

Two-dimensional concurrent HMQC-COSY as an approach for small molecule chemical shift assignment and compound identification

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Abstract Chemical shift assignment is the first step toward the structure elucidation of natural products and other chemical compounds. We propose here the use of 2D concurrent HMQC-COSY as an experiment for rapid chemical shift assignment of small molecules. This experiment provides well-dispersed ^1H - ^{13}C peak patterns that are distinctive for different functional groups plus ^1H - ^1H COSY connectivities that serve to identify adjacent groups. The COSY diagonal peaks, which are phased to be absorptive, resemble ^1H - ^{13}C HMQC cross peaks. We demonstrate the applicability of this experiment for rapidly and unambiguously establishing correlations between different functional groups through the analysis of the spectrum of a metabolite (jasmonic acid) dissolved in CDCl_3 . In addition, we show that the experiment can be used to assign spectra of compounds in a mixture of metabolites in D_2O .

Keywords 2D HMQC-COSY · Chemical shift assignment · Compound identification · Metabolite mixture

Introduction

2D COSY spectra (Aue et al. 1976) provide important information on the connectivity between different functional groups; however, 2D ^1H - ^1H COSY spectra suffer from phasing issues, arising from intrinsic differences in the relative phase of diagonal and cross peaks, and peak

overlap issues, resulting from the low proton chemical shift dispersion. Heteronuclear 2D HSQC or HMQC experiments yield better-resolved spectra of metabolites and metabolite mixtures because of the higher chemical shift dispersion of carbon; however, these experiments do not offer information on the connectivity between different functional groups. Heteronuclear multiple-bond correlation spectra, HMBC type experiments (Bax and Summers 1986) can show heteronuclear long-range “indirect” connectivities, but these are not always unambiguous. The INADEQUATE experiment (Bax et al. 1980), which makes use of direct ^{13}C - ^{13}C coupling, can provide very important information on ^{13}C - ^{13}C connectivities within the molecular skeleton, but its application is limited by the low sensitivity of the experiment at natural abundance ^{13}C .

To overcome the peak overlap issue in 2D COSY, the 3D ^{13}C -edited HMQC-COSY experiment was proposed and demonstrated on kanamycin A with natural abundance ^{13}C (Fesik et al. 1989). A ^{13}C -resolved COSY experiment involving selective excitation of a particular carbon signal by the SELINCOR technique was proposed as a means for overcoming spectral overlaps (Facke and Berger 1995). The 3D HMQC-COSY experiment was later modified with gradient-enhanced coherence order selection and further demonstrated on sucrose and menthol to facilitate assignments (Hurd and John 1991).

In 2D HMQC-COSY, the initial ^1H magnetization is transferred concurrently to ^{13}C through $^1\text{J}_{\text{CH}}$ (HMQC) and to ^1H through $^3\text{J}_{\text{HH}}$ (or $^n\text{J}_{\text{HH}}$, COSY). The ^1H - ^{13}C multiple-quantum period is exploited for both ^{13}C chemical shift coding and for achieving a maximal COSY effect. Similar information on the connectivity can be obtained from HSQC-COSY to HSQC-TOCSY (Bax and Davis 1985) experiments. For HSQC-COSY and HSQC-TOCSY, the initial ^1H magnetization is transferred sequentially to

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bonded ^{13}C 's (by HSQC) and to homonuclear J coupled ^1H 's (by COSY or TOCSY). Considering the relatively small 3-bond ^1H – ^1H homonuclear J -couplings, usually the coupling Hamiltonian has to be allowed to evolve for a considerably long time to achieve reasonable COSY or TOCSY effects. In addition, to achieve reasonable resolution along the indirect ^{13}C -dimension, magnetization on ^{13}C (single-quantum coherence) has to evolve for a long t_1 time.

Here we present 2D HMQC-COSY experiments that can be run as constant-time (Fig. 1a) or non-constant-time (Fig. 1b) versions and used for rapid spectral assignment of small molecules. In the constant-time version of 2D HMQC-COSY to achieve maximum COSY effect, given the relatively small 3-bond ^1H – ^1H homonuclear J -couplings, the coupling Hamiltonian evolves during both the ^1H – ^{13}C heteronuclear multiple quantum generation period (a–b) and the ^1H – ^{13}C multiple quantum constant-time period. In the non-constant-time version of 2D HMQC-COSY, the ^1H – ^1H homonuclear J -coupling runs in accordion mode (Mandel and Palmer 1994). The constant-time version is superior for the analysis of small molecule spectra because of its higher COSY transfer efficiency with small J -couplings and its higher resolution along the indirect ^{13}C -dimension compared to the non-constant-time version.

We illustrate the utility of this approach for the spectral assignment of a small molecule and show how the experiment can be used for identifying compounds in mixtures of metabolites.

Materials and methods

Jasmonic acid (Sigma–Aldrich) was dissolved in CDCl_3 at a concentration about 60 mM. The constant-time 2D HMQC-COSY spectrum of jasmonic acid was recorded at 25°C on a Bruker Avance 500 MHz spectrometer equipped with a z -gradient triple resonance CPTXO probe optimized for ^{13}C detection. The radio-frequency pulses on ^1H and ^{13}C were applied at 4.7 and 73 ppm, respectively. ^{13}C decoupling was carried out using GARP with a field strength of $\gamma B_2 = 3.38$ kHz. 1024×150 complex data points were collected along ^1H and ^{13}C dimensions (spectral widths of 14 and 150 ppm, respectively) with 4 scans per FID and an inter-scan delay of 3 s, resulting in a total data acquisition time of about 1 h. A mixture containing 63.8 mM alanine, 88.47 mM methionine, and 124.36 mM sodium 3-hydroxybutyrate (Sigma–Aldrich) was prepared in D_2O . The constant-time 2D HMQC-COSY spectrum of this mixture was collected at 25°C on a Bruker DMX 500 MHz spectrometer equipped with a z -gradient triple resonance TCI probe. The radio-frequency pulses on ^1H and ^{13}C were applied at 4.7 and 43 ppm, respectively.

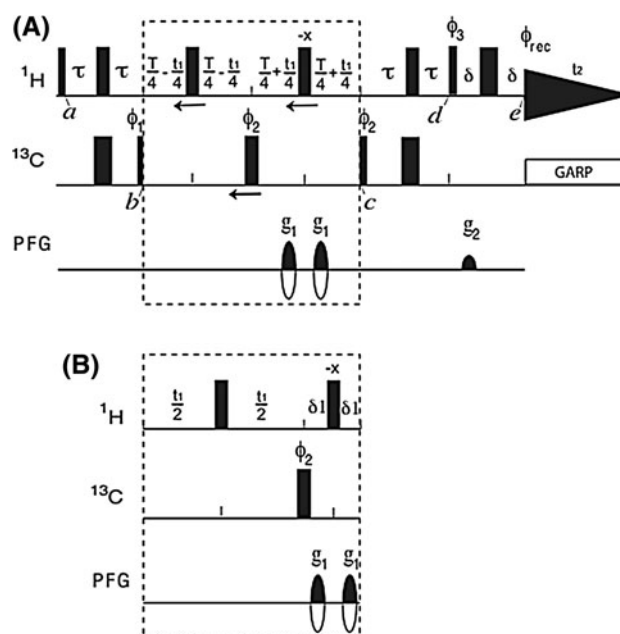


Fig. 1 **a** Constant-time 2D HMQC-COSY pulse sequence. *Narrow and wide black bars indicate 90° and 180° pulses, respectively.* The delays are $\tau = 1.8$ ms; $T = 20$ ms and $\delta = 1.21$ ms. The phase cycling is as follows: $\phi_1 = x, -x$; $\phi_2 = x, x, x, x, -x, -x, -x, -x$; $\phi_3 = y, y, -y, -y$; $\phi_{\text{rec}} = x, -x, x, -x, -x, x, -x, x$. All other radio-frequency pulses are applied with phase x except as indicated. Quadrature detection in the ^{13}C (t_1) dimension is achieved by using echo-antiecho-TPPI applied to the phase ϕ_1 and by flipping the polarity of both gradients g_1 on every another FID (as indicated by the *filled and open sine bells*). The duration and strength of the coherence selective pulsed field gradients applied along the z -axis are: g_1 : 1 ms, 26.5 G/cm and g_2 : 1 ms, 13.33 G/cm followed by a gradient recovery period of 200 μs . **b** Non-constant-time 2D HMQC-COSY pulse sequence. The constant-time period (*dashed block* in panel **a**) is replaced by a non-constant-time block. The short delay $\delta_1 = 1.21$ ms is just to accommodate the coherence selective pulsed field gradients g_1 , including the gradient recovery period of 200 μs

^{13}C GARP decoupling was carried out with a field strength of $\gamma B_2 = 3.38$ kHz. 1024×120 complex data points were recorded along the ^1H and ^{13}C dimensions (spectral widths of 14 and 80 ppm, respectively) with 4 scans per FID and an inter-scan delay of 4 s, resulting in total data acquisition time of about 1 h. The NMRPipe package (Delaglio et al. 1995) was used to process spectra with linear prediction and zero-filling along the ^{13}C dimension.

Results and discussion

NMR pulse sequences

In the constant-time 2D concurrent HMQC-COSY pulse scheme (Fig. 1a), the initial $^1\text{H}^a$ polarization is excited by a 90° pulse on ^1H followed by a multiple quantum generation period, where magnetization is transferred to $^1\text{H}^a$ – $^{13}\text{C}^a$ multiple quantum coherence at point *b*. By synchronously

shifting the 180° ^{13}C pulse (ϕ_2) and 180° ^1H pulses (as indicated by the arrows) during the constant time period T (points b – c shown in the dashed rectangle), the chemical shift of $^{13}\text{C}^a$ is encoded in time and concurrently the ^1H – ^1H homonuclear J -coupling Hamiltonian evolves. Note, that besides refocusing the chemical shift evolution of $^1\text{H}^a$, insertion of the two 180° ^1H pulses during the constant time also refocuses the evolution of other passive heteronuclear J -couplings between $^{13}\text{C}^a$ and other multiple bond coupled protons. Thus, a narrower line width along the ^{13}C dimension can be obtained. Application of a second 90° pulse on ^{13}C (ϕ_2) transfers $^1\text{H}^a$ – $^{13}\text{C}^a$ MQ coherence back to $^1\text{H}^a$. Following the 2τ reverse HMQC period, magnetization on $^1\text{H}^a$ evolves in-phase relative to $^{13}\text{C}^a$. For the entire $2\tau + T + 2\tau$ time period (point a – d), ^1H – ^1H homonuclear J -coupling Hamiltonian evolves and part of the magnetization arising from $^1\text{H}^a$ evolves to be antiphase relative to the homonuclear coupled third party proton $^1\text{H}^b$. COSY transfer from $^1\text{H}^a$ to $^1\text{H}^b$ is then achieved by the 90° pulse on ^1H (ϕ_3). The period 2δ inserted before detection serves to accommodate the refocusing gradient g_2 . The relevant coherence transfer pathway can be described as:

$$\begin{aligned}
 &H_z^a \xrightarrow{90^\circ_x} -H_y^a(a) \xrightarrow{180^\circ_H, 180^\circ_C, 2\tau} 2H_x^a C_z^a \cos[\pi J_{H^a H^b} \cdot 2\tau] \\
 &+ 4H_y^a H_z^b C_z^a \sin[\pi J_{H^a H^b} \cdot 2\tau] \\
 &\xrightarrow{90^\circ_{\phi_1}} 2H_x^a C_{-y}^a \cos[\pi J_{H^a H^b} \cdot 2\tau] \\
 &+ 4H_y^a H_z^b C_{-y}^a \sin[\pi J_{H^a H^b} \cdot 2\tau](b) \\
 &\xrightarrow{T, g_1} 2H_x^a C_{-y}^a(\omega_{C^a}, \gamma_C g_1) \cos[\pi J_{H^a H^b} \cdot (T + 2\tau)] \\
 &+ 4H_y^a H_z^b C_{-y}^a(\omega_{C^a}, \gamma_C g_1) \sin[\pi J_{H^a H^b} \cdot (T + 2\tau)](c) \\
 &\xrightarrow{90^\circ_{\phi_2}} 2H_x^a C_z^a(\omega_{C^a}, \gamma_C g_1) \cos[\pi J_{H^a H^b} \cdot (T + 2\tau)] \\
 &+ 4H_y^a H_z^b C_z^a(\omega_{C^a}, \gamma_C g_1) \sin[\pi J_{H^a H^b} \cdot (T + 2\tau)] \quad (1) \\
 &\xrightarrow{180^\circ_H, 180^\circ_C, 2\tau} H_y^a(\omega_{C^a}, \gamma_C g_1) \cos[\pi J_{H^a H^b} \cdot (T + 4\tau)] \\
 &+ 2H_x^a H_z^b(\omega_{C^a}, \gamma_C g_1) \sin[\pi J_{H^a H^b} \cdot (T + 4\tau)](d) \\
 &\xrightarrow{90^\circ_{\phi_3}} H_y^a(\omega_{C^a}, \gamma_C g_1) \cos[\pi J_{H^a H^b} \cdot (T + 4\tau)] \\
 &+ 2H_z^a H_x^b(\omega_{C^a}, \gamma_C g_1) \sin[\pi J_{H^a H^b} \cdot (T + 4\tau)] \\
 &\xrightarrow{180^\circ_H, 2\delta, g_2} H_y^a(\omega_{C^a}) \cos[\pi J_{H^a H^b} \cdot (T + 4\tau)] \\
 &+ 2H_z^a H_x^b(\omega_{C^a}) \sin[\pi J_{H^a H^b} \cdot (T + 4\tau)](e)
 \end{aligned}$$

The term $(\omega_{C^a}, \gamma_C g_1)$ arises from the coherence encoded by the chemical shift of $^{13}\text{C}^a(\omega_{C^a})$ and dephased by gradient g_1 . Gradient selection is then achieved by application of the refocusing gradient g_2 [last line in Eq. (1)]. Note that the homonuclear J -coupling evolution is ignored during the short gradient refocusing period 2δ .

The COSY diagonal peak, represented by the first term at point e , $H_y^a(\omega_{C^a}) \cos[\pi J_{H^a H^b} \cdot (T + 4\tau)]$, is 90° out of phase

relative to the COSY cross peak, represented by the second term at point e , $2H_z^a H_x^b(\omega_{C^a}) \sin[\pi J_{H^a H^b} \cdot (T + 4\tau)]$. Therefore, whereas the COSY diagonal peaks are phased to be absorptive, the COSY cross peaks show a typical pattern of antiphase dispersion (see the line shape of H12 in the dashed rectangle in Fig. 2b). The COSY diagonal peak term $H_y^a(\omega_{C^a}) \cos[\pi J_{H^a H^b} \cdot (T + 4\tau)]$ evolves under the chemical shift of H^a during detection, which will appear as normal $^1\text{H}^a$ – $^{13}\text{C}^a$ HMQC cross-peaks. The COSY cross peak term $2H_z^a H_x^b(\omega_{C^a}) \sin[\pi J_{H^a H^b} \cdot (T + 4\tau)]$, will evolve under the chemical shift of H^b during detection. It will thus show cross peaks at the chemical shift of $^{13}\text{C}^a$ and chemical shift of the coupled proton H^b . This indirect correlation provides information similar to that of an HMBC experiment. Similarly, magnetization initially from $^1\text{H}^b$, will yield a COSY diagonal peak at $^1\text{H}^b$ – $^{13}\text{C}^b$ and a COSY cross peak at $^1\text{H}^a$ – $^{13}\text{C}^b$.

Therefore, in the 2D HMQC-COSY spectrum, neighboring (^1H – ^1H homonuclear coupled) functional groups $^1\text{H}^a$ – $^{13}\text{C}^a$ and $^1\text{H}^b$ – $^{13}\text{C}^b$ will yield two COSY diagonal peaks at the chemical shifts of $^{13}\text{C}^a/{}^1\text{H}^a$ and $^{13}\text{C}^b/{}^1\text{H}^b$, respectively, which can be correlated by the two COSY cross peaks at the chemical shifts of $^{13}\text{C}^a/{}^1\text{H}^b$ and $^{13}\text{C}^b/{}^1\text{H}^a$. The COSY diagonal and cross peaks can be recognized by their line shape: the COSY diagonal peaks are phased to be absorptive, and the COSY cross peaks show an antiphase dispersive line shape. They also can be differentiated simply by comparison to conventional HSQC/HMQC spectra: the extra peaks shown in HMQC-COSY are the COSY cross peaks, giving HMBC type information. The feature of high chemical shift dispersion along the ^{13}C dimension and HMBC type information contained in the HMQC-COSY spectrum renders the HMQC-COSY experiment a robust approach for chemical shift assignments and also for identifying compounds in metabolite mixtures as demonstrated below.

In the non-constant-time version of HMQC-COSY, the constant-time period (dashed block in Fig. 1a) is replaced by non-constant-time block (Fig. 1b). The short delay $\delta_1 = 1.21$ ms is just to accommodate the coherence selective pulsed field gradients g_1 . As the chemical shift of ^{13}C is encoded in time, the effective evolution time for the ^1H – ^1H homonuclear J -coupling Hamiltonian also varies with t_1 , from $(2\tau + 4\delta_1 + 2\tau)$ to $(2\tau + t_1 + 2\delta_1 + 2\tau)$ (point a – d). ^1H – ^1H homonuclear J -coupling evolution in the accordion mode allows the COSY transfer in the non-constant-time 2D HMQC-COSY to be more efficient for very diverse values of J_{HH} .

Chemical shift assignment of jasmonic acid

We illustrate the application of this approach with data from jasmonic acid (see Fig. 2a for the structure and atom

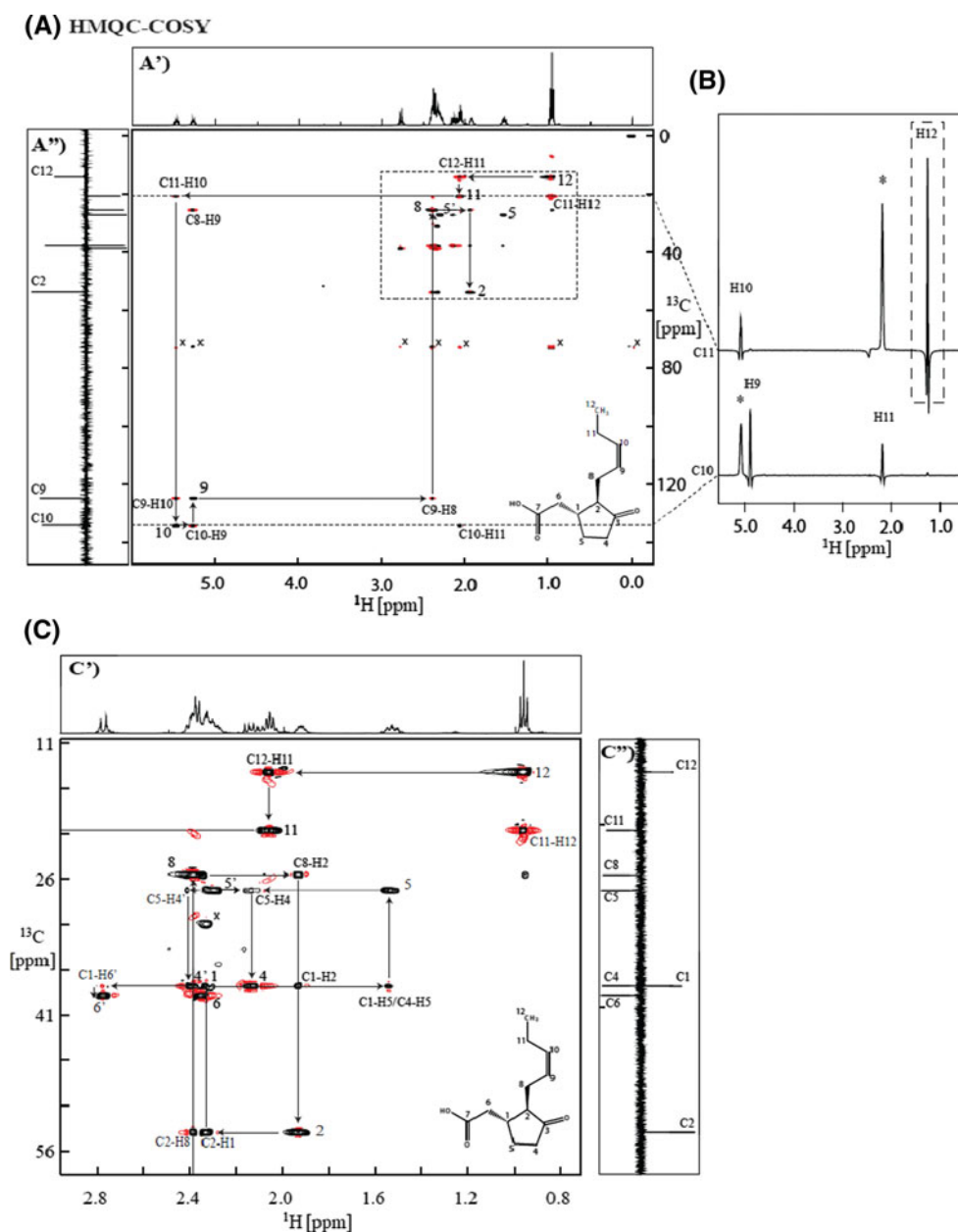


Fig. 2 a 500 MHz constant time 2D HMQC-COSY spectrum of jasmonic acid in CDCl_3 . The structure and atom nomenclature of jasmonic acid are shown at *bottom-right*. The COSY diagonal peaks are labeled with atom numbers, while the COSY cross peaks are labeled to identify the carbon and correlated proton. The *arrows* starting from 12 illustrate the stepwise path for the chemical shift assignment (see the text for details). Panels *a'* and *a''*, respectively, show for reference 1D ^1H and ^{13}C DEPT-135 spectra of jasmonic acid downloaded from BMRB (<http://www.bmrbl.wisc.edu/>). Axial artifacts are marked with "x"; the axial peaks are shifted by TPPI quadrature detection and correspond to peaks in the ^1H dimension; thus, we attribute them to ^{12}C species (98.9% abundance). **b** 1D slices along the ^1H dimension taken from the 2D HMQC-COSY spectrum at

the chemical shift of C11 and C10. The COSY diagonal peaks are marked with *asterisks* *; the COSY cross peaks are labeled with the correlated protons. While the COSY diagonal peaks are phased to be absorptive, the COSY cross peaks show a typical pattern of antiphase dispersion, as shown in the broken box. The excellent alignment of the chemical shifts of H10 and H11 from these two slices indicates the connectivity between C11 and C10. **c** Expansion of the crowded region *boxed* by a *dashed line* in **a**. The detailed pathway for chemical shift assignments is indicated by the *arrows*. Panels *c'* and *c''* show the expanded 1D ^1H and ^{13}C DEPT-135 spectra of jasmonic acid. The geminal protons attached to C4, C5 and C6, which have different chemical shifts, are labeled as H and H'

nomenclature of this compound as well as its 2D HMQC-COSY). The signal from the methyl group (C12–H12, labelled peak 12) of jasmonic acid is easily identified on the

basis of its characteristic ^1H and ^{13}C chemical shifts. The assignment of the methyl group can be confirmed by reference to 1D ^1H (Fig. 2a') and ^{13}C DEPT-135 (Fig. 2a'')

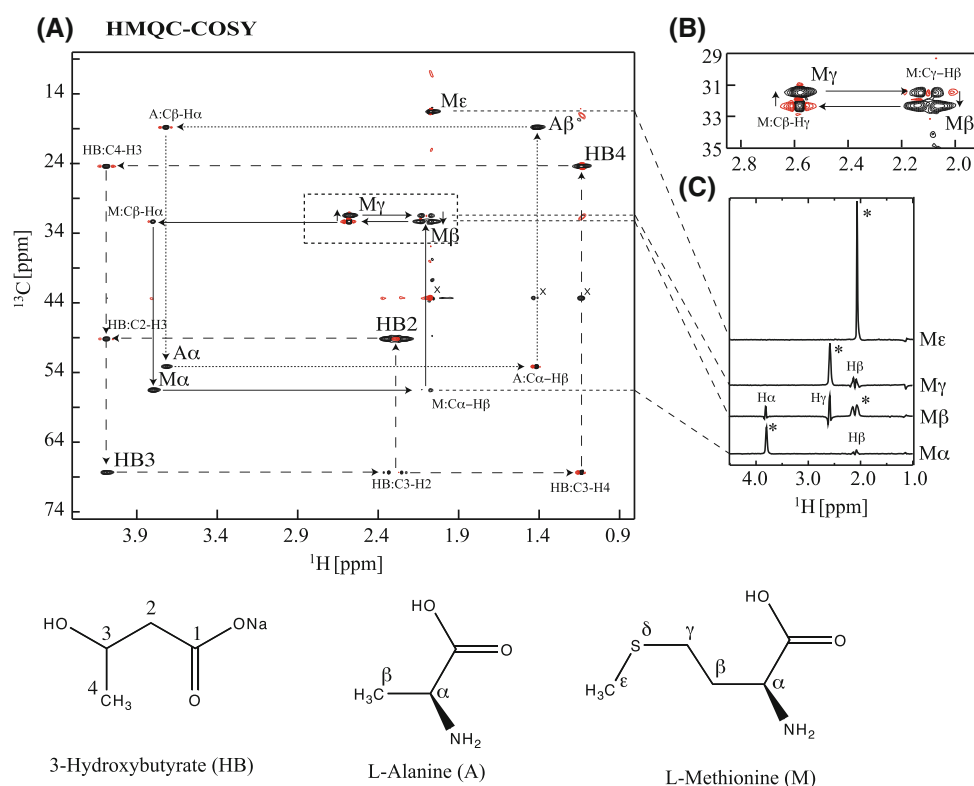


Fig. 3 **a** 500 MHz constant-time 2D HMQC-COSY spectrum of the mixture of alanine (A), methionine (M), and sodium 3-hydroxybutyrate (HB) in D_2O . The structures and atom nomenclature for these compounds are shown at the bottom of the figure. The COSY diagonal peaks are labeled with the abbreviated name of the molecule and atom designator. The COSY cross peaks are labeled with the abbreviated name of the molecule and the atom designators of the carbon and its correlated long-range proton. The spin networks of A, M, and HB are identified by the *dotted*, *solid*, and *broken* arrows. Axial artifacts are marked with “x”; their origin is as described in the caption to Fig. 2. **b** Expansion of the region enclosed by the *dashed* rectangle in

a shows the neighboring connectivity between $M C^\beta$ and C^γ . **c** Shown as an example are 1D slices along the 1H dimension taken from the 2D HMQC-COSY spectrum at the chemical shifts of $M C^\epsilon$, C^γ , C^β and C^α . The COSY diagonal peaks are marked with *asterisks* *; the COSY cross peaks are labeled with the correlated protons. The excellent alignment of the chemical shifts of H^γ , H^β and H^α from these slices and the correlation of C^β with both H^γ and H^α indicate that the spin network is connected as $C^\gamma-C^\beta-C^\alpha$. The *slice* at Met C^ϵ does not show any COSY cross peak except for the COSY diagonal peak marked by an *asterisks*. C^ϵ is thus isolated from the rest of the spin network

spectra. Walking along the 1H dimension to the left of peak 12 at the ^{13}C chemical shift of the methyl group (14.21 ppm) leads to a cross peak at $^1H = 2.058$ ppm, which corresponds to the indirect interaction of C_{12} and H_{11} ($C_{12}-H_{11}$). Then walking down from peak $C_{12}-H_{11}$ along the ^{13}C dimension leads to a cross peak at $^{13}C = 20.80$ ppm (peak 11, the direct $H_{11}-C_{11}$ correlation). Next, walking leftward from peak 11 along the 1H dimension leads to a cross peak at $^1H = 5.463$ ppm, which corresponds to $C_{11}-H_{10}$. Again, the downward arrow from peak $C_{11}-H_{10}$ along the ^{13}C dimension leads to the cross peak at $^{13}C = 134.35$ ppm, from the direct $C_{10}-H_{10}$ correlation (peak 10). This assigns C_{10} (134.35 ppm) and H_{10} (5.463 ppm). As noted above, the direct and indirect correlation peaks can be distinguished by their line shapes; these are illustrated by 1D slices along the 1H dimension taken from the 2D HMQC-COSY spectrum at the chemical shift of C_{11} (20.80 ppm) and C_{10} (134.35 ppm) (Fig. 2b). The directly correlated peaks

(COSY diagonal peaks, marked with asterisks *) are phased to be absorptive, and the indirectly correlated (long range) peaks (COSY cross peaks, labeled with the correlated protons) show an antiphase dispersive lineshape (see the inset showing H_{12}). The excellent alignment of the chemical shifts of H_{10} and H_{11} from these two slices indicates the neighboring connectivity between C_{11} and C_{10} . Figure 2c shows an expansion of the crowded region outlined by the dashed line in Fig. 2a. By following the arrows in Fig. 2a and c and by repeating the above procedure, the ^{13}C and 1H chemical shift assignments can be extended from to the rest of the molecule. Carbons C_4 , C_5 , and C_6 each have two attached protons, designated by unprimed and primed labels (e.g., H_4 and H_4'). Due to the relatively low resolution along the indirect ^{13}C dimension of 2D HMQC-COSY, C_4 and C_1 are assigned to the same chemical shift. These assignments can be easily confirmed by comparison to the ^{13}C DEPT-135 spectrum (panel 2c''), in which C_1 is positive and C_4 is

negative. Only the quaternary carbons of jasmonic acid (C3 and C7) remain undetected and unassigned by the 2D HMQC-COSY approach. They can be found by 1D ^{13}C NMR detection.

Compound identification from metabolite mixture

The 2D HMQC-COSY experiment can also be used to identify individual compounds in a mixture of metabolites. We illustrate this by reference to results with a mixture of 63.75 mM alanine (A), 88.47 mM methionine (M), and 124.36 mM sodium 3-hydroxybutyrate (HB) in D_2O . Figure 3a shows the 2D HMQC-COSY spectrum of the mixture. As before, the directly correlated peaks are absorptive while the indirectly correlated peaks have an antiphase dispersive lineshape. Following the procedure outlined above for jasmonic acid, the spin networks of A, M, and HB were identified, as illustrated by the dotted, solid, and broken arrows, respectively, in Fig. 3a and b. Slices from the 2D HMQC-COSY spectrum of the mixture taken along the ^1H dimension at the chemical shifts of M C^ϵ , C^γ , C^β and C^α (Fig. 3c) show how this spin system can be identified selectively; the excellent alignment of the chemical shifts of H^γ , H^β and H^α from these slices indicates that these groups belong to the same spin network. Because C^β shows indirect correlation with both H^γ and H^α , whereas C^γ and C^α , each show indirect correlations only with H^β , the spin network is connected as C^γ - C^β - C^α . The slice at M C^ϵ does not show any indirectly correlated peak because its attached proton is not coupled to any other group by efficient COSY transfer; thus, C^ϵ is isolated from the rest of the spin network. Having identified the spin network and associated chemical shifts, the identity of the compound can be determined from a metabolite standards database.

Conclusion

The 2D HMQC-COSY experiment presented here offers a robust approach for assigning ^1H and ^{13}C NMR signals from individual compounds or mixtures of compounds. Because of the high chemical shift dispersion along the carbon dimension, this experiment produces distinct peak patterns for different functional groups through ^1H - ^{13}C multiple quantum coherence while retaining the important information on peak connectivity through ^1H - ^1H COSY. We have implemented this method as a routine experiment for chemical shift assignment of standard compounds being added to the BMRB metabolomics database (<http://www.bmrwisc.edu/metabolomics>) in conjunction with 2D

^1H - ^{13}C HMBC for assignment of quaternary carbons. Although the spectra shown here were of compounds at high concentration (60 mM), we have used the approach with samples at 2 mM concentration with only 4 transients. With more extensive time averaging, we expect that 2D HMQC-COSY can be used for the assignment of compounds at concentrations as low as 500 μM . We anticipate that the experiment may find wider application in NMR studies of metabolites and natural products.

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