are inverted in the selected tube and a positive sign represents unperturbed Z-state. The inversion of all spins indicated in the first row of the Table 1 is a deliberate choice so that the length of the pulse sequence is identical in the two experiments used to extract the final NMR spectra.

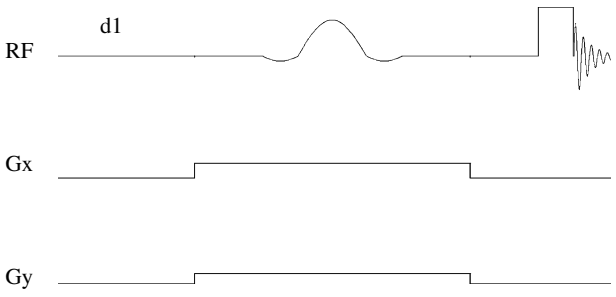

Fig. 3. A simple 1D SUSHY pulse sequence in which the fist shaped-pulse is a hyperbolic-secant inversion pulse applied in the presence of the transverse gradients whose effective gradient direction separates the two tubes (say, as in Fig. 2B). The rectangular read pulse is a 90° pulse applied in the absence of pfg pulses excites all the spins and the FID is observed again in the absence of any gradients.

Table 1
Z-state representation of spins in two tubes

Tube 1	Tube2
−	−
+	−

In Fig. 4, the bottom trace on the left (Fig. 4A) was obtained with the initial state of spins in both tubes inverted (1st row of Table 1) and the bottom trace on the right (Fig. 4B) was obtained with spins in just one tube inverted (2nd row of Table 1). Four transients were collected with a four-step phase cycle on the non-selective read pulse and the receiver. The top two traces in Fig. 4 were obtained by addition and subtraction of the two bottom traces and show the spectra from the individual tubes (Figs. 4C and D, respectively). The trace in Fig. 4C corresponds to the NMR spectrum of the tetracycline sample in D_2O and the trace in Fig. 4D is the NMR spectrum of glucose in D_2O. Since two data sets are used to extract the NMR spectra of the two tubes in Figs. 4C and D, the signal to noise in these extracted spectra is increased by $2^{1/2}$ compared to the raw signals plotted in the bottom two rows. Thus with the two-tube SUSHY experiments, the experimental time may be cut in half compared to the sequential data collection approach, in which a similar sample tube is

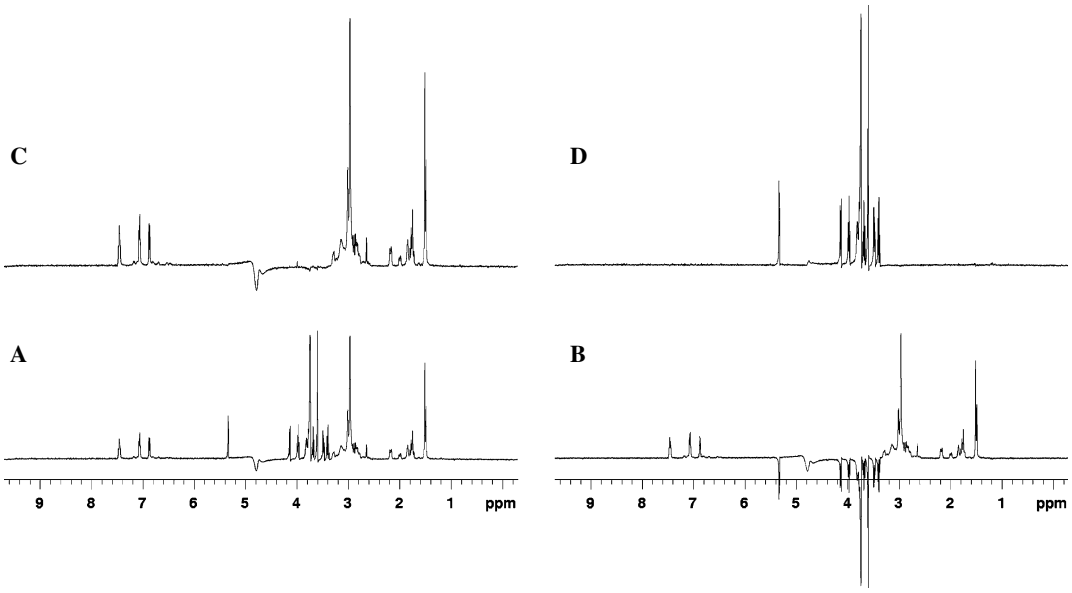

Fig. 4. SUSHY spectra obtained using the pulse scheme as in Fig. 3. The spectrum (A) was obtained after both lobes (see Fig. 2B) were inverted and phased positive and in (B) only one lobe is inverted. In both spectra (A) and (B) the inverted initial states are phased positive, so that the un perturbed signals appear as negative peaks. Spectrum (C) was obtained by adding (A) and (B) and is from the tetracycline molecule. Spectrum (D) was obtained by subtracting (B) from (A) yielding the glucose signatures. The residual protons from D_2O have been suppressed by pre-saturation before SUSHY encoding.

separately loaded and eight transients are collected. The present sample arrangement does compromise on S/N in favor of speed of data collection. The filling factor of 1.7 mm sample tubes in a 5 mm coil is poor compared to a case in which the coil diameter matches the diameter of the tube. It is desirable to take the same quantity of sample as one would use in a larger volume tube and dissolve it in lesser solvent volume, thereby increasing the concentration to gain sensitivity. This is feasible when sample availability and solubility is not a concern.

The SUSHY method can be readily extended to higher dimensional NMR experiments. A straightforward extension to a two-dimensional correlated spectroscopy (COSY) involves addition of the spatial encoding preparation part as shown in Fig. 5. In the present case, the often-used absolute-value-mode gradient-coherence-selected gCOSY sequence has been modified to include the spatially selective Hadamard encoding. Two sets of data were collected with Z-

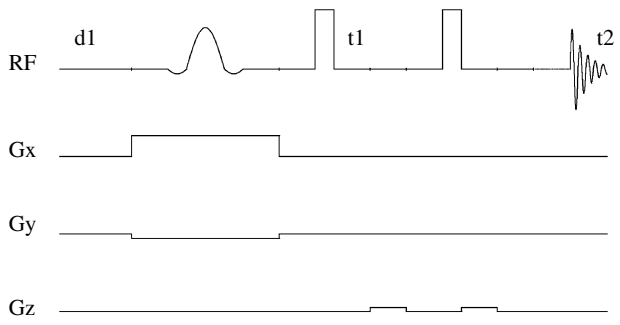

Fig. 5. SUSHY-COSY pulse sequence. The SUSHY encoding scheme as in Fig. 3 is inserted in the beginning of the standard gradient-coherence-selected COSY experiment. The two rectangular pulses are 90° pulses and the Z-pfg pulses were applied to select the coherence transfer pathways.

states prepared as in Table 1 with four transients per set and 128 t_1 increments. Addition and subtraction of the data sets yielded the desired two 2D SUSHY-COSY spectra of tetracycline (Fig. 6A) and glucose (Fig. 6B). Again, as in the 1D experiment, faster data collection is an outcome of parallel observation of NMR signals from both the samples.

SUSHY is a simple, yet powerful, method that it can be incorporated as a part of the preparation scheme in any NMR experiment to study multiple samples simultaneously. It is, however, worthwhile to pause in order to identify experimental intricacies in implementing the SUSHY method. As with any experiment, limitations posed by instrumental constraints such as non-linearity of the gradients across the sample volume and rf in-homogeneity at the ends of the sample column will lead to incomplete spatial selection due to flip angle errors of the initial encoding inversion pulse at the ends which would then lead to spectral contaminations. Also any deviation from the parallel alignment of the two tubes will also contribute to overlap of the signals even if the gradients are perfect. A careful look at the profile in Fig. 2B indicates that the separation of the two tubes are blurred as seen by the broad structures that overlap in the middle and the long tails at the either end due to the aforementioned reasons. The extracted 1D-SUSHY spectra in Figs. 4C and D and the 2D SUSHY-COSY spectra in Figs. 6A and B are, however, fairly clean. The bandwidths of the encoding shaped pulses eliminated the end effects and selected the cleanly separated portions of the samples and any contaminations, even if present, are below the detectable levels (in terms of signal to noise) in these spectra.

It is also possible to substantially eliminate the tailing effects and blurring of sample separation by selectively

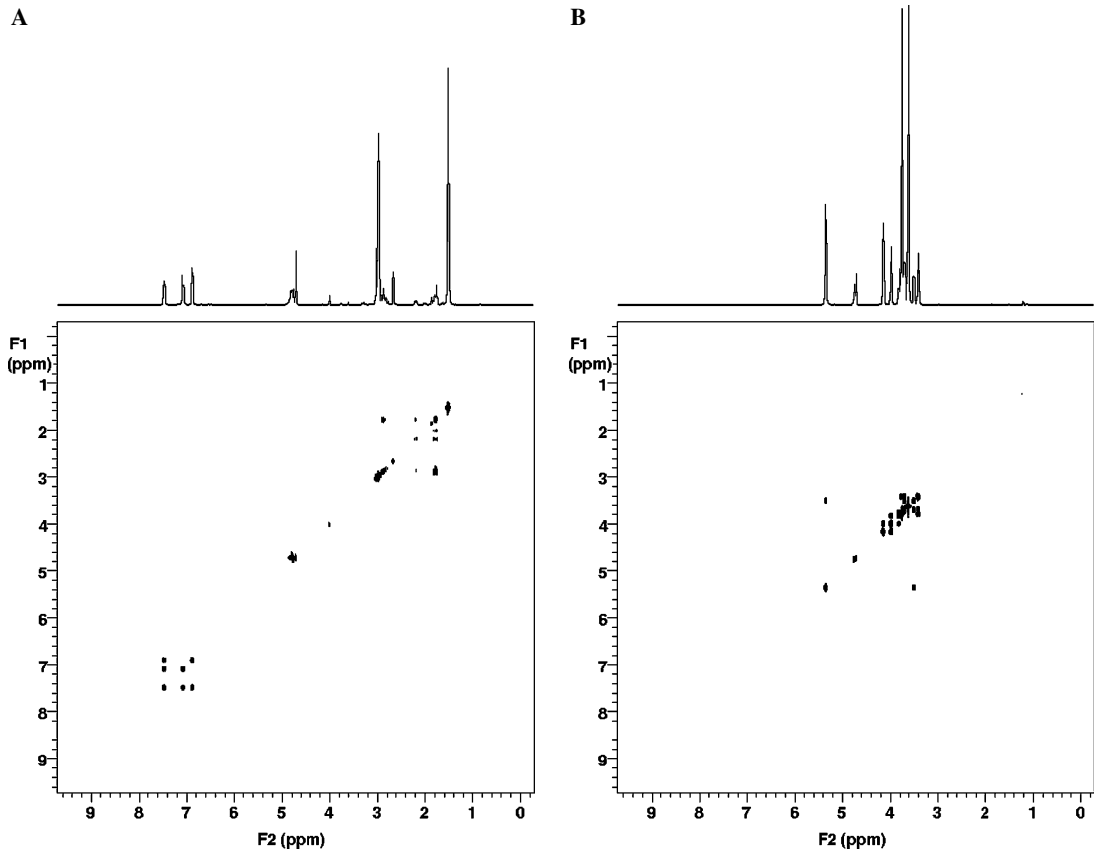

Fig. 6. SUSHY-COSY spectra of (A) tetracycline and (B) glucose along with the horizontal projection obtained using the pulse sequence in Fig. 5. Again, the solvent pre-saturation was included before the SUSHY encoding step.

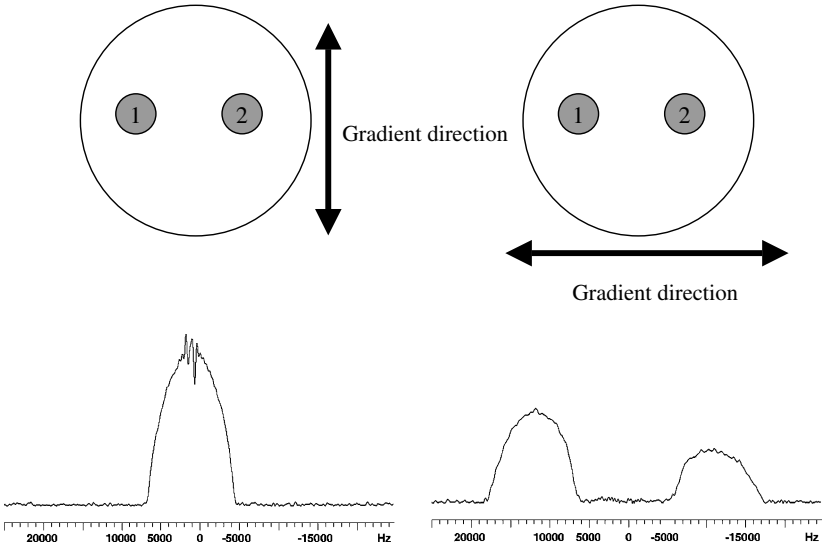

Fig. 7. Profiles as in Fig. 2 but recorded with Z axis-selective saturation of all spins at the ends of the tubes for cleaner separation of the tubes. A shaped excitation pulse (90°) was applied several times in the presence of Z-pulsed field gradients and the excitation bandwidth covered the outer edges of the two lobes in Fig. 2B for saturation.

saturating the spins at the ends of the tube by applying a selective saturation pulse in the presence of an additional pfg pulse in the Z-direction before the SUSHY preparation step. In Fig. 7, a profile of the two tubes is shown in which an end-selective (by Z-pfg pulse) saturation scheme was applied first in the gradient spin-echo profile experiment.

Table 2
Z-state representation of spin in four tubes

Alanine	Tetracycline	Glucose	Sucrose
−	−	−	−
−	−	+	+
−	+	+	−
−	+	−	+

The signals from the two tubes are cleanly separated and the profiles are much closer to the physical distribution of spins in space. End selective saturation when applied in SUSHY scheme improves the separation at the cost of reduced S/N in one-dimensional spectrum. However, the loss of signals from the end of the tubes are less detrimental

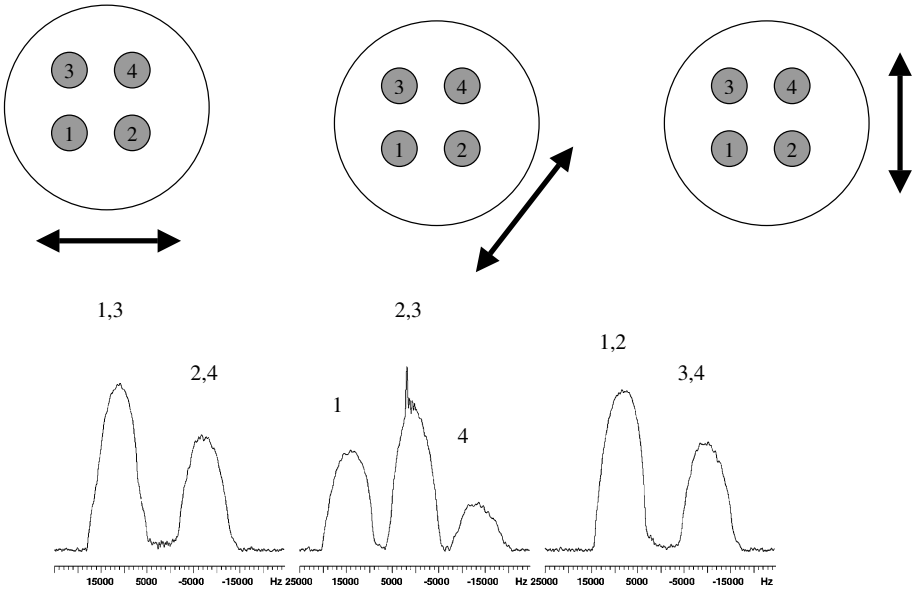

Fig. 8. A schematic representation of the orientation of the four tubes in the sample holder along with the profiles that project the NMR signals from the tubes along the gradient directions. The left most 0° projection resolves tubes 1,3 from 2,4 and the rightmost 90° projection resolves tubes 1,2 from, 3,4. The middle 45°-projection separates tubes 1 and 4 as the two outer lobes and the middle lobe is from tubes 2 and 3. Z-selective saturation is used to get the profiles.

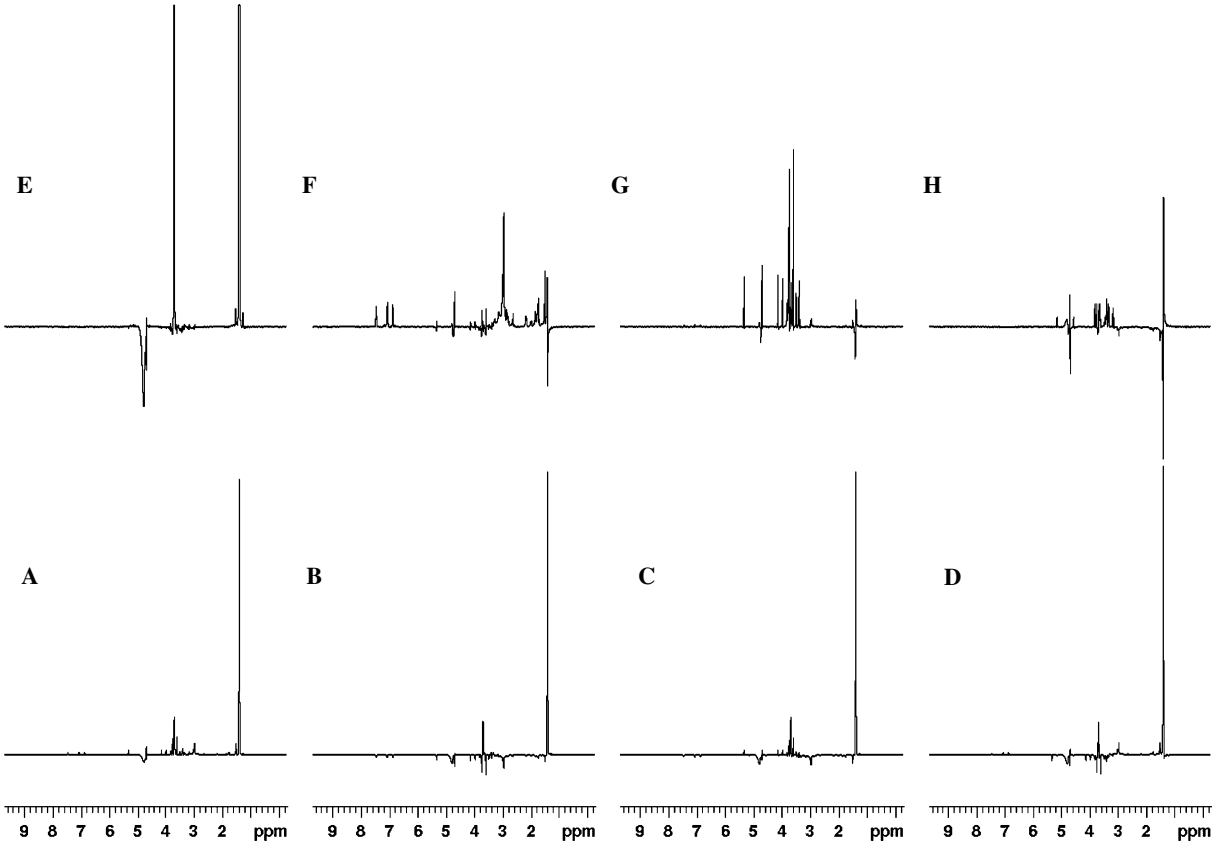

Fig. 9. 1D SUSHY spectrum from four tubes. The raw data obtained using the pulse sequence in Fig. 3 with Z axis-selective saturation and solvent presaturation and SUSHY encoding according to the Table 2 are shown in the bottom row. The top row shows extracted data of alanine (E = A + B + C + D); tetracycline (F = A + B + − C − D); glucose (G = A − B − C + D); and sucrose (H = A − B + C − D).

in 2D experiments because the undesirable pulse flip angle errors due to rf in-homogeneities arise from the sample that is outside the active volume of the coil.

Extension of SUSHY method to study more than two tubes is also straightforward. In a standard 5 mm probe there is enough room to accommodate four tubes of the dimensions mentioned above which would increase the throughput by an additional factor of two compared to the two-tube mode. A Hadamard Z-state matrix for four tubes is given in Table 2. Three gradient directions (Fig. 8) and four excitations scheme as given in the Table 2 are necessary to separate the spectra of samples in the four tubes. In Fig. 8, a cleaner separation is achieved by saturating the spins at the ends of the tubes allowing better selection by rf pulses. In the subsequent 1D and 2D SUSHY experiments, the left most gradient direction in Fig. 8 was used twice: once to get Z-state according to the first row of the Table 2 and again to get the second row (by inverting just the right lobe, tubes 2 and 4). The other two gradient directions were used only once to get the remaining two rows of encoding.

The results of 4-tube 1D SUSHY spectra are shown in Fig. 9. The bottom traces in Fig. 9 show the Hadamard excitation spectra and the top trace shows the add–subtract data yielding the SUSHY spectra from individual tubes containing alanine, sucrose, tetracycline, and glucose. All four samples were dissolved in deuterated water and the concentrations were ~10 mM. Four sets of data with four transients per Hadamard encoding were collected and data addition and subtraction yielded the desired NMR spectra. The resulting individual spectra are now equivalent to data with 16 transients. The simultaneous 2D SUSHY-COSY spectra obtained with the same set of four samples in four tubes are shown in Fig. 10.

In the four-tube mode, although the spatial separation is refined by the space selective saturation, there are still artifacts on the order of about 1–2% in the present example. They are largely due to the imbalances in the co-addition and are further exasperated by the deliberate choice of high dynamic range of the NMR signals in the various tubes. The sharp-tall alanine (methyl-) peaks are sensitive to phase and amplitude characteristics in all the Hadamard

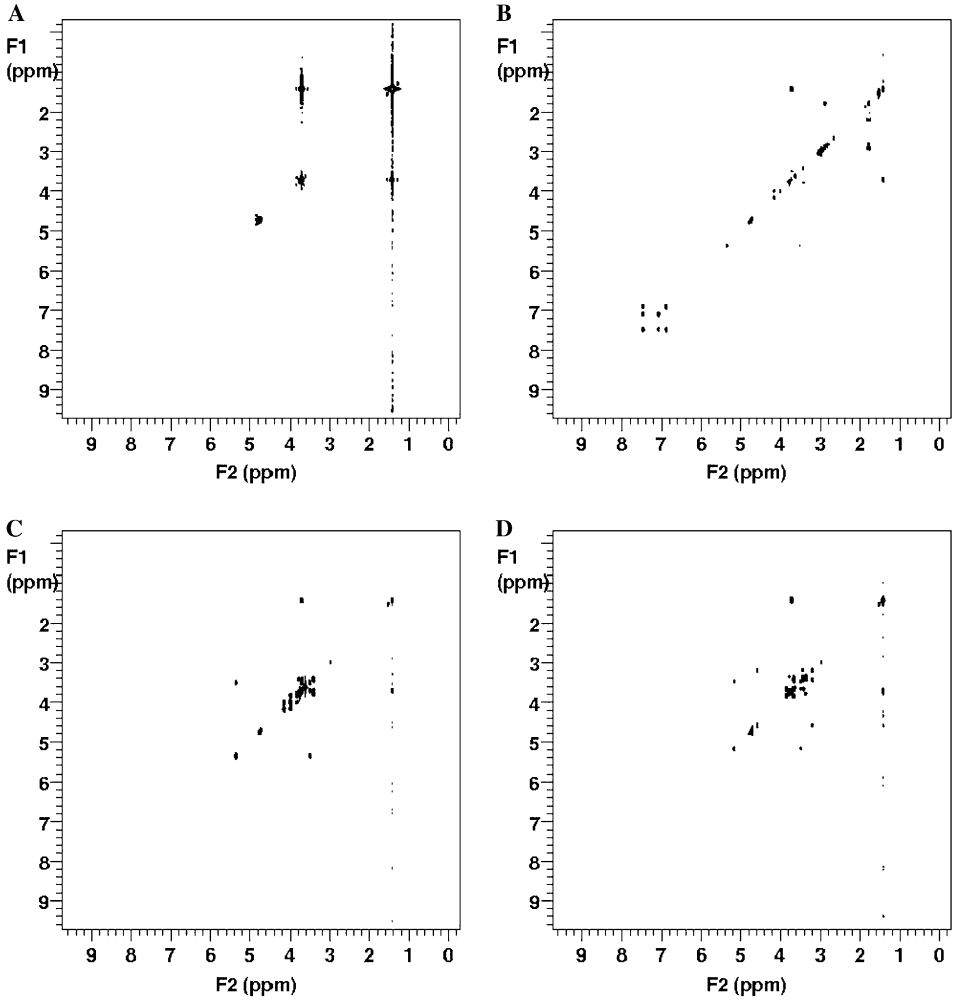

Fig. 10. SUSHY-COSY spectrum of (A) alanine, (B) tetracycline, (C) glucose, and (D) sucrose, respectively, obtained with the pulse scheme in Fig. 5 and conditions as in Fig. 10.

selected spectra and corrupt the addition–subtraction results. In the SUSHY-COSY experiments (Fig. 10) the appearance of t_1 noise is due to the truncation of the strong alanine signals in the indirect dimension and the large dynamic range of the signals of the samples chosen lead to co-addition artifacts in these data sets. But the separation is fairly complete and identifiable to individual spectra of the spin systems in each tube.

3. Conclusions

The SUSHY experiments utilizing the Hadamard excitation scheme to spatially resolved NMR spins in several tubes is a unique experiment that combines both the imaging approach for the preparation of initial states and spectroscopic detection scheme to yield high-resolution NMR spectra. The simultaneous excitation and observation of all the tubes maintains the sensitivity of an equivalent single tube NMR data but with fewer transients and increases throughput and the speed of data collection without requiring any additional unconventional hardware. The SUSHY method achieves true sample multiplexing and is also a general method that can be easily incorporated in many homo and heteronuclear NMR experiments. Extension of SUSHY to more than four tubes is not unconceivable and the imaging equivalent of SUSHY, the HSI scheme has indeed been applied to many volumes. However, in the tube mode, the size of individual tubes for sample handling would be the ultimate limit to the number of tubes that can be accommodated in a given probe.

The prototype multi-sample holder used in here did not have any special arrangement to facilitate reproducible orientation of the samples in the coil upon sample changes, but it is possible to design a spinner turbine with guides for proper positioning. Such an aligning arrangement is crucial for high-throughput sample studies so that the turbine is oriented in the same way with respect to the effective pfg axis. The use of transverse pulsed field gradients poses no additional problems in these experiments except that the gradient strengths need to be calibrated once. The gradient strengths, once calibrated for all directions, should remain the same for all samples and are only dependent on a given probe and the gradient amplifier used. Shimming is again not particularly difficult and gradient-based shimming procedures yield very good homogeneity over the sample volume and standard solvent suppression techniques work without any additional modifications as the SUSHY scheme is just added at the start of the sequence as a preparation element. The inherent advantages of the SUSHY might render itself as an application suited for high-throughput NMR when sample availability or solubility are not a major issue.

Acknowledgments

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