Simultaneous acquisition of three NMR spectra in a single experiment for rapid resonance assignments in metabolomics

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MS received 14 November 2014; revised 22 February 2015; accepted 22 February 2015

Abstract. NMR-based approach to metabolomics typically involves the collection of two-dimensional (2D) heteronuclear correlation spectra for identification and assignment of metabolites. In case of spectral overlap, a 3D spectrum becomes necessary, which is hampered by slow data acquisition for achieving sufficient resolution. We describe here a method to simultaneously acquire three spectra (one 3D and two 2D) in a single data set, which is based on a combination of different fast data acquisition techniques such as G-matrix Fourier transform (GFT) NMR spectroscopy, parallel data acquisition and non-uniform sampling. The following spectra are acquired simultaneously: (1) ¹³C multiplicity edited GFT (3,2)D HSQC-TOCSY, (2) 2D [¹H-¹H] TOCSY and (3) 2D [¹³C-¹H] HETCOR. The spectra are obtained at high resolution and provide high-dimensional spectral information for resolving ambiguities. While the GFT spectrum has been shown previously to provide good resolution, the editing of spin systems based on their CH multiplicities further resolves the ambiguities for resonance assignments. The experiment is demonstrated on a mixture of 21 metabolites commonly observed in metabolomics. The spectra were acquired at natural abundance of ¹³C. This is the first application of a combination of three fast NMR methods for small molecules and opens up new avenues for high-throughput approaches for NMR-based metabolomics.

Keywords. Metabolomics; Fast NMR spectroscopy; multiple NMR Receiver; chemical shift editing; non-uniform sampling.

1. Introduction

Metabolomics is becoming an important tool for disease diagnosis and drug toxicity in public health and medicine.^{1,2} It involves the utility of different analytical tools such as liquid chromatography (LC)-mass spectrometry (LC-MS), gas chromatography (GC)-Mass spectrometry (GC-MS) and NMR spectroscopy.^{3–5} NMR has a unique feature in terms of quantification and non-invasiveness.^{6,7} However, it is limited in terms of sensitivity and speed. While recent developments in technology such as microprobes, cryogenically cooled probes and high magnetic field strengths have addressed the problems of sensitivity, the speed of data acquisition remains a bottleneck.

An important step in the analysis of the NMR spectrum of a mixture of metabolites is the identification and resonance assignment of the individual molecules. Due to overlap of peaks in the ¹H 1D spectrum, this is typically accomplished using a set of homonuclear and heteronuclear 2D NMR spectra,

which includes two-dimensional (2D) [¹³C, ¹H] HSQC, 2D ¹H-¹H TOCSY and 2D [¹³C, ¹H] HSQC-TOCSY.^{6,7} In some cases, to reduce spectral overlap, a 3D ¹³C-edited HSQC-TOCSY experiment is used, which helps in providing improved resolution by spreading the peaks in the third dimension. However, due to its high minimal measurement time required for data acquisition, a 3D NMR spectrum is not always practically feasible for resonance assignment of small molecules, especially for those which are not enriched with ¹³C. This is because the conventional mode of sampling of multi-dimensional NMR spectra is a time-consuming process. Typically, two-dimensional (2D) experiments take minutes to hours to acquire, whereas 3D experiments, which can take up to a few days, are not generally used in metabolomics. Notwithstanding this fact, only a few attempts have been made to reduce the acquisition time of high-dimensional NMR spectra for metabolomics.⁸ The different fast NMR methods and their combinations developed during the past decade for proteins and nucleic acids such as single-scan NMR spectroscopy (ultrafast NMR),^{9–13} HADMARD encoding,¹⁴ reduced dimensional (RD)

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NMR^{15–19} and G-matrix Fourier transform (GFT) NMR,^{20–24} parallel receivers,^{25,26} covariance NMR,^{27,28} non-uniform sampling,^{29–31} rapid pulsing (reducing inter scan delay)^{32–34} and spectral aliasing³⁵ have not been satisfactorily explored in metabolomics, where their potential will provide the greatest impact.

We recently showed that the combination of two fast NMR Methods, GFT NMR and the dual receiver system, reduces measurement time of 3D NMR experiments by an order of magnitude and provides complementary information for resonance assignment.³⁶ However, assignment remains difficult if the spectra are crowded with cross peaks overlapping within a certain range of carbon and proton chemical shifts (typically 0.4 ppm for ¹³C and 0.04 ppm for ¹H). Unlike in the case of proteins, the CH, CH₂ and CH₃ groups in metabolites overlap in the similar chemical shift ranges. This is shown in figure 1, which depicts the simulated 2D [¹³C, ¹H] HSQC spectrum of 315 metabolites consisting of 902 peaks reported to be present in the blood serum. The chemical shift values were taken from Biological Magnetic Resonance Bank (BMRB).³⁷ About 30% of carbon–proton correlation peaks of CH, CH₂, CH₃ moieties (shown in different colours) have overlapping carbon chemical shifts within ± 0.2 ppm of ¹³C. While CH and CH₃ moieties are resolved to a large extent, the CH₂ moieties have overlapping shifts with CH and CH₃. This leads to challenge in assignment of resonances. To simplify the resonance assignment, we have introduced ¹³C multiplicity editing in the dual receiver experiments, which provides high resolution and separates peaks belonging to CH, CH₂ and CH₃ moieties.

Combined further with non-uniform sampling, the time required for acquiring the spectra is reduced by more than an order of magnitude compared with the conventional approach. This is demonstrated

for a mixture containing 21 of the commonly observed metabolites in the concentration range of 1 mM.

2. Experimental

2.1 Implementation of the experiments

The proposed experiments are implemented in a manner such that three FIDs from the three different experiments are acquired simultaneously. The primary source of magnetization is ¹H, which is bound to ¹²C (99%) and ¹³C (1%). The magnetization of ¹H attached to ¹³C and ¹²C are channelled through different pathways. This is depicted figuratively in figure 2 and is similar to the experiments demonstrated previously for the non-multiplicity version.³⁶ In brief, proton magnetization is first brought to a transverse plane. The ¹H spins attached to ¹³C transfer part of their magnetization to ¹³C via INEPT (Insensitive Nuclei Enhancement by Polarization Transfer) and the remaining magnetization is retained on itself. This is achieved by tuning the INEPT delay to $1/4 \cdot J_{CH}$.³⁶ The magnetization on ¹³C originating from ¹H is frequency labelled during (t_1) for joint sampling with ¹H(t_1) using a scaling factor (κ). After the chemical shift evolution period of ¹³C, the multiplicity editing block: $\tau_1 - 180^\circ_x(^1H), 180^\circ_x(^{13}C) - \tau_1$ is inserted to differentiate CH, CH₂, CH₃. The ¹³C magnetization is transferred back to ¹H and the signal is detected in proton channel. This results in the GFT spectrum. The magnetization of ¹H attached to ¹³C, which was retained is transferred to ¹³C via J-coupling and the signal is detected in carbon channel to get 2D [¹³C, ¹H] HETCOR. The chemical shift of proton (¹H) attached to ¹²C evolves during t_1 in a semi-constant time manner, and preserved as it is throughout the pulse sequence and isotropically mixed and finally signal is detected in proton channel with decoupling from ¹³C, resulting in the 2D [¹H, ¹H] TOCSY spectrum. We denote the three spectra as: DR-GFT multiplicity edited (3,2)D [¹³C, ¹H] HSQC-TOCSY, DR-(¹³C, ¹H) HETCOR, and DR (¹H, ¹H) TOCSY (figure 3). The prefix DR denotes ‘dual receiver’.³⁶

The radiofrequency (r.f.) pulse scheme is shown in figure 3. The acquisition of DR-GFT multiplicity edited (3,2)D HSQC-TOCSY is accomplished by joint increment of ¹³C chemical shift along with ¹H in a quadrature detection using the States method by varying $\phi_a = x, y$. Thus, the four FIDs collected by independently varying ϕ_a and ϕ_b . The obtained FIDs are pre-processed by multiplying with G-matrix to generate linearly combined ¹H_i chemical shift with its directly attached ¹³C_i along ω_1 axis and ¹H_i TOCSY correlation along ω_2

Figure 1. Simulated 2D [¹³C-¹H] HSQC spectrum of 315 metabolites present in the blood serum. Cross peaks of CH, CH₂, CH₃ moieties are depicted in different colours as indicated.

Figure 2. The magnetization transfer pathway implemented in the dual receiver experiments.

axis. The FIDs of 2D TOCSY and DR-GFT multiplicity edited (3,2)D HSQC-TOCSY are collected simultaneously. Separation of the two FIDs is accomplished by acquiring 2D TOCSY and DR-GFT multiplicity edited (3,2)D HSQC-TOCSY in an interleaved manner with same and opposite phases in consecutive transients, respectively. This is achieved by collecting the FID of DR-GFT multiplicity edited (3,2) HSQC-TOCSY with phase $\varphi_2 = x, -x$ (figure 3). The FID from the

two scans are added/subtracted for separating the two FIDs of 2D TOCSY and DR-GFT multiplicity edited (3,2)D HSQC-TOCSY. Note that the additional scans used above for interleaving the data sets come from the scans used for signal averaging and thus contribute to the increase in signal to noise. Thus, no additional time is invested for collecting the two data sets. The 2D [^{13}C , ^1H] HETCOR is acquired and stored in separate data set.

Figure 3. The r.f. pulse sequence of DR-GFT ^{13}C multiplicity edited (3,2)D HSQC-TOCSY, DR- ^{13}C , ^1H] HETCOR and DR- ^1H , ^1H] TOCSY is depicted. The empty thin and filled thick vertical bars represent hard 90° and 180° r.f. pulses respectively. All pulses are indicated with phases, if they are not applied along x axis. High power hard 90° ^1H pulse length is $9\ \mu\text{s}$ and $12\ \mu\text{s}$ for high power hard 90° ^{13}C r.f. pulse in the solution state. The ^{13}C r.f. carrier is placed at 40 ppm and the ^1H carrier position is set to 4.7 ppm throughout the duration of the experiment. Chemical shift evolution of ^1H during t_1 is carried out in a semi-constant time manner. The initial values ($t_1 = 0$) of t_{1a} (0), t_{1b} (0) and t_{1c} (0) are 0.9 ms, $3\ \mu\text{s}$ and 0.9 ms, respectively ($1/(8\ ^1J_{\text{HC}})$; $^1J_{\text{HC}} = 140\ \text{Hz}$) such that $T = 1.8\ \text{ms}$. For semi-constant time evolution, the values of Δt_{1b} and Δt_{1c} are based on the number of increments and the spectral width. For 512 complex points and spectral width of 20,000 Hz (25 ppm) used for the indirect (^1H) dimension in the current study, the values of Δt_{1a} , Δt_{1b} and Δt_{1c} are 25, 23.3 and $-1.7\ \mu\text{s}$. The chemical shift evolution period of ^{13}C during t_1 is co-incremented with that of ^1H with a relative scaling factor (κ). The delays used are $\tau_1 = 3.6\ \text{ms}$, $\tau_2 = 1.79\ \text{ms}$, $\tau_3 = 893\ \mu\text{s}$ and GARP is employed to decouple ^{13}C from ^1H . A ^{13}C spectral width of 200 ppm is chosen for the direct dimension (in 2D HETCOR) and 25 ppm for the indirect dimension (in DR-GFT multiplicity edited (3,2)D HSQC TOCSY and 2D ^1H - ^1H TOCSY). A mixing time of 80 ms is used for isotropic mixing of ^1H with DIPSI. The delays are $\tau_1 = 1.8\ \text{ms}$, $\tau_2 = 0.9\ \text{ms}$. Phase cycling: $\varphi_1 = y, -y$; $\varphi_2 = x, -x$; $\varphi_3 = x, -x, -x, -x$; $\varphi_4 = x, x, -x, -x$; $\varphi_5 = x, -x$; $\varphi_6 = x, x, -x, -x$; $\varphi_7 = x, x, -x, -x$; $\varphi_8 = x, -x$; $\varphi_9 = x, x, -x, -x$; $\varphi_{\text{rec}}(^{13}\text{C}) = x, x, -x, -x$; $\varphi_{\text{rec}}(^1\text{H}) = x, -x, -x, x$. The PFGs applied have Sinc shaped with strengths: $G1 = 19\ \text{G/cm}$; $G2 = 19\ \text{G/cm}$; $G3 = -19\ \text{G/cm}$; $G4 = 19\ \text{G/cm}$; $G5 = 11\ \text{G/cm}$; Quadrature detection in t_1 (^1H) out in States manner (i.e., $\varphi_a = x, y$). The ^{13}C multiplicity edited block is highlighted in red colour rectangular box.

Table 1. Experimental parameters used for data acquisition.

Experiment Name	Spectral width (ppm) and Nucleus detected				Acquisition time ($t_{\max}$) (ms)		No of complex points		Measurement time*	Signal/ Noise Ratio [§]
	Nuc.	F1	Nuc	F2	F1	F2	F1	F2		
DR-GFT ^{13}C multiplicity edited GFT (3, 2)D HSQC TOCSY	^1H	25	^1H	16	13	160	512	2048	10.5 Hrs	5.0 ± 1.7
^1H - ^1H TOCSY	^1H	25	^1H	16	13	160	512	2048		70 ± 30
2D ^{13}C - ^1H HETCOR	^1H	25	^{13}C	200	13	78	512	3072		5.5 ± 2.1

*A relaxation delay of 2.0 s was used between scans and the number of transients collected (scans) per increment is 8.

§A distribution of the S/N for different spectra is given in figure S2 of Supporting Information.

The addition of ^{13}C multiplicity edited phase to CH, CH_2 and CH_3 simplify the spectral analysis. The sign and intensity of the CH_n resonance depends on the delay ' τ_1 ' in the multiplicity editing block (figure 3; shown boxed in red colour) and given by an expression $\cos^n(2\pi J^* \tau_1)$ (where $n = 1, 2, 3$ and $\tau_1 = 1/2 * J_{\text{CH}}$, $J = 140 \text{ Hz}$). The variation of phase and intensity with different angle is shown in figure S1. The resonances of CH ($n = 1$) and CH_3 ($n = 3$) moieties possess same phase and opposite to that of CH_2 moieties ($n = 2$; due to the cosine modulation). Note that the editing of the CH, CH_2 and CH_3 groups happens only in the GFT spectra because only $^{13}\text{C}(t_1)$ is edited, which is detected in the GFT spectra. The method has been utilized for assignment of a mixture of 17 amino acid and 4 metabolites. The complete assignment of resonances is given in the Supporting Information (figure S2).

2.2 Sample Preparation and Data Collection

The sample was prepared by mixing from stock solution the desired amount of each metabolites in 100% $^2\text{H}_2\text{O}$. The components of mixture are Alanine, Arginine, asparagine, Cysteine, Glucose, Glutamine, Histidine, Lactate, Lysine, Isoleucine, Leucine, Methionine, Phenylalanine, Proline, Pyruvate, Serine, Taurine, Threonine, Tryptophan, Tyrosine and Valine. All experiments were carried out at 25°C on a Bruker Avance spectrometer operating at 800 MHz proton resonance frequency and equipped with cryogenically cooled probe and dual receiver system. Processing of all the spectra were done using NMRPipe³⁸ and analysis was carried out using XEASY.³⁹ The details of data acquisition are given in table 1.

3. Results and Discussion

Among the spectra acquired in the dual receiver set of experiments, 2D HETCOR is a central spectrum and

assignment begins from arbitrarily selecting a cross peak (C_i, H_i) from 2D HETCOR. The peak $^1\text{H}_j$ belonging to the same spin system as that of (C_i, H_i) is identified along the ω_2 axis in multiplicity edited DR-GFT multiplicity edited (3,2)D HSQC-TOCSY at $\Omega(^1\text{H}_i) \pm \kappa * \Omega(^{13}\text{C}_i)$ along ω_1 . The phase (positive or negative) of the peak provides the multiplicity of (C_i, H_i) (CH , CH_2 and CH_3). This aids in grouping all peaks belonging to one spin system. For instance, if two metabolites have similar ^{13}C chemical shifts, their spin systems will overlap. However, if they belong to different multiplicities, all TOCSY peaks for that spin system will have the same phase. The spin system thus identified is again validated by 2D [^1H , ^1H] TOCSY and the resonance peak (C_i, H_i) is assigned on 2D HETCOR. The process is repeated until all the resonances of the spin system are assigned.

The four spectra obtained for 17 amino acid and 4 metabolite mixtures are shown in figure 4. These metabolites comprise the innovative sequential media (ISM-1) used for *in vitro* fertilization.³⁶ The two DR-GFT multiplicity edited (3, 2)D HSQC-TOCSY spectra edited by ^{13}C multiplicity lead to phase discrimination between CH, CH_2 and CH_3 . All three spectra were acquired at ^{13}C natural abundance. The number of peaks observed in 2D HETCOR is 92% (61 peaks) of expected number of correlations and $\sim 90\%$ in ^{13}C multiplicity edited DR-GFT multiplicity edited (3, 2)D HSQC-TOCSY. The ^1H and ^{13}C chemical shifts of all the metabolites obtained by assigning the three spectra mapped on the 2D HETCOR spectrum is shown in figure S3 and their values are provided in table S1. The cross peaks of CH and CH_3 appear with the same phase and opposite to that of CH_2 moieties.

The transfer of magnetization is similar to the previous set of experiments and the sensitivity of the spectra is of a similar order.³⁶ However, the peaks from CH, CH_2 and CH_3 were not distinguishable in the earlier experiments. The multiplicity edited GFT spectrum helps to resolve peaks in the following way. Two spins

Figure 4. The ^{13}C multiplicity edited GFT (3, 2)D HSQC-TOCSY, 2D [^{13}C , ^1H] HETCOR, 2D [^1H , ^1H] TOCSY spectra of the 21 metabolite mixture used in the current study. Note that the editing of the CH, CH_2 and CH_3 groups happens only in the GFT spectra because only $^{13}\text{C}(t_1)$ is edited during the τ_1 delay period, which is detected in the GFT spectra.

systems that have degenerate ^{13}C shifts but different ^1H shifts of their attached protons are resolved in the GFT experiment due to the detection of the linear combination: $\Omega(^1\text{H}_i) \pm \kappa^* \Omega(^{13}\text{C}_i)$.³⁶ However, there can be a situation when two spin systems have degenerate $\Omega(^1\text{H}_{i0}) \pm \kappa^* \Omega(^{13}\text{C}_i)$ even in the GFT spectrum such that the peaks corresponding to these spin systems overlap in ω_1 dimension. However, if they belong to different CH multiplicities, the peaks corresponding to

one spin system can be resolved from the other system based on the phase (sign) of the peaks. This is exemplified in figure 5 for two spin systems: (Valine and Arginine) in the present study.

We carried out simulations to assess the overall impact of such resolution in general. For these simulations the 902 chemical shifts from 315 metabolites present in the blood sample (alluded to above) were used. An analysis reveals that $\sim 80\%$ of all ^{13}C shifts

Figure 5. The chemical shift correlation of ' $\text{H}^\beta/\text{C}^\beta$ ' peak of valine and ' $\text{H}^\gamma/\text{C}^\gamma$ ' peak of arginine overlapping along ω_1 -axis of DR-GFT ^{13}C multiplicity edited (3,2)D HSQC-TOCSY sub-spectrum ($[\Omega(^1\text{H}) + \Omega(^{13}\text{C})]$). The phase discrimination (Valine $\text{H}^\beta/\text{C}^\beta$ is a CH-system and Arginine $\text{H}^\gamma/\text{C}^\gamma$ is CH_2 -system) helps in identifying the peaks belonging to valine (green) and arginine (blue) spin systems.

observed in the 2D [^{13}C , ^1H] correlation spectrum overlap (in the ω_1 (^{13}C) dimension) within ± 0.2 ppm with ^{13}C shifts of one or more cross peaks. However, when the chemical shifts of both ^1H and ^{13}C are combined in the GFT experiment³⁶ as shown in figures 2 and 4, this overlap is reduced to $\sim 40\%$. When one further considers the discrimination of CH, CH_2 and CH_3 as shown above the percentage ambiguity reduces to $\sim 25\%$. Thus, the phase discrimination of CH_2 from CH and CH_3 in DR-GFT ^{13}C multiplicity edited (3, 2)D HSQC-TOCSY helps in speeding up resonance assignments.

Data collection can be further speeded up by adopting non-uniform sampling.^{30,31,35,40–45} This is exemplified in figure 6, which shows the construction of the ^{13}C multiplicity edited GFT (3,2)D spectrum at 50% of deletion of the points (chosen with Poisson gap sampling³¹) in the indirect dimension. The spectra with 50% NUS was acquired with half the measurement time as 100%. Considering the same measurement time the average SNR per unit measurement time of DR GFT (3,2)D ^{13}C edited HSQC-TOCSY is ~ 5.0 (provided in table 1) and 50% NUS is ~ 5.1 . Thus, very high-resolution spectra can be acquired within a short

period of time using the NUS, which will be helpful for resolving near degenerate spin systems such as those of Glucose and Maltose.⁴⁶ NUS will thus allow one to acquire high-resolution spectra without compromising on the measurement time.

4. Conclusions

In summary, we have demonstrated a new NMR-based approach applicable to metabolomics, which involves the simultaneous acquisition of three spectra in a single data set. The method is based on a combination of different fast data acquisition techniques such as G-matrix Fourier transform (GFT) NMR spectroscopy, the dual receiver system and non-uniform sampling. The spectra are obtained at high resolution and provide high-dimensional spectral information for resolving ambiguities. The addition of multiplicity editing further resolves the ambiguities between the CH, CH_2 and CH_3 groups. This opens up new avenues for high-throughput approaches for NMR-based metabolomics.

Supplementary Information

Figure S1, chemical shift assignments of metabolites mapped on to the 2D HETCOR spectrum and Table S1, list of chemical shift assignments of the metabolites obtained using the dual receiver experiments are available at www.ias.ac.in/chemsci.

Acknowledgements

The facilities provided by NMR Research Centre supported by Department of Science and Technology (DST), India, is gratefully acknowledged. HSA acknowledges support from DST (Grant no: No. IR/S0/LU-007/20110/1) and DAE-BRNS research grants.

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Figure 6. The DR-GFT multiplicity edited (3, 2)D ^{13}C HSQC-TOCSY spectra of 21 metabolite mixture are reconstructed from 50% NUS are shown in the figure. The reconstruction of spectra is done using compress sensing. The traces of 50% NUS spectra and 100% sampled (uniformly sampled) spectra are shown on the right side of the figure.

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