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Indirect Covariance NMR Spectroscopy

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Two-dimensional NMR experiments directly monitor spin−spin interactions and thereby provide unique information about biomolecular structure and dynamics.[1,2] A well-known restriction of 2D NMR experiments is the need to detect one of the spin species of interest during the NMR acquisition period. After Fourier transformation, the resonance frequencies of these spins are then displayed along the ω_2 frequency axis. Moreover, because the sensitivity of NMR experiments scales with $\gamma^{3/2}$, where γ is the gyromagnetic ratio of the detected nucleus, direct observation of low-γ nuclei leads to low sensitivity and thereby prolonged measurement times. For example, carbon-detected correlation experiments are eight times less sensitive than their proton-detected counterparts.

In this communication, indirect covariance NMR spectroscopy is introduced, which overcomes these limitations and offers a new approach to the identification of nuclear spin connectivities in molecules. The method is a modified form of covariance spectroscopy,[3,4] which yields homonuclear 2D spectra with both dimensions monitoring nuclear spin dynamics during the t_1-evolution period of the 2D NMR experiment. As the input serves a standard 2D NMR time domain data set $s(t_1,t_2)$, which is processed as follows. First, standard 2D Fourier transformation and phase correction is applied, yielding the real 2D frequency matrix $S(\omega_1,\omega_2) = \mathrm{Re} \int\int dt_1\, dt_2 \cos(\omega_1 t_1) \exp(-i\omega_2 t_2) s(t_1,t_2)$. Next, the data matrix $C(\omega_1,\omega_1')$ is computed from $S(\omega_1, \omega_2)$ using

$$C(\omega_1,\omega_1') = \int S(\omega_1,\omega_2) S(\omega_1',\omega_2)\, d\omega_2 = S \cdot S^{\mathrm{T}} \qquad (1)$$

$C(\omega_1,\omega_1')$ contains as its elements the covariances between pairs of rows of the mixed frequency time data set $S(\omega_1,t_2)$. In the third step, matrix C is subjected to the square-root operation using standard numerical linear algebraic methods yielding the final spectrum $F(\omega_1,\omega_1') = C^{1/2}$. Spectrum $F(\omega_1,\omega_1')$ is symmetric, displaying along both dimensions spin resonances whose frequencies are sampled solely during the *indirect* time dimension t_1. $F(\omega_1,\omega_1')$ generally has a cross-peak at position (ω_1,ω_1') if $S(\omega_1,\omega_2)$- and $S(\omega_1',\omega_2)$ are both cross-peaks to the same resonance in ω_2.

The method is demonstrated for a ^{1}H−^{13}C HSQC-TOCSY experiment,[5−7] with the NMR pulse sequence shown in Figure 1,

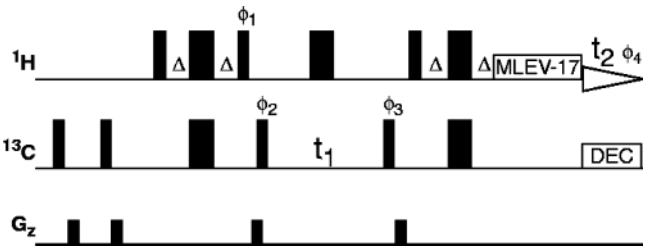

Figure 1. ^{1}H−^{13}C HSQC-TOCSY pulse sequence. It correlates ^{1}H and ^{13}C chemical shifts by a ^{1}H−^{13}C HSQC experiment[5] combined with a ^{1}H−^{1}H TOCSY transfer using MLEV-17 as mixing sequence of length τ_m = 100 ms.[6,7] Delay $\Delta = 1/(4J_{CH})$ was set to 1.6 ms. The phases of the radio frequency pulses are: $\varphi_1 = \{y,y,y,y,-y,-y,-y,-y\}$, $\varphi_2 = \{x,-x\}$, $\varphi_3 = \{x,x,-x,-x\}$, and $\varphi_4 = \{x,-x,-x,x,-x,x,x,-x\}$.

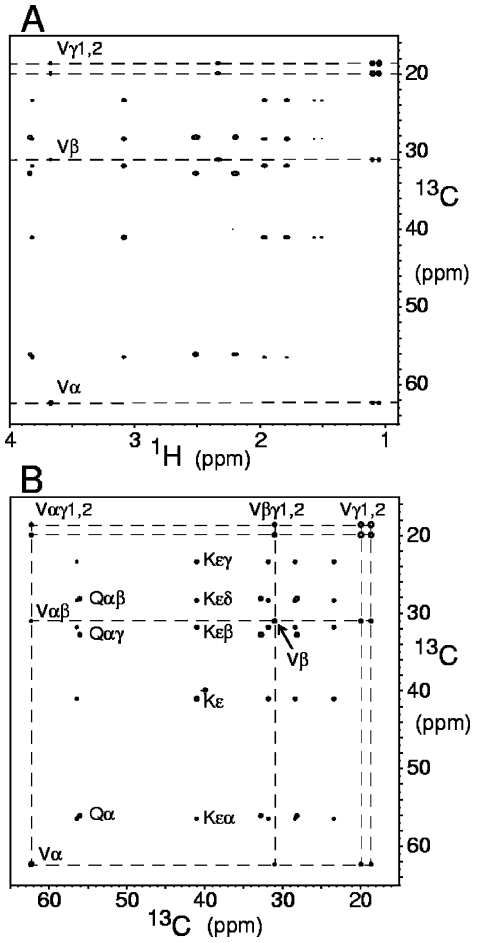

Figure 2. Indirect covariance NMR applied to ^{1}H−^{13}C HSQC-TOCSY data of a mixture consisting of the three amino acids Gln, Lys, and Val at 130 mM concentration. A data set of 2048 (^{1}H) × 1024 (^{13}C) complex data points was collected using the pulse sequence of Figure 1. (A) ^{1}H−^{13}C HSQC-TOCSY spectrum obtained after 2D Fourier transformation. (B) ^{13}C−^{13}C TOCSY obtained by the indirect covariance NMR method using eq 1. All experiments were collected at 600 MHz magnetic field strength and 298 K.

applied to an aqueous solution of the three amino acids Gln, Lys, and Val at natural ^{13}C abundance. Figure 2A shows the 2D FT HSQC-TOCSY spectrum $S(\omega_1,\omega_2)$ of the three amino acid mixture. This heteronuclear spectrum displays the ^{13}C frequencies along ω_1 and the ^{1}H frequencies along ω_2. Figure 2B depicts the indirect HSQC-TOCSY covariance spectrum $F(\omega_1,\omega_1')$ obtained by application of eq 1 to $S(\omega_1,\omega_2)$ of panel A. The two spectra have essentially the same sensitivity. The indirect covariance spectrum $F(\omega_1,\omega_1')$ is a homonuclear ^{13}C−^{13}C correlation spectrum despite the fact that it was acquired by a pulse sequence with proton detection along the direct dimension t_2 (Figure 1). The indirect covariance scheme thus overcomes the long-standing limitation that one axis of a 2D NMR spectrum must correspond to a directly

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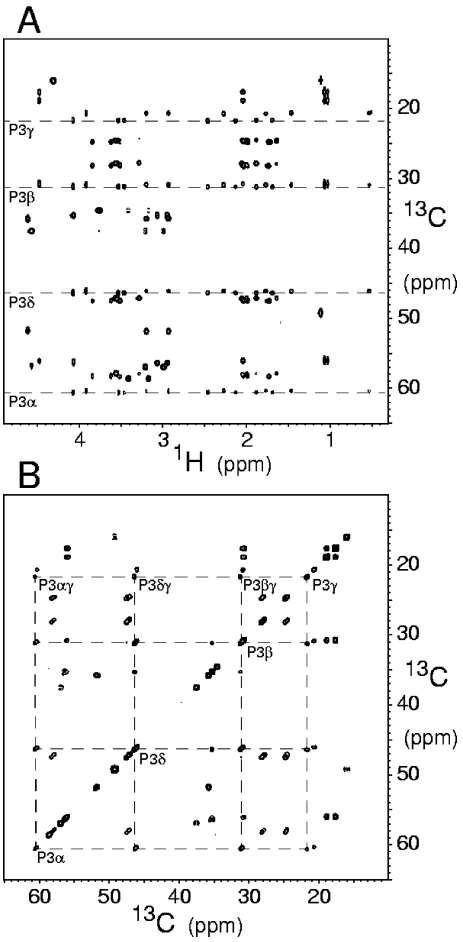

Figure 3. Indirect covariance NMR applied to HSQC-TOCSY of 0.7 mM ^{13}C-labeled antamanide, a cyclic decapeptide, in $CDCl_3$. A data set of 1024 (^{1}H) × 512 (^{13}C) complex data points was collected using the pulse sequence shown in Figure 1. (A) ^{1}H−^{13}C HSQC-TOCSY spectrum obtained after 2D Fourier transformation. (B) ^{13}C−^{13}C TOCSY obtained by the indirect covariance NMR method using eq 1. The labels and dashed lines indicate peak assignments of the carbon spins of Pro 3 taken from ref 8.

observable spin species and spin state. The spectrum contains the same spin-connectivity information as a ^{13}C−^{13}C TOCSY with direct ^{13}C detection. However, because in the indirect covariance experiment, proton instead of carbon spins are detected, it yields a sensitivity increase of $(\gamma_H/\gamma_C)^{3/2} = 8$ over a ^{13}C detected experiment.

Figure 3B shows the indirect covariance HSQC-TOCSY NMR spectrum of the 0.7 mM ^{13}C-labeled cyclic decapeptide antamanide, with sequence -Val-Pro-Pro-Ala-Phe-Phe-Pro-Pro-Phe-Phe-, in $CDCl_3$ in comparison with the standard HSQC-TOCSY spectrum (Figure 3A). The individual amino acids can be easily identified by tracing the TOCSY transfers between ^{13}C spins, as is indicated for Pro 3 in Figure 3B. The indirect covariance spectrum $F(\omega_1,\omega_1')$ is intrinsically homonuclear in nature, and it has the mathematical properties of a covariance matrix, which makes it directly amenable to principal component analysis for further analysis and interpretation.[9,10] Indirect covariance spectra are thus well suited for both manual and (semi-)automated analysis procedures.

In indirect covariance NMR spectroscopy, the spectral resolution $\Delta\nu$ along both frequency axes is determined by the sampling along the evolution t_1 time, $\Delta\nu = 1/t_{1,\max}$. Therefore, in the indirect method the desired spectral resolution determines the minimal number of required t_1 increments. A cross-peak emerges in the indirect HSQC-TOCSY covariance spectrum when in $S(\omega_1,\omega_2)$ two ^{13}C resonances exhibit a cross-peak to the same proton resonance. Thus, cross-peaks will also appear between ^{13}C resonances of different spin systems that are coupled to strongly overlapping protons. If ^{13}C spin polarization is transferred only to a single ^{1}H spin, the ^{13}C resonance will appear in the indirect covariance spectrum only as a diagonal peak. The indirect covariance method is therefore particularly useful for heteronuclear 2D NMR experiments that involve *J*-coupling, dipolar, or cross-relaxation mediated magnetization transfer from a spin S, evolving during t_1, to multiple spins I resonating during t_2. Similarly, the indirect covariance scheme can be used to monitor correlations between multiple quantum coherences that elude direct detection.

Indirect covariance NMR spectroscopy represents a new approach for the experimental analysis of molecular systems by sensitively monitoring homonuclear spin correlations in liquids and solids. It is readily applicable to a wide range of NMR experiments and molecular systems encountered in biomolecular NMR and analytical chemistry, including metabolomics.

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