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Simplified Multiplet Pattern HSQC-TOCSY Experiment for Accurate Determination of Long-Range Heteronuclear Coupling Constants[1]

Wiktor Koźmiński

Department of Chemistry, Warsaw University, ul. Pasteura 1, 02-093 Warsaw, Poland

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A new two-dimensional pulse sequence for accurately determining heteronuclear coupling constants is presented. It is derived from HSQC and HECADE techniques with B_0 gradient coherence selection. The main feature of the proposed method is spectra with only one component of the IS doublet; i.e., the final result is equivalent to a selective broadband excitation of either S_α or S_β spin states and a preservation of these states during the entire experiment. The effect is obtained by an appropriate combination of in- and antiphase coherences. It is demonstrated that heteronuclear single-bond as well as long-range coupling constants and their relative signs are readily evaluated. The proposed sequence is equally or less sensitive to a variation of heteronuclear one-bond couplings than previously published, closely related sequences. The new method is applied to a peptide sample for determination of $^3J_{N,H\beta}$. © 1999 Academic Press

Key Words: **HSQC; isotropic mixing; *J* couplings; spin-state-selective excitation.**

Scalar and dipolar coupling constants provide valuable restraints for the characterization of molecular conformation, bonding, and dynamics. However, especially in cases with coupling and linewidths of comparable magnitude, dedicated methods are required to obtain accurate values of the couplings. Established approaches, reviewed in Refs. (*1, 2*), include: (i) evaluation of coupling constants from the lineshape in one- or multidimensional spectra compared with reference spectra, (ii) quantitative *J*-correlation methods, and (iii) methods producing E.COSY multiplet patterns. Previously we proposed a new set of methods, referred to as *a*ctive-*c*oupling-pattern *t*ilting (ACT) (*3–5*). This approach, complementary to E.COSY, yields tilted cross-peak patterns even for two-spin systems and thus allows the determination of active coupling constants with high accuracy. The tilt of the multiplet patterns is obtained by appropriate coaddition of in- and antiphase coherences in the respective F_1 and F_2 domains of the 2D spectra. Recently, the new concept of spin-state-selective excitation (S^3E) in multidimensional spectra has been introduced (*6–9*). Its application to the determination of coupling constants is based on the generation and comparison of two sets of spectra with each displaying only one component of the coupling multiplet, corresponding to the resonances of molecules with a single state of a given spin. A similar approach was proposed to simplify the multiplet structure of INADEQUATE spectra (*10*), and to increase the resolution for I_2S groups in HSQC experiments (*11*). Also one-dimensional methods generating single multiplet components in the S-spin-coupled spectra have been introduced (*12, 13*). It was shown that the same effect could be obtained, with high tolerance to a magnitude of active coupling, simply by coadding the in- and antiphase multiplets in F_1-coupled HSQC and H(N)CO spectra for an accurate measurement of $^1J_{NH}$ and $^2J_{HNC'}$ in protein (*14*). Recently other methods of generation of S^3E-like spectra in the F_2-coupled HSQC (*15*) and TROSY (*16*) experiments were proposed for the determination of one-bond coupling constants.

The most important problem associated with "spin-state-selective" sequences is insufficient suppression of one of the doublet components. It was shown in Ref. (*14*) that the simplest way of generating this type of spectra, coadding two separate in- and antiphase experiments, is a less sensitive variation of coupling magnitudes than the S^3E approach. However, in the case of unlabeled material this method enables only the determination of one-bond coupling constants. Additionally, the splittings are displayed in the F_1 domain, the resolution of which can be improved only with drastically increased experimental time. Sequences proposed in Ref. (*15*) produce single multiplet component spectra with couplings displayed in F_2, but are more sensitive to a mismatch of the one-bond coupling. The sequence presented in this paper is designed for determining one- and multiple-bond heteronuclear coupling constants from the F_2 dimension and is to a large extent insensitive to one-bond coupling variations.

Figure 1 displays the pulse-sequence scheme for the proposed experiment. The new concept basically is a simplified ACT/HECADE (*3*) approach; i.e., it relies on coaddition of in- and antiphase coherences. The sequence is derived from the

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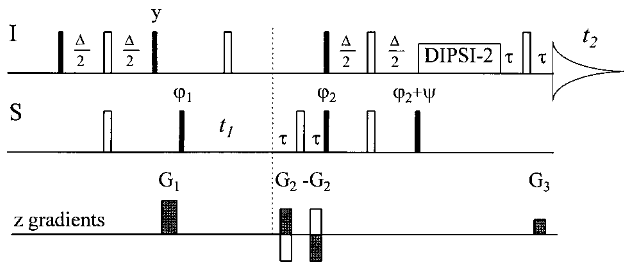

FIG. 1. Pulse sequence for simplified multiplet pattern HSQC-TOCSY experiment. Dark-filled and open bars represent $\pi/2$ and π pulses, respectively. The delay 2Δ should be tuned to $0.5/{}^1J_{IS}$. The delay τ includes the rectangular shape gradient pulse and a recovery time of 100 μs. Gradient G_1, with an amplitude of 10 G/cm and a duration of 2 ms, was used in ^1H–^{15}N experiments only. Gradients G_2 and G_3 with durations of 2.5 and 1 ms, and amplitudes of 10 and 5 G/cm, respectively, were used in ^1H–^{15}N experiments. For ^1H–^{13}C experiments G_2 and G_3 with equal length and amplitude of 10 and 5 G/cm, respectively, were used. In all cases only rectangular shaped PFG pulses were employed. Two echo and two antiecho data sets per t_1 increment were acquired, and they were combined using standard VNMR software. The basic phase cycle of $\varphi_1 = x, -x$, $\varphi_2 = x, x, -x, -x$ and receiver $x, -x, -x, x$ was applied. The upfield IS doublet line is selected by phase ψ set to $\pi/2$ in echo and $-\pi/2$ in antiecho experiments. For the opposite selection phase ψ must be reversed.

standard sensitivity-enhanced HSQC experiment (*17*) with B_0 gradients for echo–antiecho coherence selection, followed by subsequent homonuclear isotropic mixing which is required for long-range coupling measurements.

The method presented is a two-dimensional version of a sequence proposed in Ref. (*12*), with the additional improvement that the last $(\pi)_S$ pulse, at the center of the 2τ delay, is omitted, and hence the IS coupling evolution during this delay is refocused. One of the main features of this sequence, in analogy to other sensitivity-enhanced multidimensional methods (*18*), is the recovery of two orthogonal t_1 shift evolution terms. Since during the inverse INEPT step only one term is refocused and transferred to in-phase I-spin coherence, while the second term is transferred through multiple-quantum coherence into observable antiphase I-spin coherence, coaddition of the two directly results in 2D spectra that display only one single component of the IS doublets. In order to measure the coupling constants it is necessary to acquire a second spectrum showing the other doublet component. Thus, four FIDs per t_1 increment must be sampled: a heteronuclear echo and antiecho, each measured with $\psi = \pi/2$ as well as $\psi = -\pi/2$, a phase change that inverts the sign of the antiphase contribution. The two data sets with opposite ψ selection should be processed separately. Additionally, the phase ψ must be inverted synchronously with the G_2 gradients between the echo and antiecho experiments; for details see the legend to Fig. 1. Neglecting the optional application of isotropic mixing and unobservable terms created by the last $(\pi/2)_S$ pulse, the relevant components of the density matrix for an IS system at point $t_2 = 0$ can be described by the following Cartesian product-operator terms:

Data set 1 (upfield doublet components):

$$\begin{aligned}\text{echo} &= \tfrac{1}{2}(-R_{SQ}QI_x + R_{MQ}2I_xS_z)\cos(\omega_S t_1)\\ &\quad - \tfrac{1}{2}(R_{SQ}QI_y + R_{MQ}2I_yS_z)\sin(\omega_S t_1)\\ \text{antiecho} &= \tfrac{1}{2}(-R_{SQ}QI_x + R_{MQ}2I_xS_z)\cos(\omega_S t_1)\\ &\quad + \tfrac{1}{2}(R_{SQ}QI_y + R_{MQ}2I_yS_z)\sin(\omega_S t_1);\end{aligned}$$

Data set 2 (downfield doublet components):

$$\begin{aligned}\text{echo} &= \tfrac{1}{2}(-R_{SQ}QI_x - R_{MQ}Q2I_xS_z)\cos(\omega_S t_1)\\ &\quad - \tfrac{1}{2}(R_{SQ}QI_y - R_{MQ}2I_yS_z)\sin(\omega_S t_1)\\ \text{antiecho} &= \tfrac{1}{2}(-R_{SQ}QI_x - R_{MQ}Q2I_xS_z)\cos(\omega_S t_1)\\ &\quad + \tfrac{1}{2}(R_{SQ}QI_y - R_{MQ}2I_yS_z)\sin(\omega_S t_1). \qquad [1]\end{aligned}$$

R_{SQ} and R_{MQ} account for the different relaxation rates of single- and multiple-quantum coherences during the last Δ delay and $Q = \sin(\pi J\Delta)$. The requested multiplet structure is directly obtained by independent transformation of data sets 1 and 2. An additional homonuclear coherence transfer to remote I nuclei under preservation of the in- and antiphase character with respect to the S spin is possible, e.g., via isotropic mixing. However, measurements of long-range coupling constants involving nonprotonated S nuclei are not possible.

A very important criterion for the quality of all ACT and "spin-state-selective" experiments is their ability to cleanly suppress the undesired doublet component. For the experiment proposed in this paper there are four particularly relevant sources of error: (i) mismatch of the Δ delay tuned to one-bond couplings, (ii) relaxation-rate differences along the coherence pathways that lead to in- and antiphase signals, and (iii) S-spin homonuclear coupling evolution during the last Δ. The Q

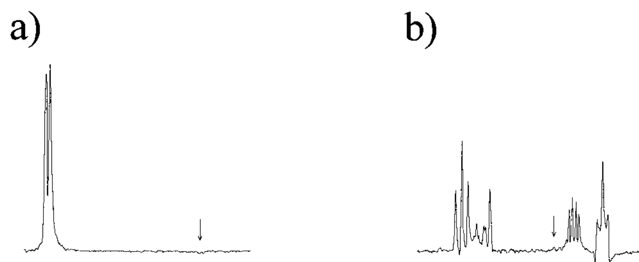

FIG. 2. Comparison of the doublet component suppression for H(1) (trace a) and H(3) (trace b) protons of the sucrose sample obtained with the proposed HSQC-TOCSY sequence. The arrows indicate the position of the suppressed line. The vertical scale is normalized. The experiment was optimized for $^1J(^{13}C$–$^1H) = 150$ Hz; however, the actual couplings are about 170 Hz for H(1)/C(1) and 150 Hz for H(3)/C(3). See the legend to Fig. 5 for experimental details.

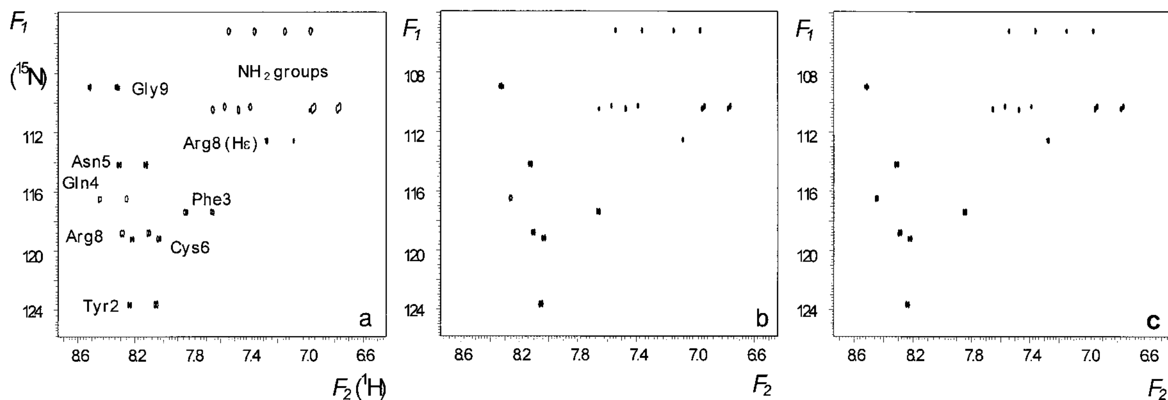

FIG. 3. Expanded amide proton region from the analog of [Me, Ala[7]]-AVP-vasopressin spectra obtained with the regular HSQC sequence (trace a) and with the proposed sequence without TOCSY mixing, tuned for upfield (trace b) and downfield (trace c) doublet-line selection. Note that this sequence does not work for I_2S systems. Contour levels for the major isomer only are shown. The spectrum was acquired with a 25 mM 90% H_2O/D_2O solution. Eight scans were coherently added for each data set for 64 t_1 increments. The maximum t_1 and t_2 times were 43 and 410 ms, respectively. A relaxation delay of 1.2 s was used. The delay Δ was tuned to a coupling of 90 Hz. The data matrix containing 64 × 2048 complex points in t_1 and t_2, respectively, was zero-filled to 512 × 8192 complex points, and cosine and exponential line broadening of 1 Hz was applied prior to Fourier transformation in t_1 and t_2, respectively.

factor in Eq. [1] affects those coherences that evolve during the last Δ delay under the $^1J_{IS}$ interaction. When the actual value of the coupling constant J is unequal to J_{target}, used for tuning Δ delays $\Delta = 0.5/J_{target}$, the selected and suppressed doublet components are attenuated and enhanced by $(1 - Q)$ respectively. Ignoring all other sources of error the intensity ratio of suppressed and selected lines is equal:

$$\left[\sin\left(\frac{\pi J}{2J_{target}}\right) - 1\right] \Big/ \left[\sin\left(\frac{\pi J}{2J_{target}}\right) + 1\right]. \qquad [2]$$

This ratio is, however, quite insensitive for any real value of J, since it reaches -0.04 for J/J_{target} equal to 0.75. For J two times larger than J_{target} a pure antiphase is observed. The same result is obtained for the IPAP method (*14*); however, for the sequences proposed in Ref. (*15*) the corresponding intensity ratio (Eq. [3]) is more sensitive to the actual magnitude of one-bond coupling and for $J/J_{target} = 0.75$ it reaches 0.2:

$$\pm\left[\sin\left(\frac{\pi J}{4J_{target}}\right) - \cos\left(\frac{\pi J}{4J_{target}}\right)\right] \Big/ \left[\sin\left(\frac{\pi J}{4J_{target}}\right) + \cos\left(\frac{\pi J}{4J_{target}}\right)\right]. \qquad [3]$$

In the case of the proposed sequence a disturbing effect of different relaxation rates of single- and multiple-quantum coherences is possible. However, since $\Delta(0.5/^1J)$ is very short in relation to ^1H transverse relaxation rates these effects are expected to be negligible. The relaxation and spin–spin coupling topology effects for this sequence are discussed in detail in Ref. (*12*) and generally for E.COSY and S^3E experiments in Ref. (*19*). Effects of homonuclear S-spin coupling evolution during the last delay Δ could be significant only in the case of ^1H–^{13}C experiments applied to a fully ^{13}C-labeled sample.

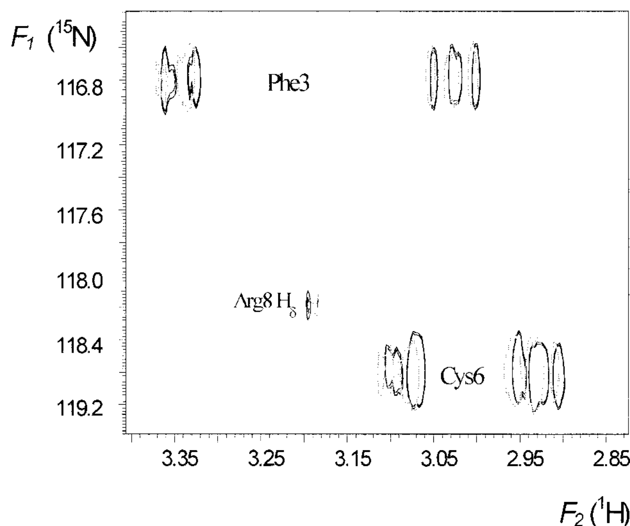

FIG. 4. Contour plot of the Cys[6] and Phe[3] H_β regions of the HSQC-TOCSY spectrum, obtained by the proposed HSQC-TOCSY sequence, of the [Me, Ala[7]]-AVP-vasopressin analog sample. Signals of the dominating isomer only are shown. Superposition of the spectra with upfield (black) and downfield (gray) doublet lines is plotted. The coupling constants correspond to the displacements of a cross peak in these two spectra. All $^3J_{N,H\beta}$ coupling constants obtained in this experiment are summarized in Table 1. Additional DIPSI-2 I-spin isotropic mixing was applied for 85 ms; 192 accumulations for each four data sets in every t_1 increment were acquired. Water signal suppression presaturation, with a $\gamma B_1/2\pi$ of about 40 Hz, during the relaxation delay was used. All other experimental parameters are the same as those for the spectra presented in Fig. 4. The data were zero-filled to 512 × 16384 complex points in t_1 and t_2, respectively.

Experimentally, the new sequence used in a ^{1}H–^{15}N experiment applied to a peptide sample produces spectra with clean suppression of one multiplet line, even with a ±25% variation of J_{target}. Additionally, the ^{1}H–^{13}C experiment applied to a sucrose sample with additional isotropic mixing revealed no significant disturbing contributions of the undesired cross-peak components even for signals with different $^{1}J_{IS}$, which is shown in Fig. 2.

As an application example, Fig. 3 displays contour plots of the ^{1}H–^{15}N correlations of a 25 mM solution of the [Me, Ala[7]]-AVP-vasopressin analog in 9/1 H_2O/D_2O, measured with the proposed sequence and a regular F_2-coupled HSQC.

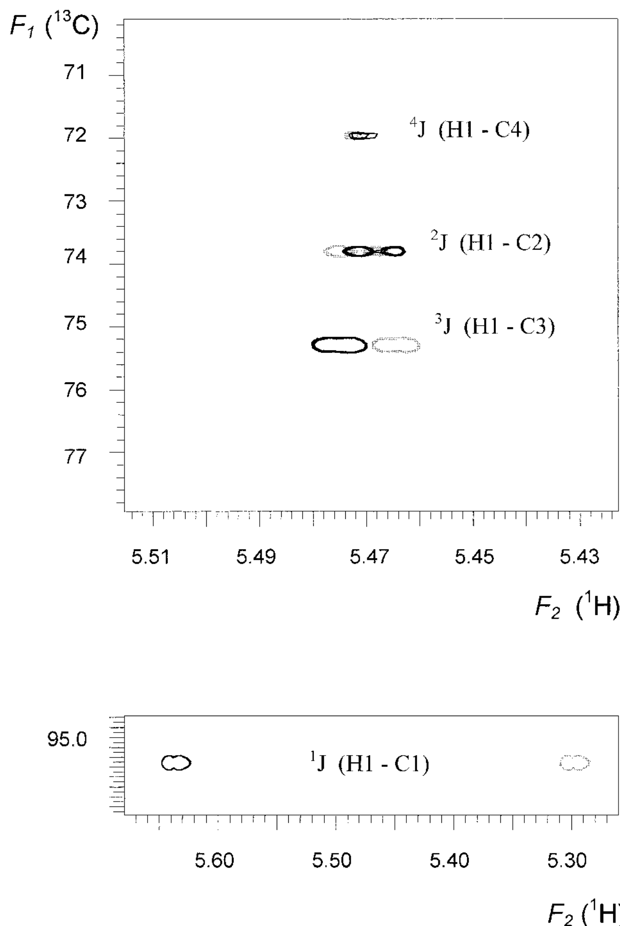

FIG. 5. Expansion of the anomeric proton region of the HSQC-TOCSY spectrum of a 0.06 M sucrose D_2O solution, obtained by the sequence depicted in Fig. 1. The spectra with selection of upfield (gray) and downfield (black) doublet lines are superimposed. The position of the doublet lines for H(1)–C(2) and H(1)–C(4), cross peaks is reversed, revealing its negative sign in relation to $^{1}J(^{13}C, ^{1}H)$. Eight scans are coherently added for each data set for 256 t_1 increments. The maximum t_1 and t_2 times were 35 and 610 ms, respectively. A relaxation delay of 1.5 s was used. The delay Δ was tuned to couplings of 150 Hz. The data matrix containing 256 × 800 complex points in t_1 and t_2, respectively, was zero-filled to 512 × 16,384 complex points, and cosine and exponential line broadening of 1 Hz was applied prior to Fourier transformation in t_1 and t_2, respectively.

TABLE 1

Coupling Constants $^{3}J_{N,H\beta}$ Obtained from HSQC-TOCSY with Simplified Multiplet Pattern Experiments, of the Major Isomer of the [Me, Ala[7]]-AVP-Vasopressin Analog

<table>
<tr><th></th><th>Tyr[2]</th><th>Phe[3]</th><th>Gln[4]</th><th>Asn[5]</th><th>Cys[6]</th><th>Arg[8]</th></tr>
<tr><td>$^{3}J_{N,H\beta}$ [Hz][a]</td><td>−3.3</td><td>−3.9</td><td rowspan="2">−2.6</td><td rowspan="2">−2.4</td><td>−3.7</td><td rowspan="2">—[b]</td></tr>
<tr><td>$^{3}J_{N,H\beta'}$ [Hz]</td><td>−1.8</td><td>−1.8</td><td>−2.1</td></tr>
</table>

Note. The accuracy is limited by the F_2 digital resolution and in the present case is estimated to be about 0.3 Hz.

[a] Downfield H_β proton signal.

[b] Not determined due to weak signal-to-noise ratio.

The ability to determine long-range coupling constants is demonstrated in the expansions of peptide and sucrose solution spectra shown in Figs. 4 and 5. For these experiments additional homonuclear isotropic mixing was used. Note that information about the relative signs of the coupling constants is obtainable by this method. Table 1 summarizes the values of $^{3}J_{N,H\beta}$ coupling constants obtained for the vasopressin sample in this experiment.

The signal-to-noise ratio of single echo and antiecho data sets acquired with the proposed method is equal to that obtainable by the standard sensitivity-enhanced HSQC experiment without S-spin decoupling during t_2.

All spectra presented were acquired at 300K on a Varian Unity Plus 500 spectrometer, equipped with a Performa I z-PFG unit and using a standard 5-mm-i.d. PFG probehead. For isotropic mixing the DIPSI-2 (*20*) scheme was used with $\gamma B_1/(2\pi) = 7$ kHz; 8-, 13-, and 26-μs high-power ^{1}H, ^{13}C, and ^{15}N $\pi/2$ pulses, respectively, were used.

In conclusion, the new sequence presented here allows for an accurate and relatively sensitive determination of heteronuclear coupling constants. The proposed method, in close analogy to HECADE sequences (*3*), is based on coaddition of in- and antiphase signals; however, there is no incremented IS coupling evolution period. In comparison to the HSQC method proposed in Ref. (*14*), the sequence presented in this paper has equal length for the acquisition of in- and antiphase signals, and moreover it is shorter by a period Δ. Additionally the couplings are displayed in the F_2 domain, which enables easy enhancement of resolution without any substantial increase in experiment time. The optimal homonuclear coherence transfer enables determination of the heteronuclear long-range couplings. In this case relative signs of the coupling constants are readily obtainable; however, the method works for IS systems only. Due to its high tolerance toward substantial variations of $^{1}J_{IS}$ couplings this experiment may be applied not only for accurate measurements of scalar and dipolar couplings of biomolecules but also for a variety of organic compounds with a significantly larger spread of isotropic one-bond coupling constants.

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