NMR Spectroscopy

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Pure In-Phase Heteronuclear Correlation NMR Experiments**

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Abstract: *A general NMR approach to provide pure in-phase (PIP) multiplets in heteronuclear correlation experiments is described. The implementation of a zero-quantum filter efficiently suppresses any unwanted anti-phase contributions that usually distort the multiplet pattern of cross-peaks and can hamper their analysis. The clean pattern obtained in PIP-HSQMBC experiments is suitable for a direct extraction of coupling constants in resolved signals, for a peak-fitting process from a reference signal, and for the application of the IPAP technique in non-resolved multiplets.*

Long-range proton–carbon correlations routinely extracted from HMBC spectra are key elements in the structural characterization of small and medium-sized molecules in solution.[1] However, the accurate quantitation of such interactions from these spectra has been problematic due to the characteristic mixed phases of the resulting HMBC cross-peaks, which lead to difficult data analysis.[2] As an alternative, the long-range optimized HSQC experiment, referred to as HSQMBC,[3] has been preferred to measure small proton–carbon coupling constants, $^{n}J(\mathrm{CH})$ ($n > 1$), although it is not free of inconveniences. In both HMBC and HSQMBC experiments, the main source of problems arises from the fact that proton–proton coupling constants, $J(\mathrm{HH})$, that have similar magnitudes to $^{n}J(\mathrm{CH})$ (typically ranging from 0–15 Hz), also evolve during the defocusing/refocusing periods. This unwanted modulation can introduce undesired effects in both the multiplet phase and the transfer efficiency. In order to avoid or minimize such interferences, several approaches have been reported such as the use of a BIRD-INEPT element,[4] CPMG pulse trains,[5] or selective 180° ^{1}H pulses.[6]

HMBC and HSQMBC experiments are usually recorded under non-refocusing conditions and the resulting cross-peaks are related to anti-phase (AP) multiplet patterns. Recently, a frequency-selective CLIP-HSQMBC experiment has been proposed to obtain undistorted pure in-phase (PIP) patterns that are easier to analyze but this approach requires the recording of multiple experiments to monitor all protons in the molecule of interest.[7] Here we report a simple and general solution to obtain heteronuclear correlation spectra that yield truly pure absorption lineshapes and IP multiplet structures for all available cross-peaks with respect to both $J(\mathrm{CH})$ and all passive $J(\mathrm{HH})$ coupling constants along the detected dimension (Figure 1). The proposal is based on a conventional HSQC pulse train with an appended adiabatic z-filter[8] applied just before a refocusing gradient perfect-echo element[9] and acquisition. Modification of HSQC and HSQMBC pulse sequences to include a PIP-module affords experiments that can be referred to as PIP-HSQC when the Δ delay is optimized as a function of $^{1}J(\mathrm{CH})$ or as PIP-HSQMBC when Δ is optimized as a function of $^{n}J(\mathrm{CH})$.

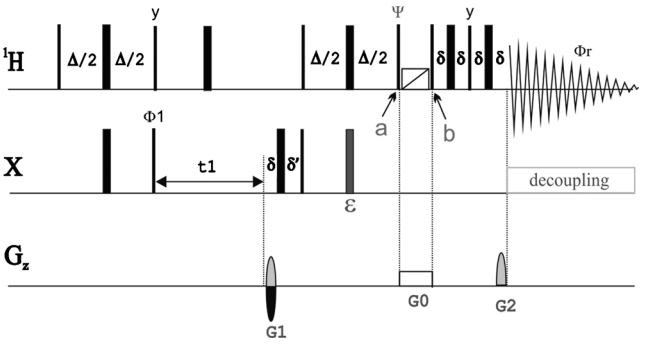

Figure 1. Pulse sequence designed to obtain pure in-phase (PIP) cross-peaks in heteronuclear correlation experiments. The delay Δ is set to $1/[2\,^{1}J(\mathrm{CH})]$ or $1/[2\,^{n}J(\mathrm{CH})]$ in PIP-HSQC or PIP-HSQMBC experiments, respectively. The z-filter includes a chirped adiabatic 180° ^{1}H pulse applied simultaneously with a purging G0 gradient, and broadband heteronuclear decoupling during proton acquisition is optional. A complete detailed description of the experiment can be found in the Supporting Information.

The HSQC scheme is easily analyzed by product operator formalism considering a heteronuclear three-spin H_1, H_2 and C system, with $J(H_1H_2)$ and $J(CH_1)$ coupling values. At the end of the refocusing INEPT period (point a in Figure 1), the magnetization of spin H_1 can be described as a mixture of IP and AP components:

$$\mathrm{H_{1x}\,c^2 s'^2 - 2\,H_{1y}C_z\,c^2 s' c' + 2\,H_{1y}H_{2z}\,c s s'^2 + 4\,H_{1x}H_{2z}C_z\,c s c' s'} \qquad (1)$$

where c is $\cos(\pi J(H_1H_2)\Delta)$, s is $\sin(\pi J(H_1H_2)\Delta)$, c′ is $\cos(\pi J(CH_1)\Delta)$, s′ is $\sin(\pi J(CH_1)\Delta)$, and Δ is the J evolution period. The last three terms explain why cross-peaks appear strongly distorted in F2-coupled HSQC spectra of a test sample of the alkaloid strychnine (**1**) (Figure 2 A). The use of the CLIP technique (a 90° ^{13}C purging pulse applied just before the acquisition)[10] or heteronuclear decoupling during acquisition

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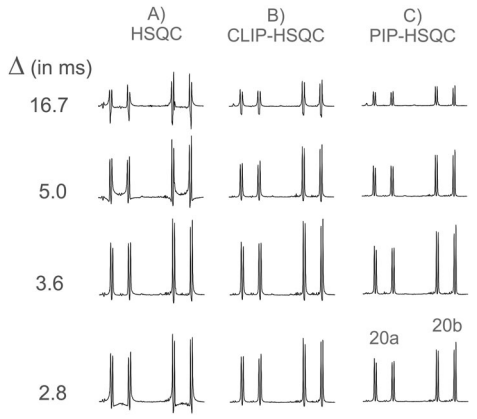

Figure 2. 1D traces extracted at the C20 chemical shift of (**1**) showing the phase distortions in A) conventional HSQC, B) CLIP-HSQC, and C) PIP-HSQC spectra with Δ optimized to several ^1J(CH) values: 2.8 ms (180 Hz), 3.6 ms (140 Hz), 5 ms (100 Hz), and 16.7 ms (30 Hz). Experimental values of the diastereotopic CH_2 group: ^2J(H20a-H20b) = 14.9 Hz, ^1J(C-H20a) = 138.8 Hz, ^1J(C-H20b) = 138.7 Hz.

efficiently removes the second and the fourth terms, but a mixture consisting of $H_{1x} + 2H_{1y}H_{2z}$ still remains (Figure 2B). In practice, due to the difference of magnitudes between ^1J(CH) and J(HH), these unwanted contributions are small and they have been traditionally omitted in cross-peak analysis in CLIP-HSQC or in conventional decoupled HSQC experiments. It is shown experimentally and by simulations that a gradient-based ^{1}H z-filter before acquisition[11] would improve the result by partially removing the double-quantum contribution in the third term but PIP peaks are still not achieved (Supporting Information, Figures S1–S6). These perturbations could become critical when measuring ^1J(CH) in the presence of large J(HH) values (Figure S3), as could be found in the measurement of Residual Dipolar Couplings (RDCs) for weakly aligned samples in anisotropic media, or in experiments involving longer Δ delays (Figures S4–S6). For instance, for J(HH) = 5 Hz and ^1J(CH) = 140 Hz, the contribution of the ZQ term is only about 3% in a 140 Hz optimized HSQC experiment. However, in the case that ^nJ(CH) = 8 Hz, this percentage increases to 75% in an 8 Hz optimized experiment.

As a further enhancement, it is shown here that all unwanted homo- and heteronuclear dispersive AP contributions are completely removed (Figure 2C) by applying a z-filter consisting of a 90°$_y$(^{1}H)–[adiabatic 180° ^{1}H pulse/purge gradient]–90°$_x$(^{1}H) element.[8] The remarkable benefits from the use of a z-filter for obtaining high-quality spectra has already been demonstrated for a number of NMR experiments.[9,12] Thus, after the 90°$_y$(^{1}H) pulse the above four components are converted to

$$-H_{1z}\,c^2s'^2 - 2H_{1y}C_z\,c^2s'c' + 2H_{1y}H_{2x}\,css'^2 - 4H_{1z}H_{2x}C_z\,csc's' \qquad (2)$$

where the second and fourth terms represent transverse AP heteronuclear magnetization and the third element represents a mixture of homonuclear ZQ and DQ coherences, which are also eliminated by the effect of the simultaneous adiabatic 180° ^{1}H pulse and the purging G0 gradient pair. As a result, only the first term representing the desired IP magnetization remains detectable after the z-filter (point b in Figure 1). To maintain the pure IP character during detection, the classical δ-180°$_x$(^{1}H)-G2 block has been replaced by a perfect gradient echo element, in the form of δ-180°$_x$(^{1}H)–δ-90°$_y$(^{1}H)–δ-180°$_x$(^{1}H)-G2, where J(HH) should be fully refocused.[9] Using gradients with a duration of 1 ms, the unwanted anti-phase J(HH) contribution should be about 3% for a J(HH) = 5 Hz using a conventional gradient echo (Figure S7).

The interference of J(HH) effects is more obvious when the size of J(CH) and J(HH) are of the same order, as found in long-range heteronuclear correlation experiments. The importance of the z-filter is illustrated with the superior IP performance of the 8 Hz PIP-HSQMBC experiment over conventional, CLIP and z-filtered HSQMBC experiments acquired under the same conditions (Figure 3). It must be emphasized that the apparent reduced sensitivity of the PIP spectrum is not due to relaxation associated to the z-filter, but rather to the elimination of all dispersive components.

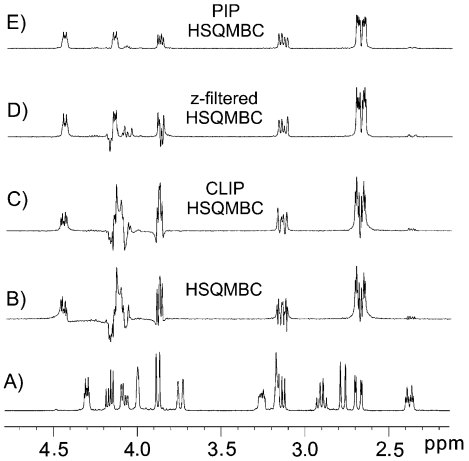

Figure 3. A) ^{1}H NMR spectrum of (**1**); B–E) 1D traces extracted at the C12 chemical shift showing the signal distortions in B) HSQMBC, C) CLIP-HSQMBC, D) z-filtered HSQMBC, and E) PIP-HSQMBC spectra (all experiments were optimized to 8 Hz (Δ = 62.5 ms)).

The inspection of some selected traces along the F2 dimension belonging to the 8 Hz PIP-HSQMBC spectrum of (**1**) clearly reveals that all cross-peaks display a clean IP character (Figure 4B). The expansion observed for the H8 and H20a cross-peaks (Figure 4C) exhibits well resolved multiplets, where the additional IP splitting (in these simple signals all observed cross-peaks become double-doublets) allows a direct and easy determination of the ^nJ(CH) value, analogous to measuring J(HH) in conventional 1D ^{1}H spectra. Note the rough proportionality between signal intensity and ^nJ(CH) values. Under these conditions, even a small coupling value of 2.3 Hz can be directly measured for the two-bond C7/H8 correlation. Experimentally, a different delay optimization can be useful in the event that expected correlations are missing (Figure S8).

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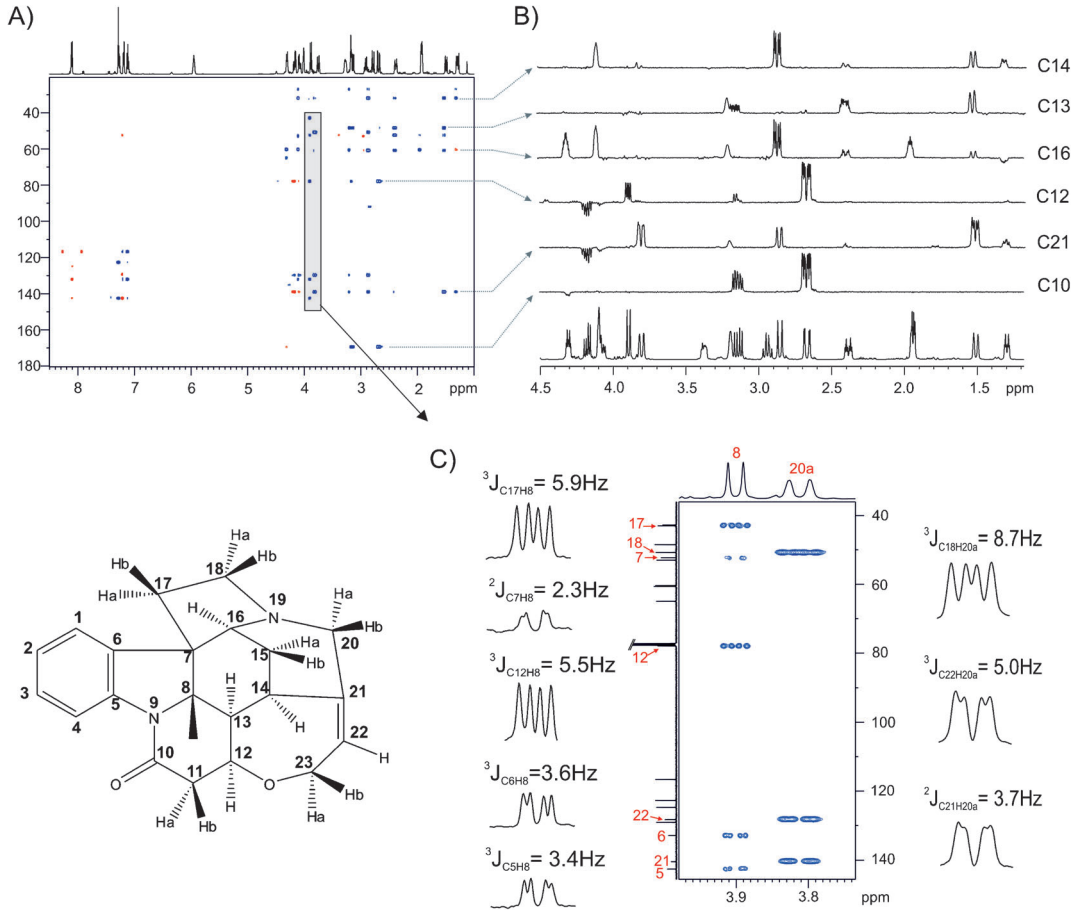

Figure 4. A) 8 Hz optimized PIP-HSQMBC spectrum of (**1**) acquired with a BIRD cluster in the initial INEPT period to minimize direct responses. B) 1D row slices taken at different ^{13}C frequencies showing in-phase multiplet patterns for all observed cross-peaks. C) Expanded area showing how the magnitude of ^nJ(CH) can be easily determined from direct analysis of undistorted and resolved IP peaks.

The extraction of ^nJ(CH) in more complex or non-resolved multiplets can be performed with several established methods: 1) measuring overall multiplet widths, 2) fitting/matching them to an external reference cross-peak obtained from the same sequence with broadband ^{13}C-decoupling during acquisition,[13] or 3) from the internal satellite lines corresponding to the direct ^1J(CH) responses, if available, without need to acquire a second reference spectrum. Alternatively, a simple approach relies on the implementation of the IPAP technique,[6] which is achieved by recording two separate IP and AP datasets as a function of the last 180° ^{13}C pulse (marked with ε in Figure 1). Figure 5 and S9–S10 compare the success of all these analytical methods from some selected cross-peaks. Similar results are obtained in experiments where the basic INEPT period has been replaced by other heteronuclear echo periods such as INEPT-BIRD,[4] CPMG,[5] or CPMG-BIRD[5] elements (Figures S11–S14).

The performance of the proposed PIP methods has been also verified under anisotropic conditions, using a sample of **1** weakly aligned in a reversible compressed poly(methyl methacrylate (PMMA) gel swollen in $CDCl_3$ (Figures S15–S17).[14] Although broader and more complex ^1H signals are typical for RDC experiments, experimental splittings arising from CH couplings in the range 110–190 Hz and to large HH

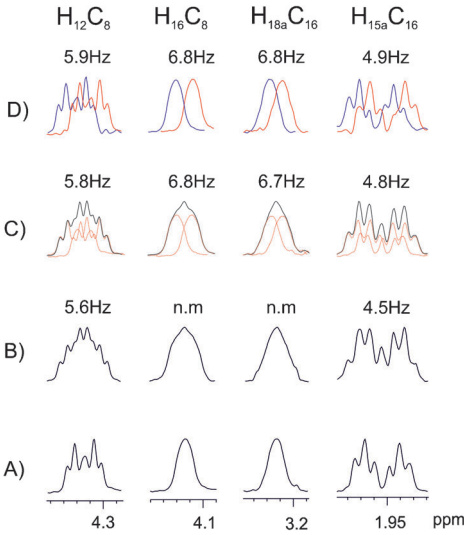

Figure 5. Comparison of several methods for the measurement of ^nJ(CH) values in non-resolved or complex multiplets. PIP-HSQMBC cross-peaks obtained A) with and B) without ^{13}C decoupling during acquisition; C) fitting process performed from the decoupled multiplets in A to match the experimental coupled multiplets in B; D) overlaid α and β multiplets obtained after IP ± AP data combination in an IPAP experiment, respectively.

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couplings up to 35 Hz can be measured (Tables S1–S3). In particular, special focus is made for the analysis and precise determination of $^{1}D(CH)$ and $^{2}D(HH)$ RDCs from undistorted cross-peaks belonging to diasterotopic CH_2 groups. The H11a/H11b protons of **1** are a good example to evaluate the more accurate measurement of their homonuclear ($^{2}D(HH) = -12.5$ Hz) and heteronuclear ($^{1}D(C_{11}H_{11a}) = +7.7$ Hz and $^{1}D(C_{11}H_{11b}) = -18.2$ Hz) RDCs, facilitating determination of their unequivocal orientation and stereoselective assignment. It is shown that errors in the measurement of up to 10% Hz can be introduced from distorted cross-peaks in conventional F2-coupled CLIP-HSQC and F2-decoupled HSQC spectra. These errors are avoided in the undistorted PIP-HSQC cross-peaks. In addition, a PIP-HSQMBC spectrum has been recorded under these challenging conditions to quantitatively measure a number of small long-range CH RDCs values, thus opening the door for the application of these parameters to superior structure determination and refinement (Figures S19–S21).

In conclusion, it has been shown that a *z*-filter is an extremely efficient tool to suppress unwanted homo- and heteronuclear anti-phase contributions in HSQC-like experiments. The proposed PIP-HSQC and PIP-HSQMBC experiments yield undistorted in-phase cross-peaks that are amenable for a more accurate extraction of small coupling constant values. All these methods can be recorded in full automation mode without any prior calibration and they offer a general implementation on a large variety of isotropic and anisotropic sample conditions. In addition, the method can be implemented in other inverse-detected NMR experiments, including HMQC-type experiments or involving other heteronuclei than carbon.

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