Perfect $^1J_{\text{CH}}$ -resolved HSQC: Efficient measurement of one-bond proton-carbon coupling constants along the indirect dimension

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ABSTRACT

A versatile $^1J_{\text{CH}}$ -resolved HSQC pulse scheme for the speedy, accurate and automated determination of one-bond proton-carbon coupling constants is reported. The implementation of a perfectBIRD element allows a straightforward measurement from the clean doublets obtained along the highly resolved F1 dimension, even for each individual $^1J_{\text{CHa}}$ and $^1J_{\text{CHb}}$ in diastereotopic H_aCH_b methylene groups. Real-time homodecoupling during acquisition and other alternatives to minimize accidental signal overlapping in overcrowded spectra are also discussed.

INTRODUCTION

One-bond proton-carbon coupling constants (the scalar $^1J_{\text{CH}}$ coupling in isotropic media or the total $^1T_{\text{CH}}$ coupling in anisotropic media) are fundamental NMR restraints in the structural determination of small molecules. In particular, residual dipolar couplings (RDCs) have become very powerful to solve challenging structural questions in molecules dissolved in weakly aligned media. Their success relies on the determination of relative orientations of specific chemical bonds with respect to a molecular alignment tensor. For instance, it is possible to unravel the relative configuration of multiple stereochemical centers without a previous knowledge of scalar J coupling constants and NOE enhancements.¹ One-bond proton-carbon RDCs ($^1D_{\text{CH}} = ^1T_{\text{CH}} - ^1J_{\text{CH}}$) are the most efficient parameters for unequivocal structural discrimination, whereas two-bond geminal proton-proton coupling constants ($^2J_{\text{HH}}$ in isotropic media and $^2T_{\text{HH}}$ in anisotropic media) have also shown an excellent complementarity for this purpose.² New strategies involving long-range proton-carbon RDCs ($^nD_{\text{CH}}$)³ or residual ^{13}C chemical shift anisotropy (RCSA)⁴ promise to be of great help in proton-deficient molecules with a prominent lack of protonated carbons. From the NMR methodological point of view, the interest has been extensively focused on the suitability of measuring $^1J_{\text{CH}}$ from the direct (F2) or the indirect (F1) dimension of HSQC spectra. In particular, the extraction of individual $^1J_{\text{CH}}$ values in diastereotopic protons H_aCH_b of methylene systems has always been a challenging task and obtaining clean J patterns for these protons has not been easy (Fig. 1). Probably, the easiest way to measure these couplings would be the F2-heterocoupled CLIP-HSQC experiment⁵ where each ^1H signal appears as a large doublet along F2 due to $^1J_{\text{CH}}$ (Fig. 1A). Recently, two improved solutions (perfectHSQC⁶ and PIP-HSQC⁷ experiments) have been proposed to remove the interference of undesired J_{HH} modulation during the INEPT periods in HSQC experiments. Both approaches afford pure in-phase multiplets with respect to $^1J_{\text{CH}}$ and J_{HH} , allowing a much accurate measurement of $^1J_{\text{CH}}$ especially when large J_{HH} values are present, as it can happen when measuring RDCs. The major drawbacks to measure accurately $^1J_{\text{CH}}$ from the F2 dimension are due to the presence of complex J_{HH} multiplets, the broader linewidths commonly observed in anisotropic conditions and the significant signal distortions which may appear due to strong coupling effects. To alleviate some of

these problems, several interferogram-based broadband homodecoupled versions of the CLIP-HSQC have been reported (Fig. 1B-C),⁸ although all of them require time-consuming 3D acquisition modes that greatly hamper its routine use. Very recently, an optimized BIRD-based real-time homodecoupled HSQC experiment has been developed to obtain simplified ^1H multiplet patterns in F2 without sacrificing sensitivity. However, diastereotopic CH_2 protons appear distorted and partially homodecoupled because the $^2J_{\text{HH}}$ splitting is not removed by the BIRD effect (Fig. 1B) and, in addition, $^1J_{\text{CH}}$ and J_{HH} modulations during the homodecoupling block could induce unexpected effects and non-accurate measurements.^{8d}

FIGURE 1

The measurement of $^1J_{\text{CH}}/^1T_{\text{CH}}$ along the F1 dimension of HSQC spectra has been another alternative approach largely explored (Fig. 1E-G).⁹ All these methods work very well for CH systems where a clean doublet is obtained, but the major criticisms come from the fact that only the sum of $^1J_{\text{CHa}} + ^1J_{\text{CHb}}$ is obtained for H_aCH_b groups, as reported in the J -scaled BIRD-HSQC (JSB-HSQC) experiment (Fig. 1E).^{9d-9g} Some alternatives have been reported to measure individual $^1J_{\text{CH}}$ values in CH_2 but they all suffer from some important practical drawbacks. Here we offer a solution to this problem by proposing a novel perfect $^1J_{\text{CH}}$ -resolved HSQC experiment that allows an accurate determination of $^1J_{\text{CH}}$ along F1 for any ^1H multiplicity and with excellent levels of resolution.

RESULTS AND DISCUSSION

The proposed pulse sequence of Fig. 2A has been derived from three early ideas: i) previous schemes where the initial fixed INEPT element in the HSQC pulse sequence was made variable to allow the modulation of $^1J_{\text{CH}}$ during the indirect dimension,¹⁰ ii) the perfectHSQC experiment⁶ that uses a perfectINEPT element^{11d} to avoid J_{HH} modulation during the INEPT periods, and iii) the use of a new perfectBIRD element, previously implemented in an interferogram-based version of the broadband homodecoupled HSQC experiment,^{8a} to efficiently remove the $^2J_{\text{HaHb}}$ modulation in methylene H_aCH_b signals

generated by a BIRD^{d,X} module.¹² This classical BIRD^{d,X} element consists of a 90°(¹H)-Δ-180°(¹H,X)-Δ-90°(¹H) cluster ($\Delta = 1/(2 \cdot {}^1J_{\text{CH}})$) that inverts both ${}^1J_{\text{CHa}}$ and ${}^2J_{\text{HaHb}}$. A simple modification referred to as BIRD^d,¹³ employing the 90°(¹H)-Δ-180°(¹H,X)-Δ-90°(¹H), 180°(¹³C) element, adds a last 180°(¹³C) pulse that re-inverts again ¹³C magnetization and therefore only ${}^2J_{\text{HaHb}}$ remains inverted. In our proposal, the initial INEPT period in a conventional HSQC scheme is replaced by a incrementing perfectBIRD element (from point a to d in Fig. 1A) which is based on the perfect-echo concept successfully implemented in many interesting applications.¹¹ It consists of a double echo building block, τ-BIRD^d-τ-90_y(¹H)-τ-BIRD^{d,X}-τ-, in order to let ${}^2J_{\text{HaHb}}$ evolve during both echoes (from point a to d in Fig. 1A) while ${}^1J_{\text{CHa}}$ only evolves during the second one (from point c to d in Fig. 1A). Thus, ${}^2J_{\text{HaHb}}$ is fully refocused at the end of the perfectBIRD and signal will be only modulated by ${}^1J_{\text{CHa}}$ (Fig. 1H). The perfect ${}^1J_{\text{CH}}$ -resolved HSQC experiment is originally designed to allow independent ${}^1J_{\text{CH}}$ (J -scale factor κ) and/or $\delta(^{13}\text{C})$ (δ -scale factor κ') evolution during two different variable t_1 periods. However, we have concentrated our study in a classical J_{CH} -resolved presentation ($\kappa = 1$ and $\kappa' = 0$) because the spectral symmetry around the $F1 = 0$ axis affords a user-friendly fingerprinting visualization, with a clear and quick ${}^1J_{\text{CH}}$ profiling for all ¹H signals that is suitable for a reliable automated extraction of ${}^1J_{\text{CH}}$ values (Fig. 2B). In addition, the use of a very reduced spectral width in $F1$ ($\text{sw}(F1) = 250$ Hz) ensures high levels of digitization and accurate measurements. This approach is highly recommendable for small molecules where the probability of signal overlap is low. In cases of accidental signal overlap due to the presence of ¹H signals with similars ${}^1J_{\text{CH}}$ and $\delta(^{1}\text{H})$, a δ -correlation presentation offering better signal dispersion along $F1$ may be advisable. Thus, the possibility to use independent κ and κ' scaling factors retains good levels of digital resolution by using reduced $\text{sw}(F1)$. Figure 2C shows the corresponding J -upscaled/ δ -downscaled perfect ${}^1J_{\text{CH}}$ -resolved HSQC spectrum of strychnine (**1**) acquired with $\kappa = 5$, $\kappa' = 0.1$ and $\text{sw}(F1) = 3000$ Hz. The use of large κ values would decrease the overall sensitivity of the experiment but digital resolution could be properly optimized i) acquiring a major number of t_1 increments, ii) setting a major κ/κ' scaling proportionality that allows the use of more reduced $\text{sw}(F1)$, or iii) using non-uniform sampling (NUS) schemes.^{9d}

FIGURE 2

Each ^1H signal in the perfect $^1J_{\text{CH}}$ -resolved HSQC spectrum displays a clean anti-phase doublet along F1 corresponding to its $^1J_{\text{CH}}$ value (Figures 3 and S1). The mismatch between $^1J_{\text{CH}}$ and the BIRD delay setting is not critical and it can only generate some residual signals at F1=0 that do not interfere with the measurement. Interestingly, note that different $^1J_{\text{CH}}$ values for each CH and all diastereotopic CH_2 protons are quickly visualized and precisely measured, as performed for H18a (145.6 Hz) and H18b (131.7 Hz) or H23a (145.2 Hz) and H23b (136.9 Hz). In other cases, it is also possible to differentiate similar values, as found for H20a (138.6 Hz) and H20b (138.2 Hz). All these measurements can be performed automatically because of the symmetry of cross-peaks with respect to the F1=0 axis (Table S1). $^1J_{\text{CH}}$ mainly depends on the *s* character of the CH bond and the electronegativity effects of substituents and neighbours nuclei. Thus, such observed differences of $^1J_{\text{CH}}$ in CH_2 can give an insight about their equatorial and axial positions and therefore can be helpful in their stereoselective assignments. It is also interesting to compare the accuracy of the measurement along F1 or F2 dimensions in terms of digital and signal resolution. Using $\text{sw}(\text{F1})$ of 250 Hz and 256 complex t_1 increments, the FID digital resolution in the perfect $^1J_{\text{CH}}$ -resolved HSQC is about 1.9 Hz/Pt. In contrast, in F2 measurements from the F2 using a ^1H spectral width of 10 ppm (6000 Hz in a 600 MHz spectrometer) and 4k data points, the FID digital resolution is about 1.5 Hz/Pt. On the other hand, signal resolution also takes into account the overall multiplet width and therefore the accuracy in perfect $^1J_{\text{CH}}$ -resolved HSQC should be better than a conventional PIP-HSQC or perfectHSQC experiment because of the absence of J_{HH} splittings. These high levels of both digital and signal resolution should be only attainable in the recent real-time homodecoupled HSQC experiment.^{8d}

FIGURE 3

The perfect $^1J_{\text{CH}}$ -resolved HSQC experiment retains the basic pulse train of the regular HSQC experiment and therefore its performance can be further improved by adding complementary features, such as solvent suppression schemes, multiplicity editing, NUS enhancement or BIRD-based real-time homodecoupling along the F2 dimension.¹⁴ As an

example, Fig. 4A shows the homodecoupled version of the perfect $^1J_{\text{CH}}$ -resolved HSQC experiment. It works well for isotropic samples, with an excellent simplification of J_{HH} coupling patterns to singlets and without a considerable increase of linewidths. Figure 4C shows the corresponding spectrum acquired combining homodecoupling and 50% NUS, demonstrating that cross-peak simplification, improved sensitivity (averaged 70% for CH and 28% for CH_2), and equivalent resolution and measurement accuracy can be simultaneously achieved with shorter acquisition times (compare internal F2 projection in 4B vs. 4C). Unfortunately, homodecoupling remains partial in diastereotopic CH_2 protons because $^2J_{\text{HH}}$ is also inverted by the BIRD element and a doublet due to an apparent $^2J_{\text{HH}}$ is still observed.

FIGURE 4

The outstanding features of perfect $^1J_{\text{CH}}$ -resolved HSQC are finally demonstrated in the challenging determination of both the magnitude and the sign of all individual $^1D_{\text{CH}}$ RDCs of **1** weakly aligned in a CDCl_3 -compatible poly(methyl methacrylate) (PMMA) gel from a single NMR spectrum.¹⁵ (Fig. 5). Despite the small $\delta(^1\text{H})$ differences between the isotropic and anisotropic cross-peaks, $^1D_{\text{CH}}$ RDCs can be directly determined from this single NMR spectrum. It is observed how the relative orientation of each diastereotopic CH_2 proton is correlated with their different $^1D_{\text{CH}}$ values. For instance, +3.8 Hz and -24.0 Hz are measured for H23a and H23b, respectively, or +7.7 Hz and -17.4 Hz for H11a and H11b, respectively. The obtained RDC data agree with those published in related works (Table S2).^{9f,10} In this type of samples, the simultaneous measurement of $^1J_{\text{CH}}$ and $^1T_{\text{CH}}$ (and consequently $^1D_{\text{CH}}$) along F1 is strongly advantageous because there is no interference of $^2J_{\text{HH}}/^2D_{\text{HH}}$ and it is performed independent of ^1H multiplet complexities and lineshapes. In such anisotropic conditions, the full collapsing of multiplets by homodecoupling should not be so advantageous because linewidths are broader and also because the presence of larger T_{HH} couplings (30-40 Hz) can difficult their efficient decoupling. The direct measurement of $^1D_{\text{CH}}$ RDCs in the equivalent J -upscaled/ δ -downscaled perfect $^1J_{\text{CH}}$ -resolved HSQC spectrum acquired with $\kappa' = 0.1$ could be also problematic depending on the ^1H and ^{13}C RCSA. For the same reasons, the attempts to perform these measurements along the F2

dimension in regular PIP-HSQC or perfectHSQC fails due to the lack of F1 resolution. The option to use highly resolved F2-spectral-aliased HSQC spectra only works for carbons having a large ^{13}C RCSA (olefinic and aromatics) but fails for most of the aliphatic carbons. The experiment would partially work for methyl protons in very strong alignment conditions because the perfectBIRD module only inverts two protons attached on the same carbon.^{8d}

FIGURE 5

CONCLUSIONS

In summary, we have reported a robust and fast approach for the accurate and automated extraction of $^1J_{\text{CH}}$ for all carbon multiplicities. The novelty relies on the selectivity of the perfectBIRD element to differentiate the evolution of $^1J_{\text{CH}}$ vs. $^2J_{\text{HH}}$ in diastereotopic CH_2 protons. The measurement is accurately performed along the indirect F1 dimension and therefore it is irrespective of ^1H multiplet complexity along F2. The experiment can be easily tuned to avoid signal overlapping by monitoring a J -upscaling/ δ -downscaling scaling factors proportionality, can incorporate real-time homodecoupling during acquisition to afford multiplet simplification and enhanced sensitivity, and NUS can also be applied to economize spectrometer time. The experiment does not need any calibration and therefore is ideal for routine application. The potential of the method has been demonstrated by measuring $^1D_{\text{CH}}$ RDCs in challenging sample conditions.

EXPERIMENTAL SECTION

NMR experiments were recorded on a 600 MHz spectrometer equipped with a triple-resonance $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ inverse probe. The temperature for all measurements was set to 298K. The isotropic sample was prepared by dissolving 20 mg of strychnine (**1**) in CDCl_3 . The anisotropic sample was prepared as described in ref. 11b of the manuscript. They consisted of 20 mg of **1** aligned in a PMMA gel swollen in CDCl_3 using the reversible

compression/relaxation method. The alignment was monitored from the ^2H quadrupolar splitting ($\Delta\nu_Q$) of the CDCl_3 signal that was set to 36 Hz.

NMR spectra were recorded with proton 90° pulses of 8.5 μs and carbon 90° pulses of 10.5 μs . For broadband carbon inversion and refocusing, 0.5 ms smoothed Chirp pulses sweeping over a frequency band of 60 kHz and a four-Chirp composite pulse of 2 ms duration were used, respectively. The interpulse Δ delays in INEPT and BIRD elements were set to 3.57 ms ($\Delta = 1/(2 \cdot ^1J_{\text{CH}})$; optimized to $^1J_{\text{CH}} = 140$ Hz) and the recycle delay to 1 s. 2 scans were accumulated for each one of the 256 t_1 increments and the number of complex data points in t_2 was set to 2048. Spectra were acquired with an spectral window of 6010 Hz (in F_2) and 250 Hz (in F_1) giving a FID resolution of 5.86 (F_2) and 1.95 (F_1) Hz, respectively. Prior to Fourier-transformation of each data, zero filling to 1024 in F_1 and a $\pi/2$ -shifted squared cosine window function (QSINE, SSB: 2) in both dimensions were applied. After applying zero filling, the digital resolution was 2.93 (F_2) and 0.24 (F_1) Hz, respectively. Linear prediction was not applied. An error of ± 0.5 Hz in the determination of $^1J_{\text{CH}}$ is assumed as a conservative estimate. Gradient ratios for G1:G2 were set to 80:20.1, measured as percentage of the absolute gradient strength of 53.5 G/cm. Sine bell shaped gradients had 1 ms of duration and were followed by a recovery delay of 100 μs (δ). All experiments were acquired and processed using the echo/anti-echo protocol where the gradient G1 was inverted for every second FID. Real-time BIRD-based homodecoupling was performed using 10.3 ms chunk length and 8 loops (2048 complex points in F_2).

The java applet jmeasurement.jar described at the end of this SI for extracting $^1J_{\text{CH}}$ automatically is available on request.

Fitting of RDC data to structures was performed using the MSpin software package (Mestrelab Research SL, Santiago de Compostela, Spain). <http://www.mestrelab.com>. The 3D structures of all compounds were initially generated using the MacroModel Suite from Schrödinger (<https://www.schrodinger.com/MacroModel/>). The diastereomers were automatically generated using the LigPrep module in MacroModel and further energy

minimized using the MMFFs molecular mechanics force field. AM1 and DFT (B3LYP/6-31G*) calculations were performed in Gaussian 09 (<http://www.gaussian.com>).

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CAPTIONS

Fig. 1 Schematic J coupling patterns for one of the diastereotopic protons' (H_a) cross-peak (with an arbitrary multiplicity along F2) belonging to a H_aCH_b methylene group in several implementations of (A-D) F2- and (E-H) F1-heterocoupled HSQC experiments. All patterns E-H could be further simplified along the horizontal F2 dimension by homodecoupling, similarly as shown in B.

Fig 2 A) General pulse scheme of the perfect J_{CH} -resolved HSQC experiment. Thin and thick rectangles represent 90° and 180° rectangular pulses, respectively, applied along the x-axis unless indicated differently. Inversion and refocusing 180° ^{13}C pulses can be applied as adiabatic pulses. The perfectBIRD element consists of two incrementing BIRD^d (from point a to b) and BIRD^{d,X} (from point c to d) pulse clusters, in order to let $^2J_{HaHb}$ evolve during both echoes while $^1J_{CHa}$ only evolves during the second one. The inter-pulse delays in INEPT and BIRD elements are optimized according to $\Delta=1/(2*^1J_{CH})$. The factors κ and κ' are used to scale up or scale down the independent $^1J_{CH}$ (J -scale κ factor) and/or $\delta(^{13}C)$ (δ -scale κ' factor) evolution during the two different variable t_1 periods. The echo/anti-echo encoding in F1 was achieved by changing the sign of G1 between successive t_1 increments. The duration of a pulsed-field gradient and of the subsequent recovery delay amounts to δ and the ratio between G1:G2 were 80:20.1. A basic two-step phase cycling is applied: $\phi_1 = x, -x$; $\phi_{rec} = x, -x$. B-C) 2D perfect $^1J_{CH}$ -resolved HSQC spectra of strychnine (**1**) in $CDCl_3$ recorded at 600 MHz proton frequency with B) $\kappa = 1$, $\kappa' = 0$ and $sw(F1) = 250$ Hz and C) $\kappa = 5$, $\kappa' = 0.1$ and $sw(F1) = 3000$ Hz, respectively. Other experimental conditions are the same for both spectra: 2048 complex data points in F2, 256 t_1 increments, 2 scans per t_1 , 1

second of pre-scan delay and Δ optimized to 140 Hz. FID resolution before processing was 1.9 Hz/pt. The experimental time for each 2D spectrum was of 14m 30s.

Fig 3 Expansion of the aliphatic area of the spectrum in Fig. 2B clearly showing the magnitudes of $^1J_{CH}$ for all CH protons and also each different diastereotopic methylene protons. Spectral resolution in F1 is 0.24 Hz/pt.

Fig 4 A) Pulse sequence of the real-time BIRD-based homodecoupled perfect $^1J_{CH}$ -resolved HSQC experiment. B-C) Comparison between the B) conventional and C) homodecoupled perfect $^1J_{CH}$ -resolved HSQC spectra of **1** recorded under the same experimental conditions as described in Fig. 2B. The only differences are the use of 50% NUS and the homodecoupled acquisition in spectrum C, saving 50% of spectrometer time. From the internal F2 projections at the top of 2D spectra, also note the improved cross-peak simplification and sensitivity enhancements achieved by signal collapsing for all CH and CH₂ protons in the homodecoupled spectrum vs. the conventional one.

Fig 5 A) Perfect $^1J_{CH}$ -resolved HSQC spectrum of **1** in compressed PMMA/CDCl₃ (2H quadrupolar coupling (CDCl₃) = 30 Hz), acquired using the pulse sequence of Fig. 1A with $\kappa = 1$, $\kappa' = 0$ and $sw(F1) = 250$ Hz. B-C) Expanded regions corresponding to the insets marked in A showing how $^1D_{CH}/2$ can be directly extracted for each resolved 1H signal. The experimental conditions were the same as described in Fig. 2B. Red boxes in B and C correspond to the isotropic components (see Figure S2 in the supporting information). Also note how the anisotropic components present a broader lineshapes.

Measurement of $^1J_{CH}$ along F2

A. CLIP-HSQC, PIP-HSQC or PerfectHSQC

B. Real-time BIRD-based Homodecoupled HSQC

C. Interferogram-based Homodecoupled HSQC using PerfectBIRD

D. Spin-state Selective HSQC, ZQ-DQ ...

Measurement of $^1J_{CH}$ along F1

E. J -scaled BIRD HSQC

F. Inverse J -INEPT

G. $^1J_{CH}/^2J_{HH}$ -resolved HSQC

H. Perfect $^1J_{CH}$ -resolved HSQC using PerfectBIRD (This work)

A)

B)

C)

Perfect $^1J_{CH}$ -resolved HSQC

- User-friendly NMR representation
- Clean doublets along F1, independent of CH_n multiplicity
- Automated determination of individual $^1J_{CH}$