

# Improved Sensitivity in Gradient-Based 1D and 2D Multiplicity-Edited HSQC Experiments

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The determination of carbon multiplicities is essential for the structural NMR assignment of small and medium-size molecules. The usual approach employs DEPT spectra (1), although carbon detection can become troublesome when sample concentration is low. For this reason, several proton-detected carbon-multiplicity-edited experiments have been proposed to improve the sensitivity of detection (2–11). In these proton-detected methods, the intensity and/or the phase of the recorded proton resonances depends on carbon multiplicity, thus rendering the structural analysis easier. Although traditional methods for proton-detected carbon multiplicity determination use the phase-cycle procedure, more-recent improved versions which use pulsed field gradients (PFG) to select the desired coherence-transfer pathway (CTP) (12) have been described (13–19). The two main advantages of gradient selection in heteronuclear inverse experiments are the effective suppression of undesired strong magnetizations, such as  $^1\text{H}-^{12}\text{C}$  or  $^1\text{H}-^{14}\text{N}$ , and the lack of need for a complete phase cycle. In this way, the use of this powerful tool results in a higher performance, giving a much better spectral quality in considerably reduced acquisition time.

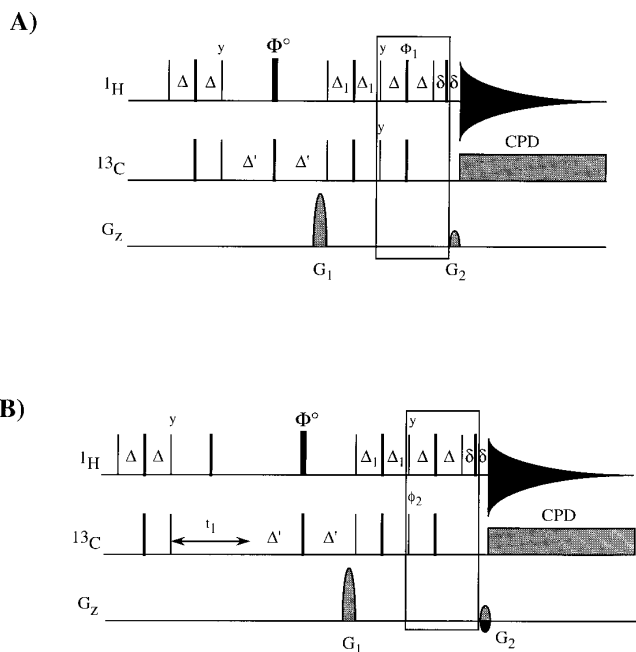
On the other hand, gradient selection is normally less sensitive than analogous phase-cycled experiments because of the selection of a single specific CTP (12, 18). Presently, only NMR experiments based on the PEP (preservation of equivalent pathways) methodology (20, 21) have been widely shown to improve the signal-to-noise ratio of multidimensional experiments in comparison to conventional phase-cycled analogues. Thus, this approach is particularly useful in 2D, 3D, and 4D HSQC-type pulse sequences (22–31) which finish with a classical reverse I–S (I is typically  $^1\text{H}$  and S is  $^{13}\text{C}$  or  $^{15}\text{N}$ ) INEPT pulse train. The reason for this increased sensitivity is that both orthogonal components of the magnetization present at the end of the typical variable evolution  $t_1$  period ( $I_z S_x$  and  $I_z S_y$ , respectively) are selected prior to acquisition. Comparisons of the relative sensitivities of the different  $^1\text{H}-^{13}\text{C}$  (24, 25, 28, 30) and  $^1\text{H}-^{15}\text{N}$  (31) HSQC and related experiments have been published.

In this work, we show how the PEP methodology can be successfully implemented in 1D and 2D pulse sequences in order to improve the sensitivity of gradient-based HSQC experiment specifically designed to edit proton spectra according to carbon-13 multiplicities. A regular gradient-enhanced 1D multiplicity-edited HSQC experiment (termed ge-1D edited-HSQC) (13, 14) can be derived from the classical HSQC pulse train in which the typical evolution period of the spin S (e.g.,  $^{13}\text{C}$ ) has been replaced by a  $\Delta' - \Phi^\circ(^1\text{H}) - 180^\circ(^{13}\text{C}) - \Delta'$  editing block (Fig. 1A with omission of pulses and delays inside the frame). In order to select the desired magnetization, two gradients with a 4:1 ratio are applied during the carbon evolution period and just before acquisition, respectively.

Using shift operators, the selected CTP ( $\Delta' = 1/2 J_{IS}$ ) can be summarized as

$$\begin{aligned} \sum_{k=1}^n I_{kz} &\xrightarrow{90_x^\circ} \frac{i}{2} \sum_{k=1}^n (I_k^+ - I_k^-) \\ &\xrightarrow{\Delta/2 - 180_x^\circ(I, S) - \Delta/2} \sum_{k=1}^n (I_k^+ + I_k^-) S_z \\ &\xrightarrow{90_y^\circ(I), 90_x^\circ(S)} -i \sum_{k=1}^n I_{kz} S^+ \xrightarrow{\Delta'} \\ &\frac{-i}{2} (-2i)^{n-1} \sum_{k=1}^n I_{kz}^{n-1} S^+ \xrightarrow{\Phi_x^\circ(I), 180_x^\circ(S)} \\ &\frac{-i}{2} (-2i)^{n-1} \cos^{n-1}(\Phi) \sum_{k=1}^n I_{kz}^{n-1} S^- \xrightarrow{\Delta'} \\ &-i \cos^{n-1}(\Phi) \sum_{k=1}^n I_{kz} S^- \xrightarrow{90_x^\circ(I, S)} \\ &\frac{-i}{2} \sum_{k=1}^n I_k^+ S_z \cos^{n-1}(\Phi) \xrightarrow{\Delta/2 - 180_x^\circ(I, S) - \Delta/2} \\ &\frac{1}{4} \sum_{k=1}^n I_k^- \cos^{n-1}(\Phi). \end{aligned}$$

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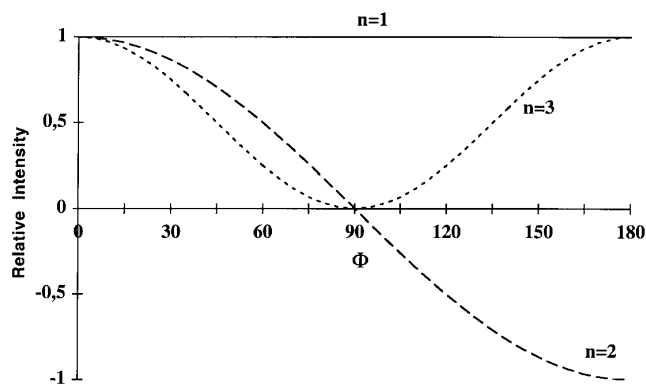
**FIG. 1.** Pulse sequences to obtain sensitivity-improved (a) ge-1D and (b) ge-2D multiplicity-edited HSQC spectra. Hard  $90^\circ$  and  $180^\circ$  pulses are indicated by vertical narrow and wide black bars, respectively. All pulses are applied from the  $x$  axis unless otherwise indicated, whereas  $\phi_1$  is usually set to  $y$ . A minimum two-step phase cycle has been applied in which the first  $90^\circ$  carbon pulse and the receiver have been inverted in alternate scans. The delay  $\Delta$  is set to  $1/4J_{IS}$ ,  $\Delta'$  depends on the editing purpose, and  $\Delta_1$  is optimized according to the desired  $I_nS$  multiplicity (see text for details). Longitudinal pulsed field gradients with a 5% truncated Gaussian shape and 1 ms of duration ( $\delta$ ) are indicated by shaded shapes on the line  $G_z$ . The PFG recovery time was  $100 \mu\text{s}$ . Gradient strengths are 20 and 5 G/cm for  $G_1$  and  $G_2$ . Carbon decoupling during proton acquisition was achieved by applying a GARP pulse train. Omission of the pulses and delays inside the frame results in the conventional gradient-based experiments, while if they are applied as specified (PEP methodology), improved-sensitivity experiments are obtained. All experiments have been experimentally implemented in a Bruker ARX400 spectrometer using an inverse broadband probehead incorporating a Z-gradient spoil.

Several conclusions can be extracted from the above expression. A dependence of the  $I_nS$  ( $n = 1-3$ ) signal intensity with respect to the term  $\cos^{n-1}(\Phi)$  appears when  $\Delta' = 1/2J_{IS}$  (Fig. 2). Thus, multiplicity differentiation can be easily accomplished when  $\Phi^\circ = 180^\circ$  ( $I_S$  and  $I_3S$  protons have positive intensities, whereas protons belonging to  $I_2S$  systems appear as negative peaks; see Fig. 3A) while on the other hand, a clean subspectrum for  $I_S$  spin systems can be obtained when  $\Phi^\circ = 90^\circ$ .

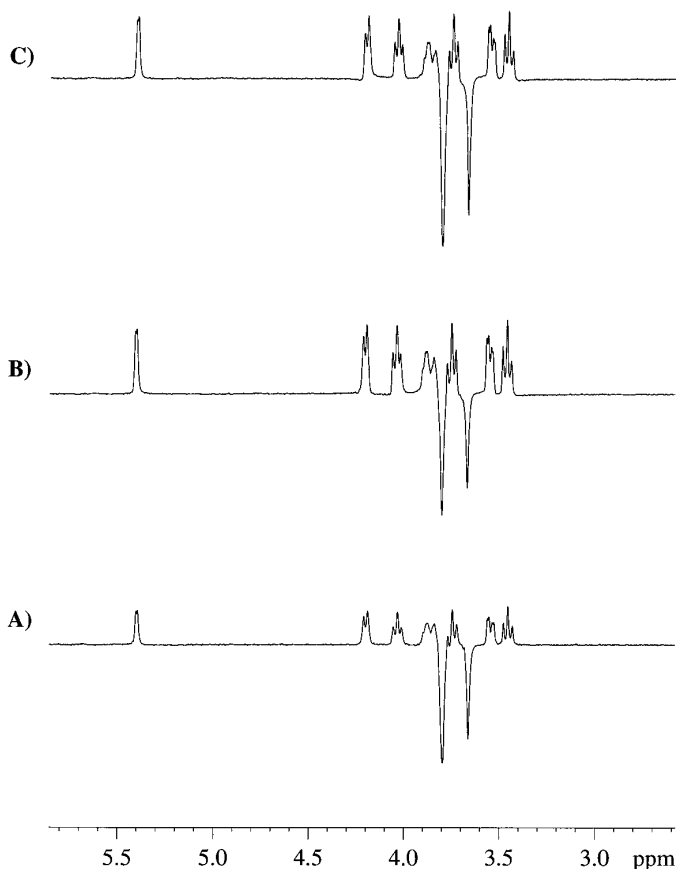
However, as known for other 1D experiments (18), the gradient-based approach presents a decrease of the signal-to-noise ratio by a factor of 2 (represented by the coefficient  $\frac{1}{4}$  in the last term) when compared with the analogue phase-cycled experiment. In order to regain this loss, we show here how the PEP methodology can be easily incorporated by simply adding a second reverse INEPT block (the pulses and delays inside the frame in the schemes of Fig. 1) into

the ge-1D edited-HSQC pulse train (Fig. 1A). During the first reverse INEPT block, the  $I_zS_y$  term is refocused to in-phase  $I_x$  magnetization whereas the  $I_zS_x$  term exists as a mixture of zero- and double-quantum coherences ( $I_yS_x$ ). The two simultaneous  $90^\circ$  pulses of the second reverse INEPT block convert the in-phase magnetization into longitudinal magnetization, whereas the MQC is converted to antiphase magnetization ( $I_z$  and  $I_yS_z$ , respectively). After the refocusing period and the final  $90^\circ(I)$  pulse applied on the  $x$  axis, transverse magnetization is obtained in the form  $I_y \cos(\omega_s t_1) + I_x \sin(\omega_s t_1)$ . In this way, sensitivity-improved 1D multiplicity-edited HSQC (termed as ge-1D edited-HSQC-PEP) spectra can be obtained in no extra time under the same experimental conditions (Fig. 3). However, as known for multidimensional HSQC-type experiments, it is not possible to achieve the maximum improved sensitivity for all multiplicities at the same time (24). Thus, the maximum theoretical signal-to-noise increase by a factor of 2 is experimentally obtained for  $I_S$  spin systems when  $\Delta_1 = 1/4J_{IS}$  (Fig. 3A vs 3B), whereas other  $I_nS$  systems ( $n > 1$ ) are unaffected. On the other hand, a maximum sensitivity gain about 1.4 is achieved for  $I_2S$  spin systems when  $\Delta_1 = 1/8J_{IS}$  (Fig. 3A vs 3C), although  $I_S$  systems will not reach the maximum gain (only about a factor of 1.7). In general, it is not usually critical to improve the sensitivity of  $I_3S$  systems, and therefore, an average value of  $\Delta_1 = 1/6J_{IS}$  would achieve overall improvements for all multiplicities [theoretically, 1.5 for  $I_S$ , 1.37 for  $I_2S$ , and 1.25 for  $I_3S$  as described in Ref. (24)]. Experimentally, the extra additional delays of the new proposed sequence practically do not affect the sensitivity via  $T_2$  relaxation losses but some attention should be paid when very different  $^1J_{IS}$  are present.

The editing capabilities of the proposed 1D sequence could be extended in cases where  $I_3S$  signals are missing. For instance, this method can be used to distinguish NH from  $\text{NH}_2$  protons. Different approaches would emerge to obtain these clean  $I_2S$ -edited subspectra with improved sensitivity ( $\Delta_1 = 1/8J_{IS}$ ): (i) subtraction of two spectra acquired with  $\Phi^\circ = 180^\circ$  and  $\Phi^\circ = 0^\circ$  ( $\Delta' = 1/2J_{IS}$ ); (ii) acquisition



**FIG. 2.** Signal dependence of different  $I_nS$  multiplicities on  $\Phi$  in ge-1D and ge-2D HSQC-edited experiments ( $\Delta' = 1/2J_{IS}$ ).



**FIG. 3.** ge-1D  $^1\text{H}$ - $^{13}\text{C}$  edited-HSQC spectra of a 0.2 M sucrose sample in  $\text{D}_2\text{O}$  acquired with the pulse sequences of Fig. 1A: (A) conventional spectrum (without the frame) using  $\Phi = 180^\circ$  and  $\Delta' = 2\Delta_1 = 3.4$  ms; (B) sensitivity-improved spectrum (with the frame) acquired as (A) and optimized for CH protons ( $\Delta_1 = 1.7$  ms); (C) sensitivity-improved spectrum acquired as (B) but optimized for  $\text{CH}_2$  protons ( $\Delta_1 = 0.9$  ms). In all experiments, eight scans preceded by two dummy scans were acquired using a prescan delay of 1 s and an interpulse delay  $\Delta$  of 1.7 ms. All spectra have been plotted with the same vertical scaling.

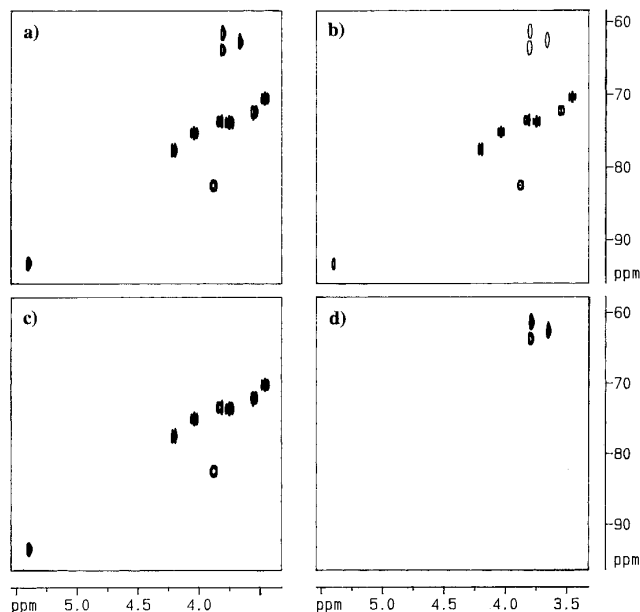
of a simple spectrum with  $\Phi^\circ = 90^\circ$  and  $\Delta' = 1/4J_{\text{IS}}$ ; or (iii) recording a single spectrum with  $\Phi^\circ = 180^\circ$  and  $\Delta' = 1/2J_{\text{IS}}$  but setting the phase  $\phi_1$  to  $x$ .

The same principles described above for the 1D experiments can be further expanded to 2D analogues in which carbon multiplicity information and heteronuclear chemical-shift correlation are achieved in a single experiment. A gradient-enhanced HSQC version incorporating a carbon  $z$  filter has already been proposed for this purpose (19). PEP methodology can be also inserted in this experiment according to Ref. (26), yielding a sensitivity increase by a factor of 2 for IS systems. Furthermore, improved results are achieved employing the widely used HSQC-PEP pulse train (22). In this method, phase-sensitive data are recorded applying the echo-antiecho approach (32). Thus, two independent signals corresponding to the phase-modulated N- and P-type data are separately recorded and stored in different memory

blocks for each  $t_1$  point with the sign of the last gradient inverted along with cycling of the phase of the last carbon  $90^\circ$  pulse ( $y$  and  $-y$ , respectively). After this, the desired pure amplitude-modulated signals are obtained by adding and subtracting the two phase-modulated data sets and further processed in the usual way. This version gives experimental results in terms of sensitivity similar to those of the conventional HSQC-PEP experiment (22). In addition, all predictions described for the HSQC experiment can be extrapolated to our proposed sequence (24).

Figure 4 shows several 2D  $^1\text{H}$ - $^{13}\text{C}$  edited-HSQC spectra recorded with the improved-sensitivity pulse sequence of Fig. 1B in which several editing possibilities are reported depending on the  $\Phi^\circ$  and  $\Delta'$  settings. As predicted theoretically, an experimental gain by 2 is also achieved in the 2D version for IS systems when  $\Delta_1 = 1/4J_{\text{IS}}$ .

In summary, a simple approach based on the PEP methodology to achieve sensitivity improvements in gradient-based 1D and 2D multiplicity-edited HSQC experiments has been proposed. In cases when resolution is of interest, the 1D version should be chosen. On the other hand, a simple modification of the ge-2D HSQC-PEP experiment allows one to obtain carbon multiplicity information and heteronuclear



**FIG. 4.** ge-2D  $^1\text{H}$ - $^{13}\text{C}$  edited-HSQC chemical-shift-correlation spectra of sucrose acquired with the pulse sequence of Fig. 1B incorporating the frame. (a) All CH and  $\text{CH}_2$  protons appear positive when  $\Phi = 0^\circ$  and  $\Delta' = 2\Delta_1 = 3.4$  ms; (b) positive peaks for CH and negative for  $\text{CH}_2$  when  $\Phi = 180^\circ$  and  $\Delta' = 2\Delta_1 = 3.4$  ms; (c) a CH subspectrum is obtained if  $\Phi = 90^\circ$  and  $\Delta' = 2\Delta_1 = 3.4$  ms; (d) a  $\text{CH}_2$  spectrum is obtained if  $\Phi = 180^\circ$ ,  $\Delta' = 1.7$  ms, and  $\Delta_1 = 0.9$  ms. For all experiments, two scans per  $t_1$  value were acquired according to the echo-antiecho procedure (see text). The final time-domain data matrix has a size of  $1024 \times 64$  complex points. Zero filling was applied in  $\omega_1$  to a size of 128 complex points. Prior to Fourier transformation, data were multiplied with a cosine window function along both dimensions.

chemical-shift correlation with improved sensitivity in a single experiment. This version becomes a suitable tool for both routine and high-demanding applications.

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