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NMR profiling of biomolecules at natural abundance using 2D ^1H – ^{15}N and ^1H – ^{13}C multiplicity-separated (MS) HSQC spectra

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Abstract

2D NMR ^1H – X ($\text{X} = ^{15}\text{N}$ or ^{13}C) HSQC spectra contain cross-peaks for all XH_n moieties. Multiplicity-*edited* ^1H – ^{13}C HSQC pulse sequences generate opposite signs between peaks of CH_2 and CH/CH_3 at a cost of lower signal-to-noise due to the ^{13}C T_2 relaxation during an additional $1/^1J_{\text{CH}}$ period. Such CH_n -editing experiments are useful in assignment of chemical shifts and have been successfully applied to small molecules and small proteins (*e.g.* ubiquitin) dissolved in deuterated solvents where, generally, peak overlap is minimal. By contrast, for larger biomolecules, peak overlap in 2D HSQC spectra is unavoidable and peaks with opposite phases cancel each other out in the edited spectra. However, there is an increasing need for using NMR to profile biomolecules at natural abundance dissolved in water (*e.g.*, protein therapeutics) where NMR experiments beyond 2D are impractical. Therefore, the existing 2D multiplicity-*edited* HSQC methods must be improved to acquire data on nuclei other than ^{13}C (*i.e.* ^{15}N), to resolve more peaks, to reduce T_2 losses and to accommodate water suppression approaches. To meet these needs, a multiplicity-*separated* ^1H – X HSQC (MS-HSQC) experiment was developed and tested on 500 and 700 MHz NMR spectrometers equipped with room temperature probes using RNase A (14 kDa) and retroviral capsid (26 kDa) proteins dissolved in 95% $\text{H}_2\text{O}/5\%$ D_2O . In this pulse sequence, the $1/^1J_{\text{XH}}$ editing- period is incorporated into the semi-constant time (semi-CT) X resonance chemical shift evolution period, which increases sensitivity, and importantly, the sum and the difference of the interleaved $^1J_{\text{XH}}$ -active and the $^1J_{\text{XH}}$ -inactive HSQC experiments yield two separate spectra for XH_2 and XH/XH_3 . Furthermore we demonstrate improved water suppression using triple *xyz*-gradients instead of the more widely used *z*-gradient only water-suppression approach.

Keywords

MS-HSQC; Multiplicity editing; Natural abundance; Triple-axis gradient water suppression

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5. Disclaimer

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

1. Introduction

There is an increasing need to fingerprint therapeutic biomolecules by solution NMR in formulated drug products at natural abundance [1–8]. Two-dimensional *hetero*-nuclear ^1H -X (X = ^{15}N or ^{13}C) spectral patterns are sensitive to chemical structure and therefore can detect structural change at the level of individual nuclei. These methods are superior to *homo*-nuclear two-dimensional spectra because of their increased chemical shift dispersion, less *homo*-nuclear J -splitting, and concomitant reduced peak overlap [2]. Two-dimensional methods have become even more powerful with the advent of multiplicity-*edited* ^1H - ^{13}C HSQC, HMQC, HMBC and H2BC experiments [9–19]. Multiplicity-*edited* pulse sequences use a dedicated period of $1/^1J_{\text{CH}}$ to edit CH_2 and CH/CH_3 peaks into the opposite phases in the same spectrum. Most edited experiments were developed for and applied to small molecules at ^{13}C natural abundance dissolved in deuterated solvents and are therefore multiple-quantum based experiments. Additionally, generally, small molecules contain fewer overlapping resonances and have longer T_2 values compared to larger protein molecules.

For larger molecules like proteins, HSQC-based experiments have higher sensitivity than HMQC-based experiments, because, due to the transverse ^1H magnetization during t_1 , the HMQC-based experiments have additional T_2 decay [20]. Interestingly, among all published ^{13}C multiplicity-*edited* 2D experiments only the HSQC version was demonstrated on ubiquitin dissolved in D_2O [16].

Experimental protein NMR spectra are typically collected in H_2O , where some protein backbone amide NH peaks overlap with side-chain NH_2 peaks. In addition, CH_n peaks also overlap with CH_m (where $n \neq m$) peaks, especially in spectra where peaks are folded. Finally, XH_n peaks in un-folded peptides and proteins may overlap with peaks from un-structured regions of a folded protein. Unresolvable 2D cross-peaks, though edited, will adversely affect chemical shift assignment and, concomitantly, chemometric analysis of 2D-NMR data acquired on biomolecules at natural abundance. Thus, to resolve potentially overlapped XH_n peaks, we present a multiplicity-*separated* (MS) HSQC experiment. Here, the most important feature of multiplicity-separation is achieved by separately collecting the $^1J_{\text{XH}}$ -active and the $^1J_{\text{XH}}$ -inactive spectra in an interleaved fashion; then, the sum and the difference are calculated directly to yield separation. This new approach absorbs the previously separate editing period of $1/^1J_{\text{XH}}$ into a modified semiconstant time (semi-CT) chemical shift evolution period, reducing the total time that X magnetization spends in the transverse plane, and minimizing signal loss due to T_2 relaxation. In addition, water suppression can be readily achieved in 95% $\text{H}_2\text{O}/5\%$ D_2O using either flip-back pulses [21] or triple-axis gradients. Herein, we demonstrate the new experiments on a 14-kDa natural abundant RNase A and a 26-kDa ^{15}N -labeled retroviral capsid within a reasonable amount of spectrometer time (1–2.5 days for the natural abundance sample) using a room-temperature probe-head.

2. Results and discussion

2.1. The $^1J_{\text{XH}}$ -active and the $^1J_{\text{XH}}$ -inactive semi-CT HSQC pulse sequences

The original multiplicity-*edited* ^1H - ^{13}C HSQC pulse sequence [16,17], commonly found in vendor pulse sequence libraries, has the signature spin chemical shift and $^1J_{\text{CH}}$ evolution shown in Eq. (1). Eq. (1) depicts the ^{13}C antiphase H_zC_y magnetization evolution between the first and the last INEPT periods,

$$\text{H}_z\text{C}_y \xrightarrow{t_1} \text{H}_z\text{C}_y \cos \omega_{\text{C}} t_1 \xrightarrow{1/J_{\text{CH}}} \text{H}_z\text{C}_y \cos \omega_{\text{C}} t_1 \cos^n \pi \quad (1)$$

where t_1 is the ^{13}C chemical shift evolution period, ω_{C} is the ^{13}C Larmor frequency and n is the number of ^1H attached to ^{13}C . Depending on the value of n , the sign of the H_zC_y magnetization will be positive for CH_2 and negative for CH/CH_3 cross-peaks. The maximum amount of time that ^{13}C magnetization spends in the transverse plane is $t_{1\text{max}} + 1/{}^1J_{\text{CH}}$.

To keep the editing feature and reduce T_2 losses, we incorporated an extra $1/{}^1J_{\text{XH}}$ time, 8–11 ms, into the t_1 chemical shift evolution period, *i.e.*, $t_{1\text{max}}$ of 40–50 ms. The semi-CT method is selected for this purpose. The semi-CT was originally used for ^1H chemical shift evolution during INEPT transfers to ^{13}C in order to minimize ^1H T_2 losses [22,23]. Here, we modify the semi-CT during the *hetero*-nuclear X chemical shift evolution to generate the multiplicity-*separated* spectra. Briefly two semi-CT HSQC pulse sequences were run in an interleaved fashion. The difference between the two sequences lies in where the ^1H 180° pulse is applied (Fig. 1). In the $^1J_{\text{XH}}$ -active experiment, the ^1H 180° pulse is applied at the red position and properly decrementing t_a and incrementing t_b and t_c (Table 1), the net $^1J_{\text{XH}}$ evolution time is maintained constant as $1/{}^1J_{\text{XH}}$ (Eq. (2)). Therefore, the editing feature is retained and the t_1 chemical shift labeling is properly maintained. The maximum time that *hetero*-nuclear X magnetization spends in the transverse plane is $t_{1\text{max}} + (1/{}^1J_{\text{XH}})/(N+1) \approx t_{1\text{max}}$, where N is the number of real points in t_1 acquisition.

$$\begin{aligned} \text{H}_z\text{X}_y &\xrightarrow{t_a} \text{H}_z\text{X}_y \cos \omega_{\text{X}} t_a \cos^n \pi J_{\text{XH}} t_a \xrightarrow{180^\circ\text{X}, t_b} \text{H}_z\text{X}_y \cos \omega_{\text{X}} (t_a - t_b) \quad (2) \\ &\times \cos^n \pi J_{\text{XH}} (t_a - t_b) \xrightarrow{180^\circ\text{H}, t_c} \text{H}_z\text{X}_y \cos \omega_{\text{X}} (t_a - t_b - t_c) \\ &\times \cos^n \pi J_{\text{XH}} (t_a - t_b + t_c) \\ &= \text{H}_z\text{X}_y \cos \omega_{\text{X}} (\Delta t_1) \cos^n \pi J_{\text{XH}} (1/J_{\text{XH}}) \end{aligned}$$

In parallel the $^1J_{\text{XH}}$ -inactive experiment has the ^1H 180° pulse being applied at the blue position (Fig. 1) with a new introduction of variable delay t_d which is incremented within the t_c period (Table 1). The modification results in the net $^1J_{\text{XH}}$ evolution time of 0 (Eq. (3)), ensuring the ^1H -decoupling throughout the full t_1 period. The $^1J_{\text{XH}}$ -inactive spectrum yields

the same sign for all XH_n cross-peaks. The t_1 chemical shift labeling is identical to the $^1J_{XH}$ -active spectrum.

$$\begin{aligned}
 H_z X_y &\xrightarrow{t_a} H_z X_y \cos \omega_X t_a \cos^n \pi J_{XH} t_a \xrightarrow{180^\circ X, t_b + t_c - t_d} H_z X_y \cos \omega_X (t_a - t_b - t_c + t_d) \cos^n \pi J_{XH} (t_a - t_b - t_c + t_d) \\
 &\xrightarrow{180^\circ H, t_d} H_z X_y \cos \omega_X (t_a - t_b - t_c + t_d - t_d) \cos^n \pi J_{XH} (t_a - t_b - t_c + t_d - t_d) \\
 &\times \cos \omega_X (t_a - t_b - t_c + t_d - t_d) \cos^n \pi J_{XH} (t_a - t_b + t_c + 2t_d) \\
 &= H_z X_y \cos \omega_X (\Delta t_1) \cos^n \pi J_{XH} (0)
 \end{aligned} \quad (3)$$

2.2. MS-HSQC spectra

Since both the $^1J_{XH}$ -active and the $^1J_{XH}$ -inactive experiments have the identical pulse execution time or T_2 loss, the post-acquisition sum and difference of the two at a 1:1 ratio yields the XH_2 only and the XH/XH_3 only spectrum (Figs. 2–4 and S2) without any need to scale relative intensities or further correct phases. The approach of directly adding and subtracting to obtain the desired spectra is similar to the in-phase-anti-phase (IPAP) HSQC experiments for residual dipolar coupling (RDC) measurement [24].

For experiments on proteins at natural abundance the current multiplicity-*separation* is an efficient and practical choice because chemical shift assignments from MS-HSQC spectra are more interpretable than those from multiplicity-*edited* spectra and an improved S/N is obtained relative to the standard multiplicity edited HSQC sequence. We tested both 1H - ^{15}N (Figs. 1A and 2) and 1H - ^{13}C (Figs. 1B, 4A and S2) MS-HSQC experiments on an RNase A sample at natural abundance dissolved in 95% H_2O /5% D_2O . For example, the NH peak *a* of RNase A, would commonly be treated as a minor impurity peak in the $^1J_{NH}$ -active (edited) spectrum (Fig. 2A) or as a tail of peak *b* in the $^1J_{NH}$ -inactive (regular) spectrum (Fig. 2B). Only under the separation method we can confidently identify peak *a* as an NH peak (Fig. 2C) and peak *b* as an NH_2 peak with correct intensity (Fig. 2D).

The 1H - ^{15}N MS-HSQC experiments were also demonstrated on a larger ^{15}N -labeled 26-kDa retroviral capsid protein (Fig. 3). The capsid protein shape is highly asymmetric, with an anisotropy of 1.8. Indeed, its averaged ^{15}N T_2 relaxation rate and Stokes radius correspond to that of a 32 kDa symmetric protein [25]. Table 2 shows sensitivity comparisons of the standard HSQC, the standard multiplicity-*edited* HSQC and the new semi-CT HSQC (Table 2). Due to the reduction in the time that ^{15}N magnetization spends in the transverse plane, the MS-HSQC yielded an average of 10% increase in peak S/N from the standard edited HSQC (Table 2 and Fig. S1). The MS-HSQC is at 85% of the S/N of the standard HSQC spectra, indicating a protein sample should be roughly 15% more concentrated to obtain S/N similar to a standard HSQC, if an MS-HSQC spectrum is desired. Based on the comparison we expect the MS-HSQC is generally applicable to a similar molecular mass range as the standard HSQC.

Natural abundance ^1H - ^{13}C MS-HSQC experiments were performed on the RNase A sample. The $^1J_{\text{CH}}$ -active and the $^1J_{\text{CH}}$ -inactive ^1H - ^{13}C HSQC spectra were collected with CH_2/CH_3 peaks folded (Figs. S2A and S2B). The difference and sum yielded the CH/CH_3 only (Figs. S2C and 4A) and the CH_2 only spectra (Fig. S2D). Importantly, the $^1J_{\text{CH}}$ values vary among CH_n moieties, *i.e.*, the $^1J_{\text{CH}}$ values of the sp^2 ring CH are 220–155 Hz, the $^1J_{\text{CH}}$ of the sp^3 groups are 140–150 Hz for CH, 135–145 Hz for CH_2 and 125–135 Hz for CH_3 , and are directly correlated to their respective ^{13}C chemical shifts [26]. Since all amino acid aromatic groups have CH only, a standard HSQC can be collected for them without any need of multiplicity separation. For aliphatic CH_n moieties, we have used the value of 125 Hz to match CH_3 peaks. Notably, the CH_2 only spectra contain undetectable CH/CH_3 peaks at a S/N threshold of 7.8 (Fig. S2D). However, there are still 3 CH_2 clusters/peaks with attenuated intensity in CH/CH_3 sub-spectra (red-dashed circles in Figs. 4 and S2C). In practice the $^1J_{\text{CH}}$ value might be set higher to 135 Hz, closer to the CH_2 $^1J_{\text{CH}}$ values to minimize the imperfect cancelation. Another alternative approach demonstrated by Heikkinen et al. is to add several MS-HSQC spectra with varied $^1J_{\text{CH}}$ values, because the sum of these spectra are less sensitive to $^1J_{\text{CH}}$ variations [27].

2.3. Water suppression

The MS-HSQC methods developed here (Fig. 1) also minimize sample manipulation steps, (*e.g.*, freeze-drying a biomolecule sample and re-dissolving in D_2O for ^1H - ^{13}C HSQC data) because all NMR experiments can be performed in 95% $\text{H}_2\text{O}/5\%$ D_2O . Water suppression was demonstrated on two general versions of HSQC, the standard HSQC [28] (Fig. 1A) and the sensitivity-enhanced version [29,30] (Fig. 1B). For the standard HSQC experiment, we used low-power shaped pulses to achieve a more complete water flip-back scenario, where water magnetization has been kept along $+z$ throughout the pulse sequence except for the first INEPT transfer, where water magnetization was along the y -axis. The complete $+z$ -flip-back is more robust than the typical flip-back pulse used in vendor libraries. Specifically, in typical flip-back experiments, the water magnetization is aligned along the $-z$ -axis for a period of $t_1/2$, which increases the likelihood of radiation damping, especially at longer t_1 acquisition times.

Here, for the sensitivity-enhanced HSQC (Fig. 1B) water suppression was achieved by using xyz -axis gradients after the first INEPT and during the coherence selection without using any shaped pulses. This allows the observation of $\text{C}_\alpha\text{--H}_\alpha$ correlation in the ^1H - ^{13}C MS-HSQC spectrum (Fig. 4A), which can be obscured by poor water suppression. The xyz -axis gradient is essential since the same HSQC experiment, when performed without using xy -gradient but otherwise identical experimental parameters, yielded poor water suppression spectrum which precluded the observation of $\text{C}_\alpha\text{--H}_\alpha$ correlations (Fig. 4B).

3. Conclusion

Here, the application of a new MS-HSQC experiment on biomolecules at natural abundance dissolved in 95% $\text{H}_2\text{O}/5\%$ D_2O was demonstrated. The new experiment generates separated spectra containing XH_2 peaks only or XH/XH_3 peaks only with higher sensitivity than the multiplicity-*edited* HSQC [14–16]. The separation feature is not an issue for $^{15}\text{N}/^{13}\text{C}$ -

labeled proteins, *e.g.*, a 3D HNCQ spectrum can be used to separate the side-chain NH₂ peaks and reveal any backbone NH peaks that may be hidden underneath them. However, for proteins at natural abundance, the simplified 2D MS-HSQC can improve the accuracy of comparisons between molecules made by chemometric analysis of specific chemical structures (*e.g.*, backbone NH *vs.* side-chain NH₂) of biomolecules in their native state [31]. This is crucial when profiling protein therapeutics for backbone structure as the side-chain NH₂ peaks add no information to such an evaluation and are more susceptible to formulation differences. Thus, the separated HSQC spectra with good water suppression should be amenable to NMR analysis of biotherapeutics in their formulation buffers.

The experiment described here will also work on isotope-labeled molecules for collecting ¹H-¹⁵N HSQC spectra (with the decoupling of C_α/C' if ¹³C-enriched). Similar to protein spectra in natural abundance, this approach can resolve the ambiguities in chemical shift assignments and serve to improve resolution in 2D spectra, which in turn will guide 3D assignments with higher confidence. Finally, the MS-HSQC can reveal more interacting NH peaks in titration studies if such peaks happen to be buried by the NH₂ resonances, and when combined with spin relaxation HSQC, allow a more complete set of measurements on the structure and dynamics of a biomolecule.

4. Materials and methods

4.1. Protein sample preparation

The RNase A powder at natural abundance (GE Healthcare Bio- Sciences, Pittsburgh, PA) was dissolved in 2– diluted PBS buffer (Quality Biological, Gaithersburg, MD) to the desired concentration of 1.7 mM. The ¹⁵N-labeled retroviral capsid protein, 0.1 mM in PBS buffer, was prepared as described [32]. For both samples, 570 µl protein solution was mixed with 30 µl D₂O (Cambridge Isotope Laboratories, Tewksbury, MA) then 550 µl of these solutions were loaded into 5-mm Wilmad 535-pp tubes (Wilmad-LabGlass, Vineland, NJ).

4.2. NMR spectroscopy

All NMR experiments were performed at 25 °C. The experimental temperature was calibrated using pure methanol. The pulse sequence details and acquisition parameters are in Fig. 1 caption and Table 1. We include the pulse sequence codes (Supplementary material) of Fig. 1A and B using the templates of the Bruker pulse programs *hsqcxfp3gpplhwg* and *hsqcetgpsl*, respectively. For the ¹H-¹⁵N HSQC experiment, which was performed on a Bruker Avance III, 500 MHz spectrometer, equipped with a room temperature QXI probe and a z-axis gradient, the total experimental time is 63 h using the pulse sequence in Fig. 1A. For the ¹H-¹³C HSQC experiment, which was performed on a Bruker Avance II 700 MHz spectrometer, equipped with a room temperature QXI probe and a xyz-axis gradient, the total experimental time is 21 h using the pulse sequence in Fig. 1B.

4.3. Data processing

The interleaved raw FID file was separated then added and subtracted using NMRPipe [33] scripts (Supplementary material). The resulting time domain FID were polynomial filtered, apodized with a cosine function and zero-filled to 4 k data points before Fourier transform

along both dimensions. The both frequency domain were baseline corrected with a polynomial function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jmr.2014.11.011>.

Fig. 1.

The pulse sequences for the $^1J_{\text{XH}}$ -active/inactive semi-CT HSQC experiments, which can be either the standard HSQC (A) or the sensitivity-enhanced version (B). Narrow and wide rectangles correspond to hard pulses with flip angles of 90° and 180° , respectively. Filled and open bells are 90° sinc-shaped water-selective pulses with a duration of 1.5–2.0 ms. The pulses in red and blue are only applied for the $^1J_{\text{XH}}$ -active and the $^1J_{\text{XH}}$ -inactive experiment, respectively. The open rectangles denoted CPD are low power decoupling scheme in either WALTZ-16 for ^{15}N or GARP for ^{13}C . Pulses are x -phase by default. Phase cycles are listed as follows, $\phi_1 = x$; $\phi_2 = x, -x$; $\phi_3 = (x)_4, (-x)_4$; $\phi_4 = (x)_2, (-x)_2$; $\phi_5 = (x)_8, (-x)_8$; $\phi_{\text{rec}} = (x, -x)_2, (-x, x)_2$ for (A) and $\phi_1 = x$; $\phi_2 = y, -y$; $\phi_3 = (x)_4, (-x)_4$; $\phi_4 = (x)_2, (-x)_2$; $\phi_5 = (y)_2, (-y)_2$; $\phi_{\text{rec}} = (x, -x, -x, x, -x, x, x, -x)$ for (B). The fixed delay durations are listed as follows, $\tau_1 = 1/(4^1J_{\text{XH}})$, $\tau_2 = 1.25$ ms and $\tau_3 = 1/(8^1J_{\text{XH}})$. The varied delays t_{a-d} during the X spin chemical shift evolution are listed in Table 1. All gradient pulses are sine-shaped and along z -axis by default except for the xyz gradient pulses G_1 , G_2 , G_4 and G_5 of (B). The duration and strength for gradient pulses are as follows, $G_1 = 1$ ms, 10 G/cm; $G_2 = 1$ ms, 5 G/cm; $G_3 = 0.5$ ms, 10 G/cm for (A) and $G_1(xyz) = 5$ ms, 12.5 G/cm; $G_2(xyz) = 1$ ms, 40 G/cm; $G_3 = 0.5$ ms, 5 G/cm; $G_4(xyz) = 1$ ms, 20 G/cm; $G_5(xyz) = 1$ ms, 30 G/cm for (B). Quadrature detection in (A) was achieved via States-TPPI method such that phase ϕ_1 and ϕ_2 of the X pulses were incremented 90° for every FID in t_1 acquisition. Quadrature detection in (B) was achieved via the Echo-Antiecho method such that the second FID for each increment of t_1 was collected with signs of gradient pulses G_2 and phase ϕ_5 of the 180° pulse switched simultaneously. The ^1H carrier frequency was at water resonance of 4.76 ppm and the ^{15}N and ^{13}C carrier frequencies were at 117 ppm and 57.0 ppm, respectively. The spectral width of ^1H was 12 ppm and the number of direct time domain complex points was 1024. The ^{15}N acquisition time was 42.1 ms and the spectral width was 30 ppm. The

^{13}C acquisition time was 50.7 ms and the spectral width was 14 ppm. The $^1J_{\text{XH}}$ -active and the $^1J_{\text{XH}}$ -inactive spectra were collected in interleaved manner by switching the position of the 180° ^1H pulse between the red and the blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

The sub-region of the ^1H - ^{15}N MS-HSQC spectra of RNase A at natural abundance dissolved in 95% H_2O /5% D_2O . The positive and the negative peaks are shown in black and red, respectively. The spectra of the $^1J_{\text{NH}}$ -active (A) and the $^1J_{\text{NH}}$ -inactive (B) were collected using the pulse sequence in Fig. 1A. The post-acquisition difference (C) and sum (D) spectra showed multiplicity separation between peaks of NH and NH_2 , *e.g.*, peaks *a* and *b* within the dashed box. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. The ^1H - ^{15}N MS-HSQC spectra of a ^{15}N -labeled 26 kDa retroviral capsid protein dissolved in 95% H_2O /5% D_2O . The post-acquisition difference (A) and sum (B) spectra showed multiplicity separation between peaks of NH and NH_2 .

Fig. 4.

The ^1H - ^{13}C MS-HSQC spectra for moieties of CH/CH₃ of RNase A at natural abundance dissolved in 95% H₂O/5% D₂O. The positive and the negative peaks are shown in black and red, respectively. The two spectra were collected with identical experimental parameters except for the use of the *xyz*-axis gradient pulses (A) and the *z*-axis only ones (B). The red dashed-circles indicate CH₂ cross-peaks that were not completely removed due to $^1J_{\text{CH}}$ mismatch. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

The varied delays in the $^1J_{\text{XH}}$ -active/inactive semi-CT HSQC experiments.

Varied delay	In-/de-crement value	Resulting value
t_a	$t_a = (1/2^1J_{\text{XH}})/(N + 1)^a$	$1/(2^1J_{\text{XH}}) - t_a$
t_b	$t_b = t_1/2 + t_a^b$	t_b
t_c	$t_c = t_1/2$	$1/(2^1J_{\text{XH}}) + t_c$
t_d	$t_d = t_1/2$	t_d

^a N is the number of real points in the indirect t_1 dimension.

^b $t_1 = 1/\text{SW}$, where SW is the spectral width of the X (^{15}N or ^{13}C) spin.

Table 2

Feature comparison among the three ^1H - ^{15}N HSQC spectra collected on a 26 kDa ^{15}N -labeled protein.

Type	Pulse program	Cross-peak sign			Averaged S/N^a	Experimental time ^b
		NH	NH ₂	Separation		
Standard	Bruker <i>hsqcpt3gpplhwg</i>	+	+	No	32.4	3 h 18 min
Standard multiplicity-edited	<i>hsqcpt3gpplhwg</i> with an extra $1/J_{\text{NH}}$ period after t_1	+	-	No	24.6	3 h 21 min
Semi-CT	The $^1J_{\text{NH}}$ -active version in Fig. 1A	+	-	Yes	27.1	3 h 12 min

^a A total of 137 isolated cross-peaks were picked on all spectra and peak S/N were calculated using Sparky (T.D. Goddard and D.G. Kneller, University of California, San Francisco). The averaged S/N are shown.

^b The sample is a 0.1 mM ^{15}N -labeled retroviral capsid protein (26 kDa). All HSQC spectra were collected on a Bruker 500 MHz spectrometer equipped with a room-temperature probe.