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Covariance Spectroscopy in High-Resolution Multi-dimensional Solid-state NMR

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Abstract

Covariance spectroscopy (COV), a statistical method that provides increased sensitivity, can be applied to two-dimensional high-resolution solid-state NMR experiments, such as homonuclear spin-exchange spectroscopy. We proposed the alternative States sampling scheme to the experimental time by 50%. By combining COV with other processing methods for non-uniform sampling (NUS), many different three-dimensional experiments can be performed with substantial increases in overall sensitivity. As an example, we show a three-dimensional homonuclear spin-exchange / separated-local-field (SLF) spectrum that enables the assignment of resonances and the measurement of structural restraints from a single experiment performed in a limited amount of time.

There are many advantages to performing three- and higher dimensional experiments in NMR spectroscopy. This is an area that is well developed for high-resolution solution NMR, but has lagged somewhat for solid-state NMR, especially of proteins, largely because of the broader widths of single-line resonances and the complex line shapes of powder patterns, and the generally lower signal-to-noise ratios of the spectra. For example, a three-dimensional combination of homonuclear spin-exchange and separated-local-field (SLF) spectroscopies can provide many distance and angular structural restraints, and full or partial resonance assignments in a single experiment[1–4]. Therefore, it has the potential to accelerate and improve protein structure determination by solid-state NMR. However, using conventional uniform sampling (US) of the time domain signals in three dimensions, it often requires many days of signal averaging to perform. To reduce the total amount of time required to perform three- and higher-dimensional NMR experiments, several methods of processing US[5] and non-uniform sampled[6–13] (NUS) signals have been proposed as replacements for conventional Fourier transformation of complete US free induction decays (FIDs). However, most of these methods require a minimal signal-to-noise ratio >10 for successful spectral reconstructions, and even this modest ratio may not be readily available for many of the protein samples of greatest interest. In addition, sparse data sets are typically required to avoid the introduction of spectral artifacts.

Here we describe applications of covariance (COV) spectroscopy to reconstruct spectra whose resonances have signal-to-noise ratios <10 and relatively broad linewidths. These include the use of sampling schemes based on the States method[14], which has the further

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benefit of reducing the time spent acquiring signals in half. We also demonstrate that it is possible to combine the NUS method of Compressed Sensing (CS)[10; 11] with COV to reconstruct a three-dimensional homonuclear spin-exchange/ separated-local-field spectrum with only 11.7% sampling compared to 100% US sampling without losing resolution, and with a diagonal in only one of the two-dimensional spectral planes. The COV and CS processing were performed using Matlab (Mathworks, http://www.mathworks.com) scripts.

COV[15–19] is a statistical method that generates the indirect dimension according to the covariance of the direct dimension, saving the time required to acquire a full data set in the indirect dimension. The spectrum is generated according to equation (1) [17]:

$$\mathbf{C}_{ij}=\frac{1}{N_1}\sum_{k=1}^{N_1}\left(\mathbf{S}(k,i)-\langle\mathbf{S}(i)\rangle\right)\left(\mathbf{S}(k,j)-\langle\mathbf{S}(j)\rangle\right) \quad \text{equation (1)}$$

N_1 is the number of point in indirect dimension, **S** is the one-dimensional frequency spectrum at evolution time t_k for the indirect dimension, *i* and *j* are the indices of the frequencies, and the angle bracket of **S** is the average over time t_k at frequency ω_i. It has been shown that the covariance of a frequency pair represents the off-diagonal element in a two-dimensional Fourier transform spectrum[17; 18], and the variance of each frequency represents the diagonal element. The frequency evolutions in the t1 dimension are treated as trials to find correlations between signals. Some advantages are immediately apparent. The spectral width and the resolution of the indirect dimension are identical to those of the direct dimension, however they are independent of the dwell time and N_1 for isolated cross-peaks because the covariance matrix is symmetric and calculated from the data acquired in the direct dimension. Therefore, baseline and phase corrections, apodization, or other signal processing methods are not required in the t1 dimension. Consequently, high-resolution homonuclear correlation spectra can be obtained with a quite small value of N_1, as long as N_1 is large, which can be verified 'on the fly'[20].COV spectra can be obtained from either homo- or hetero-nuclear correlations, with the latter referred to as indirect covariance NMR spectroscopy[16].

We acquired the $^{15}N/^{15}N$ homonuclear spin-exchange spectrum of the 46-residue membrane-bound form of uniformly ^{15}N-labeled Pf1 coat proteins in macrodiscs, which consist of a phospholipid bilayer surrounded by a 'belt' protein[21]. Macrodiscs are large enough to magnetically align the protein-containing bilayers and to 'immobilize' the proteins in all directions while still enabling them to undergo rapid rotational diffusion about the bilayer normal. Both macrodiscs and bicelle samples have excess water present, therefore the proteins and lipids are fully hydrated. Moreover, macrodiscs possess the additional advantages over bicelles in that they are "detergent-free" and therefore have less potential to cause distortions of the protein structures [21]. Protein-containing bilayers aligned on glass plates also do not have detergent present, but are more difficult to keep fully hydrated due to heating from the radiofrequency irradiations.

According to equation (1), a homonuclear two-dimensional correlation spectrum can be obtained even when $N_1 = 1$; however, due to the absence of sufficient evolution for statistical verifications, the correlations will be incorrect. Similar situations (Figure 1E and 1H) are found for highly truncated sampling (Figure 1B).

To reduce the experimental time without building up incorrect correlations, we alternately sample real and imaginary parts of the signals (Figure 1C) because when COV is applied with the States method, the sign does not rely on quadrature detection. In this way it is possible to both maintain spin evolution and reduce the experimental time by 50%. To

demonstrate the idea, we randomly generated six ^{15}N resonances with different chemical shifts (23.4, −40.9, −34.6, −23.4, −7.7, 27.9 ppm), with 100 Hz line widths at a field strength corresponding to a 1H resonance frequency of 700 MHz. Notably, one pair of resonances is symmetric about the carrier frequency. We calculated 48 complex points with 100 μs dwell time in the indirect dimension as a conventional sampling scheme, and 24 complex points under the same conditions as an example of a highly truncated scheme. The signals were generated according to the alternative States sampling scheme. These simulated signals were processed with COV. Comparing to the covariance spectra with reasonable sampling points (Figure 1D), the highly truncated sampling (Figure 1E) results in artifacts due to the short evolutions. In contrast, the alternate (Figure 1F) States sampling provides only the correct correlations.

The spectrum of the membrane-bound form of the uniformly ^{15}N labeled coat protein in aligned bilayers obtained with conventional sampling and processed by COV is shown in Figure 1D. On larger membrane proteins, whose samples contain a smaller total amount of protein in the same volume, the sampling in the indirect dimension must be reduced in order to acquire the data for a two-dimensional spectrum with an adequate signal-to-noise ratio in a reasonable amount of time. The sensitivity enhancement by COV has been reported to be >20% when the dwell times of both dimensions are identical[15; 19]. In our example, the signal-to-noise ratio resulting from COV is almost twice that from Fourier transformation. The dwell time in the direct dimension (20 μs) is smaller than that in the indirect dimension (100 μs), which results in the better sensitive in the frequency domain, and this benefit is brought to the indirect dimension via COV. In this spectrum, the average signal-to-noise ratio of the resonances obtained after Fourier transformation is <10. Notably, other NUS methods generally fail for spectra with such broad line widths and limited signal-to-noise ratios. We applied the alternative States sampling that also reduces the experimental time required to obtain the same signal-to-noise ratio. The result in Figure 1I shows that it is possible to reconstruct the spectrum with a similar signal-to-noise ratio, while highly truncating sampling (Figure 1H) already started to generate artifacts.

The tolerance of COV to low signal-to-noise ratios makes it feasible to accelerate the acquisition three-dimensional US/NUS spectra with low to moderate signal-to-noise ratios. We demonstrate this with a single crystal sample of ^{15}N N-acetyl leucine (NAL), which has four unique molecules in the unit cell, which results in four signals. The three-dimensional ^{15}N/^{15}N spin-exchange/^{1}H-^{15}N SLF experiment employs mismatched Hartmann-Hahn (MMHH) spin diffusion for the homonuclear spin-exchange and SAMPI4 for measuring the heteronuclear dipolar couplings associated with individual chemical shift frequencies[22]. MMHH relies on the proton bath to assist spin-exchange among the dilute nuclei; therefore long-range correlations can be observed under this mechanism. Since the magnetizations tend to evenly distribute among the dilute spins via the proton bath, the magnetizations also have the opportunity to be deposited into other reservoirs of couplings, and it would make the spectra ambiguous for samples with weak signals[23; 24]. With this concern, we only applied MMHH to the single crystal spectrum, and applied a conventional homonuclear spin exchange (proton-driven spin diffusion, PDSD) to coat protein in bilayer samples. We reconstructed the ^{1}H-^{15}N dipolar coupling dimension and ^{15}N/^{15}N correlations by CS and COV, respectively, where the CS reconstructions are based on the NUS schemes that we previously optimized[13]. The reconstructed spectrum is shown in Figure 2A, and the strong diagonal noise resulting from COV was readily removed by subtracting the average values (Figure 2B)[25]. Spectral planes extracted from the three-dimensional spectrum for the four resonances are shown in Figure 3. They consist of doublets from the heteronuclear dipolar couplings associated with each of the resonances correlated via MMHH. 50% and 23.4% of points were sampled for the dipolar coupling and the indirect chemical shift dimensions, respectively, which results in 11.7% sampling compared to 100%

US for the three-dimensional experiment. The line widths and line shapes of the resonances in the reconstructed spectral planes are comparable to those obtained from the corresponding two-dimensional experiments.

In conventional two-dimensional NMR[24; 26–30] approaches to structure determination, at least two separate experiments would be needed in order to make resonance assignments and measure structural restraints. By combining homonuclear spin exchange and SLF spectroscopies, the correlations and measurements of dipolar couplings and chemical shifts are obtained from a single spectrum. Moreover, the covariance spectra have intrinsically high-resolution and sensitivity, which makes this a general approach that can be incorporated into a variety of higher-dimensional experiments. The combination of benefits has the potential to substantially shorten the total amount of time spent performing experiments and signal averaging while improving the quality of structural restraints in structure determination of biopolymers by solid-state NMR. It is particularly applicable to membrane proteins studied by oriented sample (OS) solid-state NMR in stationary, aligned samples or in unoriented samples undergoing magic angle spinning.

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Highlights for Lin and Opella 2013

- Covariance spectroscopy enables spectral reconstruction with low signal-to-noise.
- Covariance and compressed sensing can be combined for three-dimensional NMR.
- A three-dimensional spectrum was obtained with 11.7% sampling.

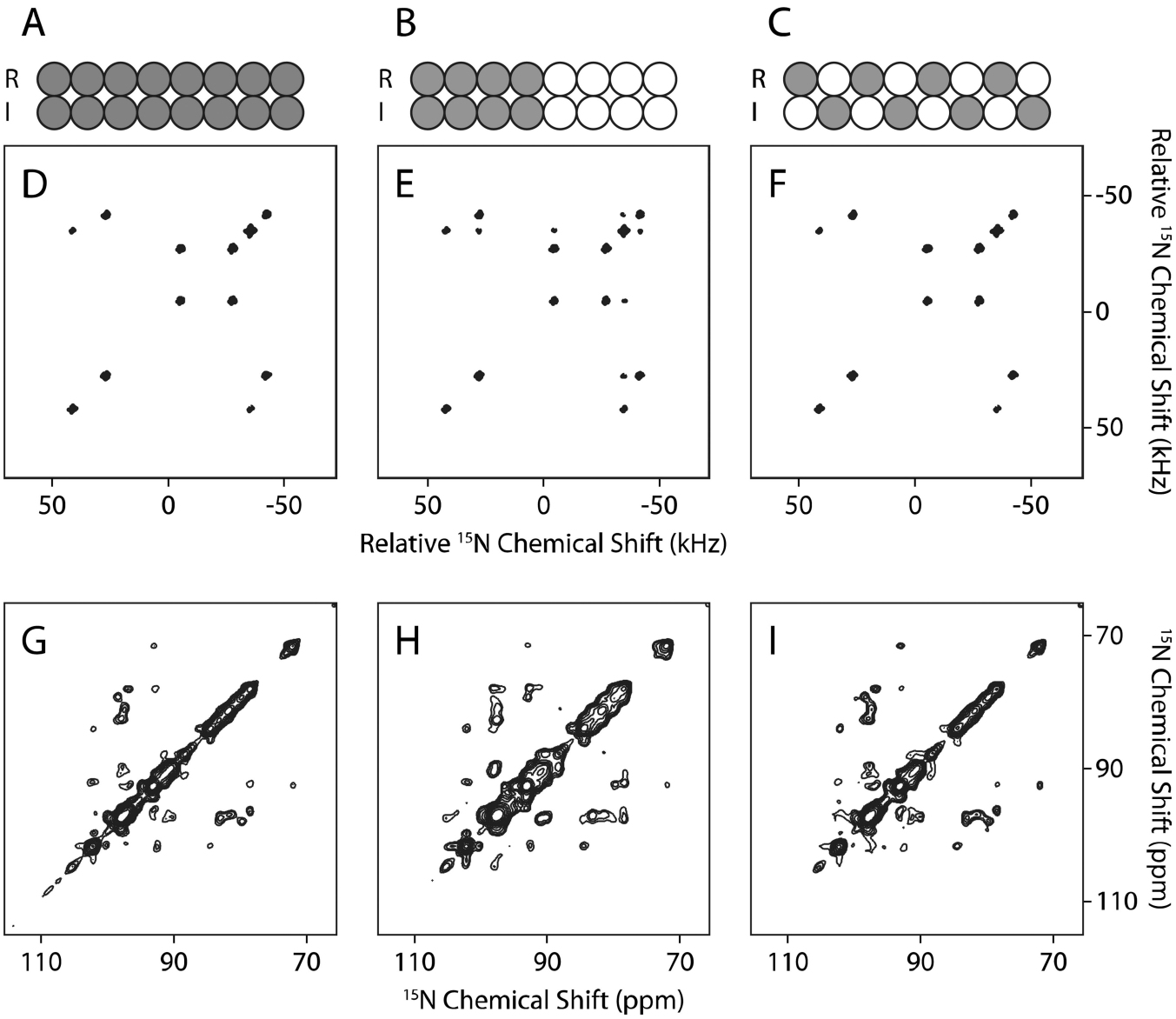

Figure 1.

A., B., and C. are schematic diagrams of the sampling schemes. A. Conventional sampling. B. Highly truncated sampling. C. Alternative States sampling. D., E. and F are the simulated spectra generated from A., B. and C. sampling schemes, respectively. G., H., and I. are the ^{15}N/^{15}N homonuclear spin exchange spectra of the membrane-bound form of uniformly ^{15}N-labeled Pf1 coat protein in magnetically aligned (with the normal perpendicular to the field) macrodiscs, which are reconstructed by the sampling schemes, A. to C., respectively. R and I represent the real and imaginary points in the indirect dimension, and the filled and unfilled circles indicate the sampling and non-sampling points. 96 complex points were acquired in the regular covariance spectrum and 48 complex points were taken for demonstrating other sampling schemes (B and C). 3mg of ^{15}N-labeled Pf1 coat proteins were incorporated into 30mg DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) macrodiscs at pH 4.9[21]. The protein spectra were obtained by the conventional homonuclear PDSD (proton-driven spin diffusion) spin-exchange with a 3 sec mix time at 30°C, and the strength of B_1 field was 50 kHz. 1024 complex points were

acquired with 20μs dwell time in the direct dimension, and 96 complex points were acquired with 100μs dwell time in the indirect dimension. The spectrum resulted from 500 scans of signal averaging for each time domain point. The experiments were performed on a Bruker Avance spectrometer with ^{1}H and ^{15}N frequencies of 699.70 and 70.90MHz by the home-built double-resonance MAGC probe[31]

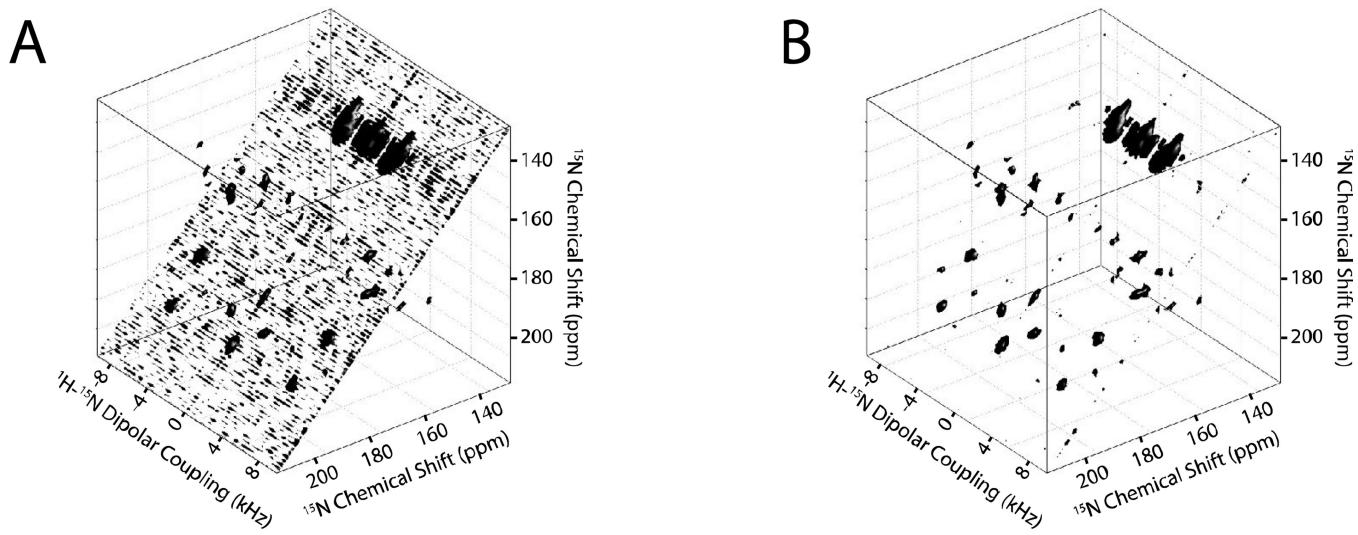

Figure 2.

A. The reconstructed ^{1}H-^{15}N/^{15}N/^{15}N three-dimensional spectrum of a ^{15}N-labeled NAL single crystal. B. The same spectrum without diagonal noise, which was removed by subtracting their average value. COV was applied to ^{15}N/^{15}N correlations for the reconstruction. 50 complex points were acquired using the alternate phase sampling scheme, which corresponds to 23.4% sampling, and the dipolar coupling dimension was reconstructed from 50% sampling (44 real points) by compressed sensing. The experiment was performed with 4 msec mix time under 38% MMHH conditions with a B_1 field of 52 kHz. The dwell time in the direct dimensions was 20 μs with 1024 complex points, and the dwell time in the t2 dimension was 100 μs. The spectrum resulted from 8 scans of signal averaging for each time domain point. The experiments were performed on a Bruker Avance spectrometer with ^{1}H and ^{15}N frequencies of 699.70 and 70.90MHz by the home-built double-resonance MAGC probe[31]

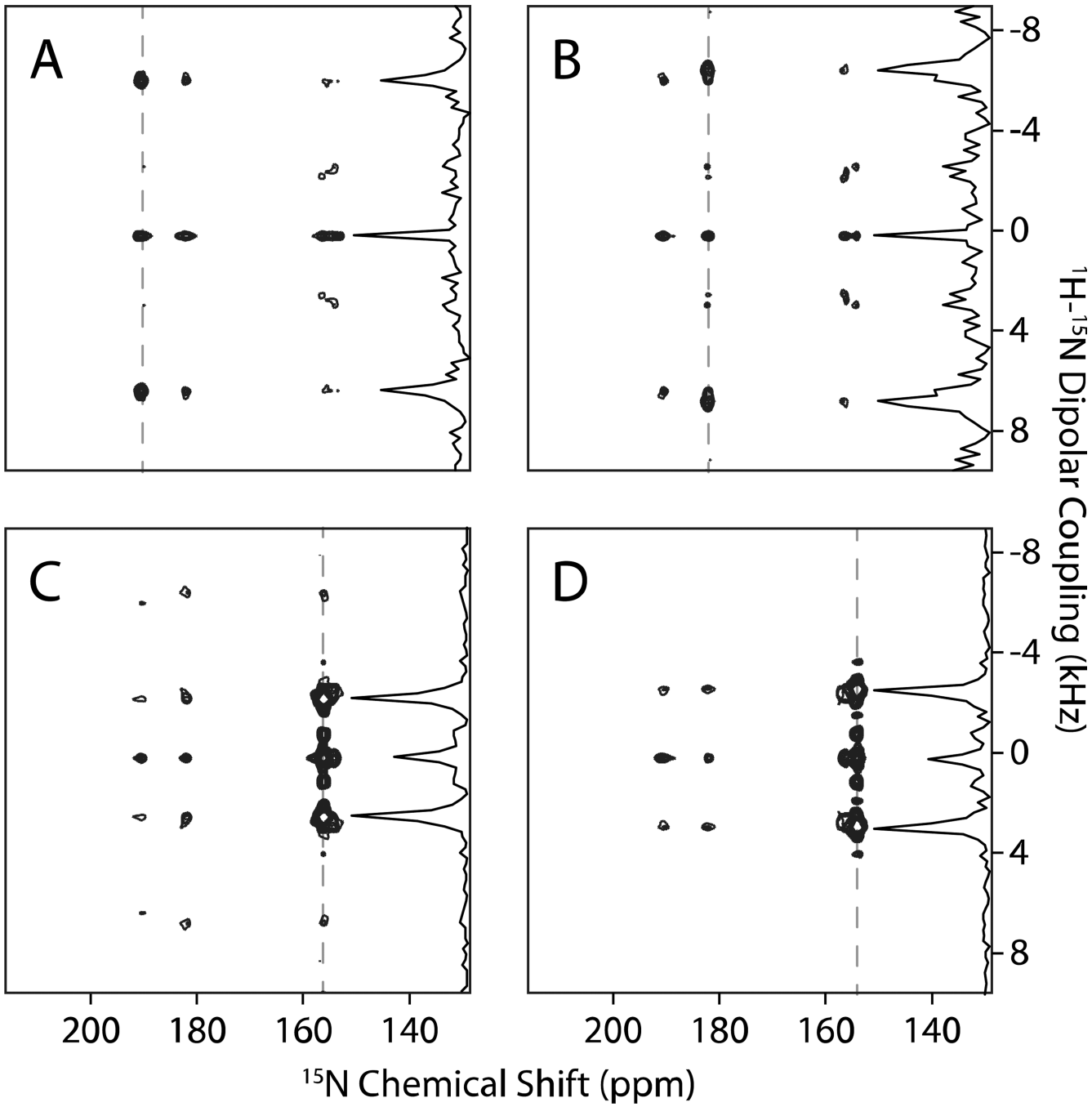

Figure 3.
Spectral planes extracted from the there-dimensional spectrum of the resonances in Figure 2. A. 192 ppm. B. 184 ppm. C. 157 ppm. D. 155 ppm. Artifacts generated in the SAMPI4 experiments for the small dipolar frequencies are found in low dipolar coupling regions (near 0 kHz) in C. and D. The signal-to-noise ratio of the lowest contour in each spectrum is 6. The dash lines in the spectra indicate the slices shown on the sides.