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Spin-edited 2D HSQC–TOCSY experiments for the measurement of homonuclear and heteronuclear coupling constants: Application to carbohydrates and peptides

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Abstract

Simple modifications of the sensitivity-improved HSQC–TOCSY pulse sequence are proposed for the easy determination of the sign and the magnitude of homonuclear and heteronuclear coupling constants. Whereas in well-resolved regions, a clean two-component E.COSY-like pattern allows a direct measurement from a single 2D spectrum, separate acquisition of equivalent single-component TROSY/anti-TROSY spectra becomes highly interesting when spectral crowding complicates the spectral analysis. It is also demonstrated that an additional restricted planar mixing element after the isotropic TOCSY process completely retains all spin-editing features and permits the accurate measurement of the sign and the size of the corresponding homonuclear proton–proton coupling constants. Among others, the proposed techniques are particularly suited for molecules presenting a great number of CH and NH spin systems. Examples and practical details of the implementation of these techniques on standard carbohydrates and peptides at ^{13}C and ^{15}N natural abundance are provided.

Keywords: E.COSY; Coupling constants; TROSY/anti-TROSY; HSQC–TOCSY; Spin-state selection

1. Introduction

Heteronuclear-edited correlation experiments of the type 2D ^{1}H–X HSQC–TOCSY or HMQC-TOCSY have been developed into powerful NMR tools for structural studies of molecules in which the analysis of homonuclear TOCSY spectra can fail due to undesired overlapping effects [1]. In particular, the gradient-enhanced version of the HSQC–TOCSY experiment allows (i) a careful analysis and straightforward interpretation from clean spectra, (ii) can take profit of the sensitivity-enhanced features traditionally associated to the regular HSQC pulse scheme, and (iii) offers excellent dispersion/resolution properties associated to the much better resolved F1 X dimension [2]. In addition to the classical structure elucidation strategy based on ^{1}H and X chemical shift assignments, typically applied for ^{13}C and ^{15}N in molecules such as carbohydrates and peptides, the regular HSQC–TOCSY pulse scheme has also been largely modified in many different ways with the aim to measure heteronuclear long-range coupling constants, $^{n}J(XH)$ $n > 1$, in both natural abundance and labelled molecules [3–21].

Basically, all these X-edited TOCSY experiments contain two independent building blocks. First, an isotope-edited block in which magnetization is first transferred from ^{1}H to the heteronucleus X and then come back to ^{1}H again via $^{1}J(XH)$. The resulting in-phase ^{1}H magnetization obtained at the end of this initial step is suitable for further propagation via a homonuclear

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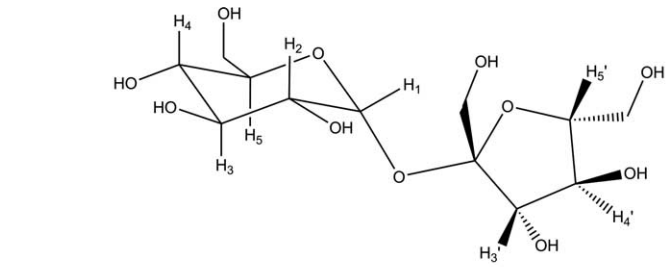
^{1}H–^{1}H TOCSY transfer to other relayed protons. Since the initial heteronuclear coherence transfer is based on the large and rather uniform $^{1}J(HX)$ values, this approach is very useful to determine even small, several-bond away $^{n}J(XH)$ coupling constants which are difficult to obtain using other NMR methods, such as HMBC [4] or long-range optimized HSQC experiments [5], in where the inter-pulse delays are directly optimized on small $^{n}J(XH)$ values and where the final measurements are often performed on highly complicated anti-phase or mixed-mode multiplet patterns. On the contrary, the main drawbacks of HSQC–TOCSY-type experiments rely in the fact that only couplings on protonated heteronucleus can be measured and the overall sensitivity largely depends of the transfer efficiency during the TOCSY step [3].

Several years ago, w_1-edited 2D TOCSY experiments, in which the indirect detection was based on ^{1}H evolution instead of X evolution, were proposed for this purpose. The original experiment, known as HETLOC experiment [6,7], has been further improved in several ways, sometimes using rather sophisticated but useful pulse schemes to measure $^{n}J(XH)$ [8–11]. In these experiments, a conventional 2D ^{1}H–^{1}H correlation map is obtained displaying the satellite lines of the proton resonances as a diagonal peaks and the resulting cross-peaks showing the typical E.COSY-style patterns from which the sign and the magnitude of the corresponding $^{n}J(XH)$ can be extracted. Because only the relative signal displacement between spin-edited signals along the detected dimension is measured, small values even than the natural linewidth can be determined with excellent accuracy. The major inconvenient of such experiments is the poor chemical shift dispersion in the F1 ^{1}H dimension that requires high digitization ratios in this dimension and, therefore, long acquisition times.

Based on the principles of the HETLOC experiment, several related ^{1}H–X heteronuclear correlation approaches have been also reported [12–17] offering (i) a much better dispersion in the indirect F1 dimension, (ii) the absence of diagonal peaks, and (iii) the achievement of the finest signal-to-noise ratios by combining the different available magnetization components with pulsed field gradient coherence-order selection. A very simple approach to measure these $^{n}J(XH)$ should be from a w_2-X-coupled HSQC–TOCSY spectrum, in which the active coupling, found into the final multiplet pattern as an additional in-phase splitting in the detected dimension, can be measured from computer-aided coupled/decoupled multiplet analysis [18,19]. On the other hand, a phase-cycled 2D HSQC–TOCSY experiment using spin-state selection in both dimensions was described several years ago [20]. Most recently, an improved E.COSY-type approach termed HSQC–HECADE was proposed in which the evolution of the X chemical shift and the heteronuclear coupling took place in independent evolution periods [15,16]. Other useful approaches based on spin-state selection have been also reported [8,13,14,17] and, in particular, selective 1D versions are very attractive tools for such measurements in small-to-medium-sized molecules because offer excellent accuracy, individual optimization parameter settings, enhanced resolution and reduced experimental time [21].

The current paper is concerned with the development of more robust NMR experiments to measure both homonuclear and heteronuclear coupling constants in a variety of conditions. We propose modifications of the sensitivity-improved gradient-enhanced 2D HSQC–TOCSY pulse scheme that yields a set of different selective spin-state cross-peak pattern correlation spectra with optimal sensitivity and without extra experimental setup requirements than the original decoupled experiment. The original pulse timing is fully preserved and no additional delays are introduced into the new basic pulse schemes, the inter-pulse delays can be particularly optimized for IS spin systems if required and accurate J-coupling measurements are reliable from several spin-edited cross-peak patterns generated in fully coupled 2D heteronuclear ^{1}H–X correlation maps. As it is known, the extraction of J-coupling values from E.COSY patterns are highly valuable in spectra showing clean well-resolved resonances but, unfortunately, the number of peaks is doubled when compared to the equivalent decoupled experiment. It will be shown that the individual edition of each E.COSY component, the corresponding αα and ββ cross-peaks, into two separate spectra using a double-coherence TROSY-like transfer can be of enormous utility because strongly minimizes the overlapping problems and also reduces the spectral resolution requirements. Combined with these techniques, the incorporation of an optional restricted planar mixing transfer also allows the additional measurement of the corresponding proton–proton coupling constants, $J(HH)$ [22–24]. This restricted element do not alter the spin editing E.COSY or TROSY-like features and, therefore, the determination of the sign and the magnitude of these $J(HH)$ is made exactly in the same conditions as performed for $^{n}J(XH)$. Although the proposed experiments are limited to IS spin systems, it can be of general interest in molecules having a great number of CH and NH spin systems as, for instance, in carbohydrates and peptides. For instance, in the particular case of peptides, a complete set of heteronuclear and homonuclear coupling constants, namely $^{2}J(N–H^{\alpha})$, $^{3}J(N–H^{\beta})$, $^{2}J(C^{\alpha}–NH)$, $^{2}J(C^{\alpha}–H^{\beta})$, $^{3}J(C^{\alpha}–H^{\gamma})$, $^{3}J(NH–H^{\alpha})$, and $^{3}J(H^{\alpha}–H^{\beta})$, can be measured with good precision starting from the backbone NH and $C^{\alpha}H^{\alpha}$ spin systems. The proposed NMR methods have been successfully tested and results are provided for two classical standard compounds such as sucrose, **1** , and cyclosporine, **2** (see Scheme 1).



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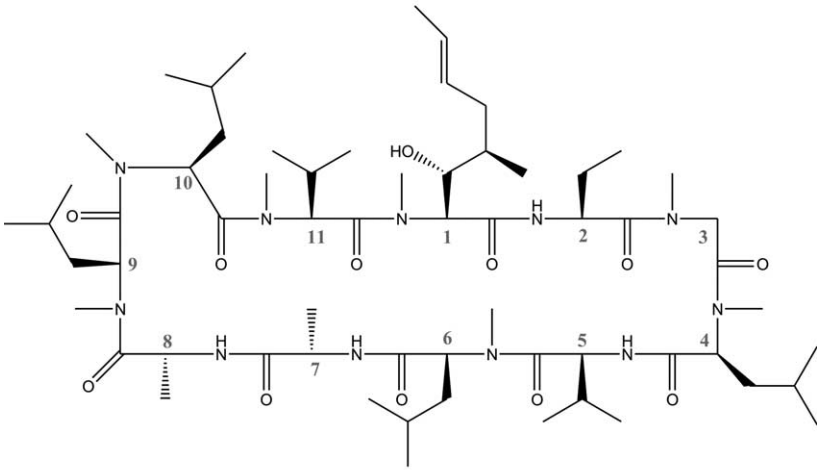

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Scheme 1.

2. Results and discussion

Fig. 1 shows the sensitivity-enhanced spin-edited 2D HSQC–TOCSY pulse schemes designed to measure ^{n}J(XH) and J(HH) coupling constants. Both schemes consist of three independent steps where data can be separately recorded at the different marked a, b, and c times (in each case, the acquisition should be performed by adding the last gradient echo period for proper refocusing). First, an out-and-back ^{1}H-to-X-to-^{1}H transfer based on the regular HSQC–PEP pulse scheme in which the last retro-inept mixing transfer has been modified accordingly in each version to achieve a specific spin-state transfer selection via ^{1}J(XH) (point a). Second, a conventional homonuclear mixing TOCSY process, executed with a DIPSI-2 pulse train, that preserves all proton magnetization components thanks to its isotropic properties. The resulting data acquired at point b afford spin-edited cross-peaks from which the size and the sign of ^{n}J(XH) can be determined if proton is acquired without X decoupling. At this point, the overall duration of the proposed schemes is kept at a minimum length and, therefore, the additional sensitivity T_2 losses with respect to the original sequence can be neglected. Finally, a restricted planar mixing element (see marked box in schemes of Fig. 1) can be optionally inserted at the end of the sequence to measure J(HH) under the same experimental conditions described above if data is acquired with X-decoupling (point c). Although this additional double retro-INEPT sequence has a overall duration of $1/J$(XH) ms, about 7 ms for CH and 11 ms for NH, this is not a critical factor in small and medium-size molecules. As major features of the proposed spin-edited HSQC–TOCSY J(CH) and J(HH) versions, heteronuclear coupling can evolve freely in the indirect t_1 and in the acquisition t_2 periods and maximum sensitivity is obtained by using the gradient-enhanced coherence-order echo/anti-echo protocol for both data acquisition and data processing. The maximum sensitivity gains traditionally associated to the PEP protocol can be fully reached because the interpulse delays in all INEPT clusters can be finely adjusted for IS spin systems.

For a stepwise understanding of each involved transfer, Tables 1 and 2 summarize the different magnetization components present in each time point of the two proposed approaches. All available coherence transfer pathways and the particular spin-state labelling are completely preserved during the entire pulse sequence. The most important aspect of the pulse sequence of Fig. 1A is the last 90°(X) pulse (marked with an asterisk) placed at the end of the double retro-INEPT block.

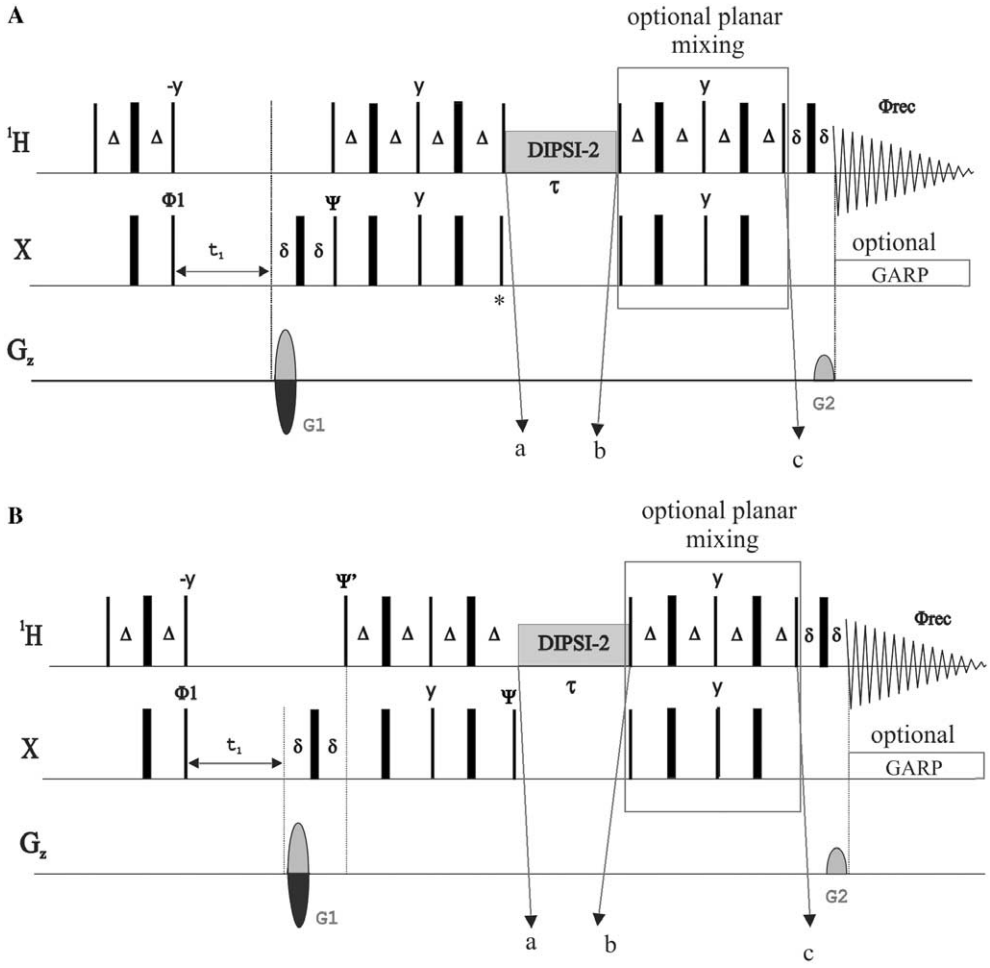

Fig. 1. Pulse schemes of the sensitivity-enhanced 2D (A) E.COSY-type and (B) TROSY-type HSQC–TOCSY experiments for the determination of heteronuclear ^{1}H–X and homonuclear ^{1}H–^{1}H (with the optional planar mixing box and the optional X GARP decoupling during acquisition) coupling constants. After the TOCSY transfer of duration τ (point b) the experiment is suitable to extract ^nJ(HX) ($n > 1$) coupling constants from protonated X nuclei. At point c, the experiment is suitable for the measurement of J(HH) in IS spin systems. Thin and thick vertical lines represents hard 90° and 180° pulses, respectively, with phase x unless otherwise indicated. When the 90°(X) pulse marked with an asterisk (*) is not applied, the pulse sequence becomes a conventional HSQC–PEP experiment. A minimum basic two-phase cycle is applied in which the first X 90° pulse ($\phi_1 = x, -x$) and the receiver ($\phi_{rec} = x, -x$) are inverted each alternate scan. The phases Ψ and Ψ' are set to x and y, respectively. The delay Δ is set to $1/(4*^1J_{XH})$. Pulsed field gradients of duration δ are inserted as indicated for coherence transfer pathway selection and the G1:G2 strength ratio is set to γ_H/γ_X:1. The echo/anti-echo gradient-coherence selection protocol is applied in which for each t_1 increment, pure N- and P-type data are collected alternately by inverting the polarity of G1 gradient with the pulse together with phases Ψ and Ψ', stored separately and then combined accordingly to generate pure absorptive line shapes in both dimensions.

Table 1
Product operator components present at different times of the E.COSY-type HSQC–TOCSY (Fig. 1A) following the evolution time t_1 (for $\Delta = 1/(4*^1J_{XH})$)

Term No.	t_1[a]	$90_x(^1H)\ 90_x(X)$	2Δ	$90_y(^1H)\ 90_y(X)$	2Δ	$90_x(^1H)$	$90_x(X)$[b] (time a)	TOCSY (time b)
I	$H_{1z}S_x \cos(\Omega t_1) \cos(\pi J t_1)$	$H_{1y}S_x$	$H_{1y}S_x$	$H_{1y}S_z$	H_{1x}	H_{1x}	H_{1x}	H_{2x}
II	$S_y \cos(\Omega t_1) \sin(\pi J t_1)$	S_z	S_z	S_x	$H_{1z}S_y$	$H_{1y}S_y$	$H_{1y}S_z$	$H_{2y}S_z$
III	$H_{1z}S_y \sin(\Omega t_1) \cos(\pi J t_1)$	$H_{1y}S_z$	H_{1x}	H_{1z}	H_{1z}	H_{1y}	H_{1y}	H_{2y}
IV	$S_x \sin(\Omega t_1) \sin(\pi J t_1)$	S_x	$H_{1z}S_y$	$H_{1x}S_y$	$H_{1x}S_y$	$H_{1x}S_y$	$H_{1x}S_z$	$H_{2x}S_z$

[a] Trigonometric factors are shown only on this column.
[b] This pulse is optional. If applied, an E.COSY coupling pattern is obtained. See text and Fig. 1A for more details.

If this pulse is omitted, only the terms I and III will be detected because the other terms II and IV would represent non-observable multiple-quantum coherences (see Table 1). Thus, this option is equivalent to a regular HSQC–PEP experiment recorded with full coupling in both dimensions and it generates a typical fully coupled

Table 2
Product operator components present at different times of the TROSY-type HSQC–TOCSY (Fig. 1B) following the evolution time t_1 (for $\Delta = 1/(4^{*1}J_{XH})$)

Term No.	t_1[a]	$90_y(^1H)$	2Δ	$90_x(^1H)$ $90_y(X)$	2Δ	$90_x(X)$ (time a)	TOCSY (time b)
I	$H_{1z}S_x \cos(\Omega t_1) \cos(\pi J t_1)$	$H_{1x}S_x$	$H_{1x}S_x$	$H_{1x}S_z$	H_{1y}	H_{1y}	H_{2y}
II	$S_y \cos(\Omega t_1) \sin(\pi J t_1)$	S_y	$H_{1z}S_x$	$H_{1y}S_z$	H_{1x}	H_{1x}	H_{2x}
III	$H_{1z}S_y \sin(\Omega t_1) \cos(\pi J t_1)$	$H_{1x}S_y$	$H_{1x}S_y$	$H_{1x}S_y$	$H_{1x}S_y$	$H_{1x}S_z$	$H_{2x}S_z$
IV	$S_x \sin(\Omega t_1) \sin(\pi J t_1)$	S_x	$H_{1z}S_y$	$H_{1y}S_y$	$H_{1y}S_y$	$H_{1y}S_z$	$H_{2y}S_z$

[a] Trigonometric factors are shown only on this column.

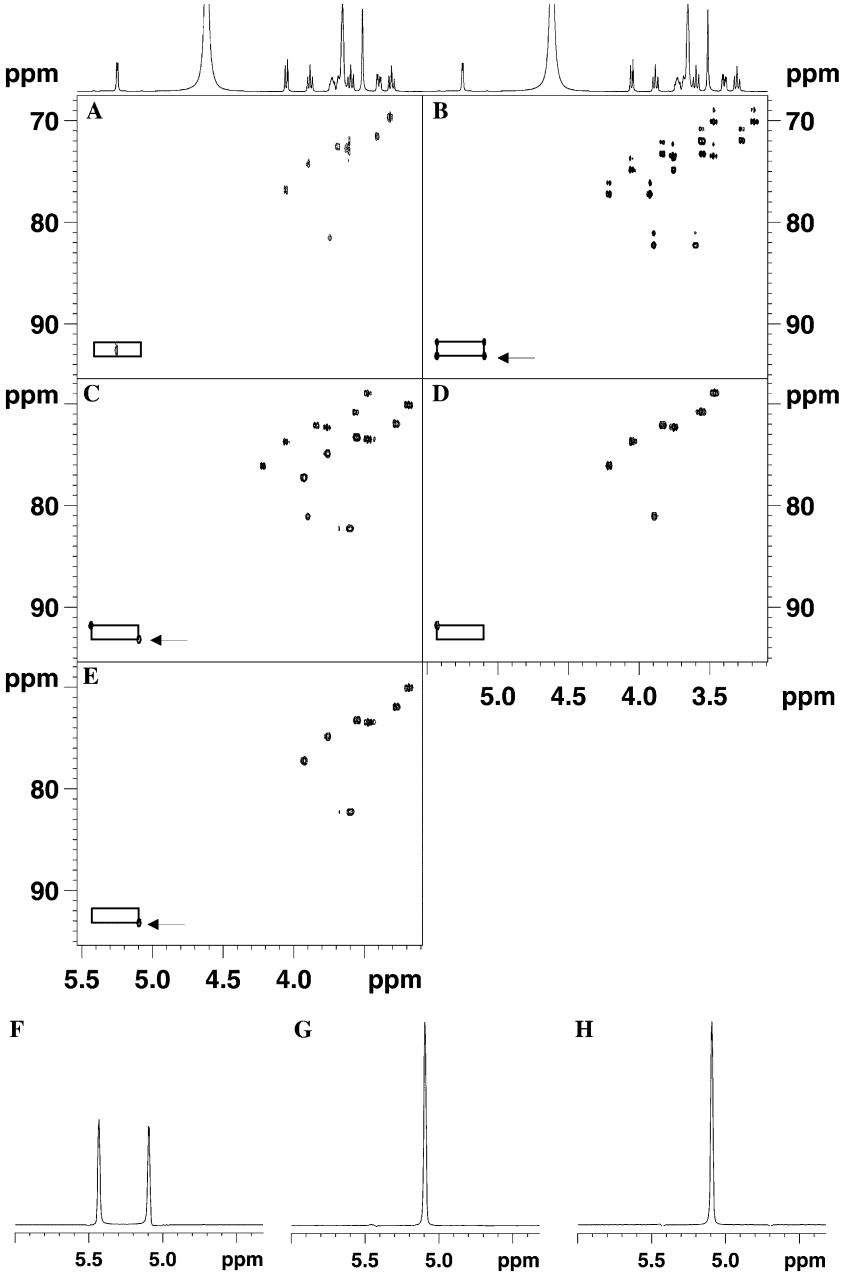

Fig. 2. 2D ^1H–^{13}C HSQC spectra of **1** recorded at time point a using the pulse sequences of Figs. 1A and B: (A) fully decoupled and (B) four-component coupling pattern spectra acquired with pulse sequence 1A without the last editing 90°(X) marked with an asterisk; (C) two-component E.COSY coupling pattern acquired exactly as (B) but adding the marked editing 90°(X) pulse; (D–E) Single-component TROSY-type spectra acquired with $\Psi = y/\Psi' = x$ and $\Psi = -y/\Psi' = -x$, respectively, using the sequence Fig. 1B. (F–H) 1D slices were taken from spectra of Fig. 2B (fully coupled), 2C (E.COSY), and 2E (TROSY), respectively, to show sensitivity comparisons.

four-component multiplet with an unwanted 50% of sensitivity loss (see Fig. 2B and the corresponding 1D trace in Fig. 2F). These two observable terms (I and III) correspond to antiphase-to-in-phase transfer and this is the reason why the widely accepted HSQC–PEP pulse scheme offer maximum attainable sensitivity when acquired under decoupling conditions in both dimensions (Fig. 2A). If the editing 90°(X) pulse is applied, as shown in Fig. 1A, all four terms become observables and a full spin-state-selective to spin-state-selective coherence transfer takes place. The result is an interesting simplified two-component E.COSY-like multiplet pattern for the direct auto-correlation peaks with maximum sensitivity ratios (see Fig. 2C and the corresponding 1D trace in Fig. 2G).

Similar magnetization components are also obtained when analyzing the TROSY-type HSQC version (Fig. 1B). However, a detailed product operator analysis shows that the resulting components come from different pathways (Table 2) and can be understood as the addition of two independent spin-state-selection to in-phase transfer (terms I + II) and spin-state-selection to anti-phase transfer (terms III + IV), respectively. The result is a double coherence transfer providing single-component TROSY or anti-TROSY cross-peaks in separate spectra, as a function of the Ψ and Ψ' phases (Figs. 2D and E, respectively, and the corresponding 1D trace in Fig. 2H). Improved sensitivity in TROSY/anti-TROSY spectra can be also achieved if separate in-phase and anti-phase E.COSY spectra are recorded by introducing a S^3E element just prior to the t_1 period in Fig. 1A and with further addition/subtraction data processing. Alternatively, it is also possible to achieve spin-selection only in the F2 dimension by inserting a 180° X pulse in the middle of the t_1 period (not shown in scheme of Fig. 1B). In this way, only the terms I and III in Table 2 should be considered and both contribute to the detected signal. This approach affords sensitivity-enhanced F2-spin-selected 2D spectra as described early for other related experiments [8,13,14,17].

Once this clean spin-edited selection procedure is achieved by any of the described E.COSY-type or TROSY-type blocks (at point a), the isotropic properties associated to a TOCSY mixing process assures that this spin-editing information is propagated without alteration to other relayed protons (point b). The different ^1H–^{13}C spin-edited HSQC–TOCSY spectra of **1** are shown in Fig. 3. Very clean spectra are obtained with excellent sensitivity and from which accurate $^nJ(CH)$ values can be reliable extracted by direct analysis of multiplet displacements along the detected F2 dimension.

As mentioned above, the major inconvenient of the E.COSY-type HSQC–TOCSY methodology (Fig. 3A) is the increased number of cross-peaks compared to a conventional decoupled spectrum that can complicate the spectral analysis if accidental resonance overlapping occurs. To circumvent this complication, the use of the TROSY/anti-TROSY-like approach can be a very interesting alternative. As theoretically predicted, the sensitivity of each individual TROSY-peak (Figs. 3B and C) is the same as the E.COSY counterpart and, in practical terms, the E.COSY spectrum is the sum of the two separate TROSY spectra. Whereas the main advantage of the E.COSY approach is that coupling constants can be extracted from a single spectrum, the advantages to use separate TROSY approach is clearly appreciated. In addition to avoid problematic overlapping, the requirements for high resolution levels in the indirect F1 dimensions are strongly reduced and this is an important factor when deciding the number of points to be indirectly sampled and, consequently, to fix the

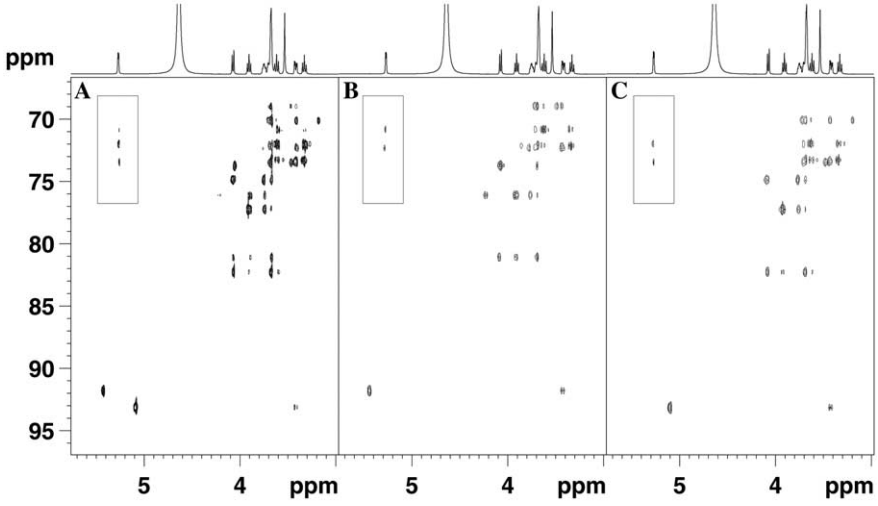

Fig. 3. 2D ^1H–^{13}C spin-edited HSQC–TOCSY spectra of **1**: (A) E.COSY and (B, C) TROSY and anti-TROSY spectra acquired as described in Figs. 2B–D, respectively, but with an additional TOCSY step.

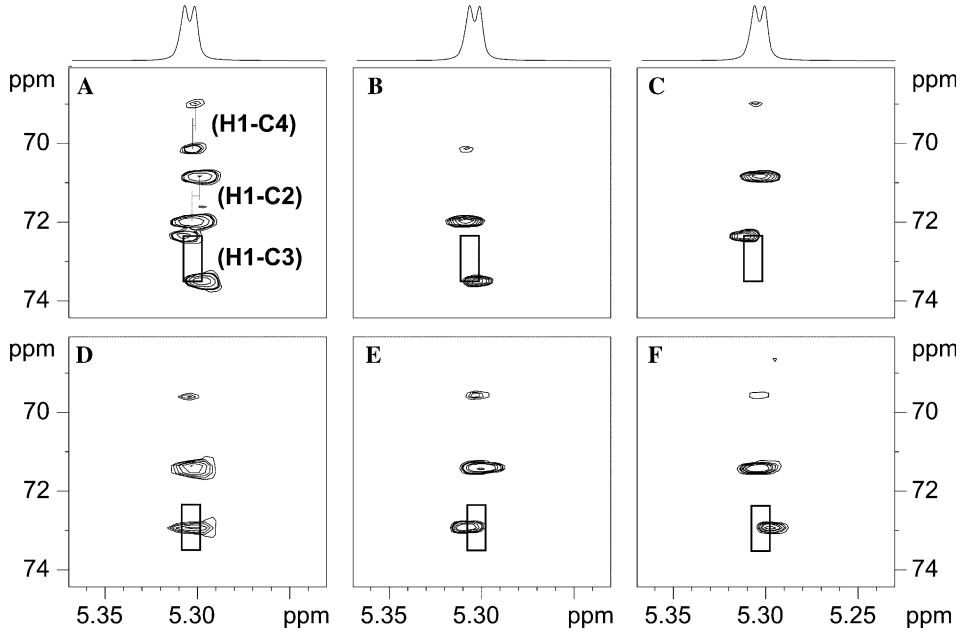

Fig. 4. Different multiplets patterns obtained from several versions of the HSQC–TOCSY-J(CH) experiment of **1** acquired as described in caption of Fig. 3 but with better resolution in the F1 dimension to resolve overlapped cross-peaks. (A) E.COSY (Fig. 1A); (B, C) TROSY/anti-TROSY (Fig. 1B); (D) conventional w1-decoupled (19); (E,F) w_1-decoupled version acquired as spectra B and C, respectively, but inserting a hard 180° ^{13}C pulse in the middle of the t_1 period. For clarity, a reference box selecting the H1–C3 cross-peak highlights the coupling pattern.

overall acquisition time. The positive effect to increase the F1 resolution by a factor of 2 in E.COSY-type spectra (Fig. 4A) and a summary of the different coupling patterns that can be achieved using the different versions of the HSQC–TOCSY experiment are exemplified on the different 2D spectra shown in Fig. 4.

As an extra feature of the proposed spin-edited HSQC–TOCSY schemes, it is also possible to add a second heteronuclear planar mixing element after the TOCSY transfer period which allows for the measurement of homonuclear J(HH) coupling constants without supplementary requisites. This element (marked into a box in Fig. 1) achieves the full re-conversion of the anti-phase $H_{2x,y}X_z$ heteronuclear components to anti-phase $H_{2x,y}H_{1z}$ homonuclear components whereas all in-phase $H_{2x,y}$ terms are unaffected (see Table 3). Because all final terms do not include any X magnetization component, the proton signal is best detected under X decoupling in order to avoid the presence of extra passive in-phase heteronuclear splitting in the resulting multiplet. Fig. 5 shows the highly resolved H1 cross-peak region of the TROSY-type spectra of **1** recorded at times b (HSQC–TOCSY-J(XH)) and c (HSQC–TOCSY-J(HH)) under the same experimental conditions. The analysis of the most congested area, for instance around 3.3–3.8 ppm in carbohydrates as shown in Fig. 6, is a clear example how separate TROSY editing can avoid undesired resonance overlapping in more complex spectra and how the J(HH) version yields much more cleaner spectra because of direct correlation peaks are strongly attenuated and only residual contributions are usually present.

It should be pointed out that the proposed experiments also work finely on other highly interesting heteronuclei, such as ^{15}N. They can be used to extract the sign and the magnitude of ^{n}J(NH) and J(HH) from NH spin systems such those found in peptides. Fig. 7 shows the N–H$^{\alpha}$ and NH–C$^{\alpha}$ areas of 2D E.COSY-type ^{1}H–^{15}N and ^{1}H–^{13}C HSQC–TOCSY J(NH) and J(HH) spectra, respectively, recorded on **2**, in which the, ^{2}J(C$^{\alpha}$–NH) and ^{3}J(NH–H$^{\alpha}$) values can be easily determined. Note that all ^{2}J(N–H$^{\alpha}$) values are positive. On the other hand, the analysis of the aliphatic ^{13}C region in peptides also permits the simultaneous determination of a great number of coupling values, such as ^{2}J(C$^{\alpha}$–H$^{\beta}$),

Table 3
Product operator components present at different times in the optional planar mixing transfer (Figs. 1A and B) after the TOCSY mixing process (for $\Delta = 1/(4*^{1}J_{XH})$)

Term No.	TOCSY (time b)	$90_x(^1H)$ $90_x(X)$	2Δ	$90_y(^1H)$ $90_y(X)$	2Δ	$90_x(^1H)$ (time c)
I	H_{2x}	H_{2x}	H_{2x}	H_{2z}	H_{2z}	H_{2y}
II	$H_{2y}S_z$	$H_{2z}S_y$	$H_{2z}H_{1z}S_x$	$H_{2x}H_{1x}S_z$	$H_{2x}H_{1y}$	$H_{2x}H_{1z}$
III	H_{2y}	H_{2z}	H_{2z}	H_{2x}	H_{2x}	H_{2x}
IV	$H_{2x}S_z$	$H_{2x}S_y$	$H_{2x}H_{1z}S_x$	$H_{2z}H_{1x}S_z$	$H_{2z}H_{1y}$	$H_{2y}H_{1z}$

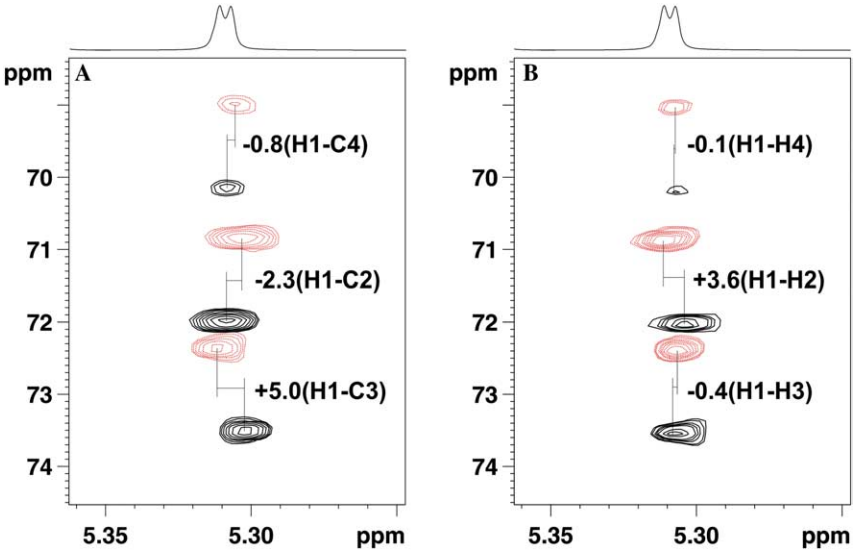

Fig. 5. Part of the TROSY-type HSQC–TOCSY (A) J(CH) and (B) J(HH) spectra of **1** acquired as described in Fig. 4. The measured values are given in Hz with an experimental error of less than 0.6 Hz.

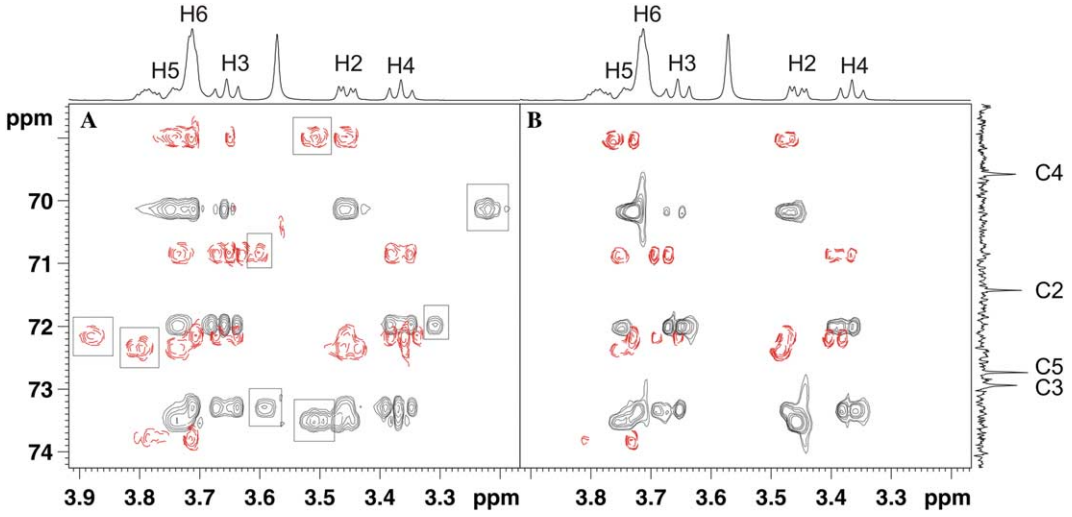

Fig. 6. Part of the separate 2D ^{1}H–^{13}C TROSY (black solid lines)/anti-TROSY (red dashed lines) HSQC–TOCSY correlation spectra of **1** acquired as described in Fig. 5 for the measurement of the sign and the magnitude of (A) ^{n}J(CH) and (B) J(HH) coupling constants, respectively, using the TROSY version (Fig. 1B). To make clear, direct correlation peaks are marked into a box in (A) but they are strongly minimized in (B).

$^{3}J(C^{\alpha}–H^{\gamma})$, $^{4}J(C^{\alpha}–H^{\delta})$, $^{3}J(H^{\alpha}–H^{\beta})$, and $^{4}J(H^{\alpha}–H^{\gamma})$ in addition of the simultaneous confirmation and validation of ^{1}H, ^{13}C, and/or ^{15}N chemical shift assignments (Fig. 8). It is also interesting to note that the magnitude of other homonuclear passive couplings can be simultaneously extracted from the J(HH) version. All upfield/downfield H^{α}–N cross-peaks in Fig. 7D are decoupled with respect to the active $J(H^{\alpha}–NH)$ coupling constant and therefore the $J(H^{\alpha}–H^{\beta})$ coupling constant can be also measured if there is enough resolution. For instance, the H^{α} proton at 4.57 ppm belonging to the val-5 residue shows an apparent triplet about 9.2 Hz in the conventional proton spectrum. Clearly, this pattern is splitted in two very clean doublets in the edited spectra allowing the easy determination of the two different $J(H^{\alpha}–NH)$ and $J(H^{\alpha}–H^{\beta})$ values (see Fig. 9B).

The overall duration of both proposed E.COSY and TROSY approaches are exactly the same, only some pulses and/or phases change, and the possible deleterious effect due to undesired T_2 relaxation upon the addition of the last mixing planar can be neglected for small molecules (compare 1D slices in Fig. 9).

3. Methods and materials

All experiments were performed on a BRUKER DRX-500 spectrometer at 298 K equipped with a

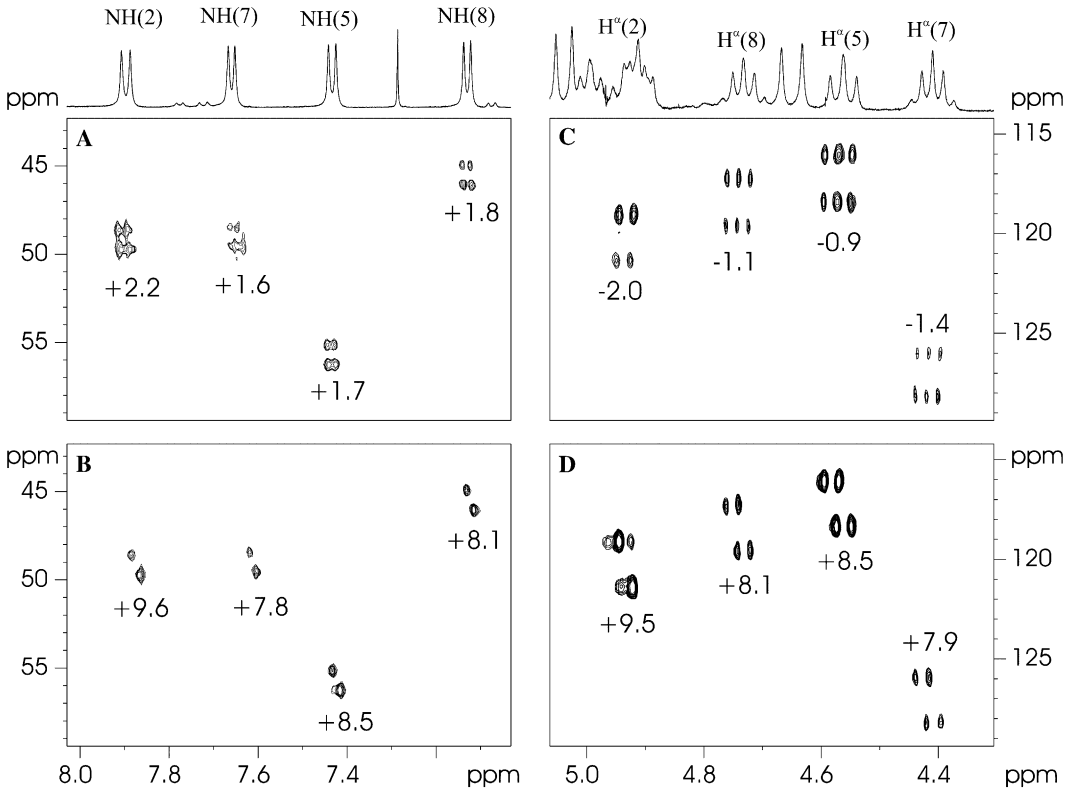

Fig. 7. Expanded (A, B) C^{α}–NH and (C, D) N–H^{α} regions of the 2D ^{1}H–X E.COSY-type HSQC–TOCSY spectra of **2** acquired using the pulse sequences of Fig. 1A for (A, B) X = ^{13}C and (C, D) X = ^{15}N, respectively: (A) measurement of $^{2}J(C^{\alpha}–NH)$ by performing ^{1}H acquisition without ^{13}C decoupling at point b; (B) measurement of $^{3}J(HN–H^{\alpha})$ by performing the ^{1}H acquisition with ^{13}C decoupling at point c; (C) measurement of $^{2}J(N–H^{\alpha})$ by performing ^{1}H acquisition without ^{15}N decoupling at point b; (D) measurement of $^{3}J(HN–H^{\alpha})$ by performing the ^{1}H acquisition with ^{15}N decoupling at point c.

5 mm inverse triple-resonance TXI probe incorporating *Z*-gradients. All spectra were acquired and processed with TOPSPIN v1.2 (pulse programs are available on request for this platform). Samples of 0.1 M Sucrose in D_2O, **1**, and 50 mM cyclosporine in $CDCl_3$, **2**, were chosen as model samples. The isotropic homonuclear TOCSY mixing process consists of a 7 kHz DIPSI-2 pulse train in all experiments. All 2D ^{1}H–^{13}C HSQC spectra of Fig. 2 have been recorded with no ^{13}C decoupling during ^{1}H acquisition (except spectrum of Fig. 2A which was decoupled in both dimensions), recycle delay = 1 s, inter-pulse delay $\Delta(=1/4*^{1}J(CH))$ set to 1.75 ms, number of t_1 increments = 128, number of data points in t_2 = 1K, and number of scans = 2. Sine-shaped pulsed field gradients of 1 ms of duration were set to a 80:20.1 ratio. The spectral windows in both dimensions were 2000 Hz (F2) and 7500 Hz (F1), respectively. Data were processed applying a zero filling in the F1 dimension and a 90°-shifted qsine window function in both dimensions, consisting of 1024*1024 points. The total experiment time was 10 min for each 2D spectrum. The 2D ^{1}H–^{13}C HSQC–TOCSY spectra of Fig. 3 were recorded and processed exactly as described in caption of Fig. 2 but with an additional 40 ms DIPSI-2 mixing time and 4 scans for each one of the 128 t_1 increments. The total experiment time was 20 min for each 2D spectrum. The 2D ^{1}H–^{13}C HSQC–TOCSY spectra of Fig. 4 were recorded as described before but using 256 t_1 increments of 2K data points each one to increase the F1 resolution. The total experiment time was about 40 min for each 2D spectrum.

Experimental conditions for ^{1}H–^{13}C HSQC–TOCSY spectra on **2** (Figs. 7A, B and 8A, B): recycle delay = 1 s, inter-pulse period Δ set to 1.75 ms ($1/4*^{1}J(CH)$), number of t_1 increments = 256, number of data points in t_2 = 4K, number of scans = 64. Gradients of 1 ms of duration are set to a 80:20.1 ratio. Spectral widths: 5000 Hz (F2) and 12,600 Hz (F1). The overall acquisition time was about 6 h for each spectrum. Experimental conditions for ^{1}H–^{15}N HSQC–TOCSY spectra on **2** (Figs. 7C and D): recycle delay = 1 s, inter-pulse period Δ set to 2.7 ms ($1/4*^{1}J(NH)$), number of t_1 increments = 64, number of data points in t_2 = 4K, number of scans = 160. Gradients of 1 ms of duration are set to a 80:8.1 ratio. Spectral widths: 4000 Hz (F2) and 1600 Hz (F1). The overall acquisition time was about 4 h for each spectrum. The duration of the TOCSY process in all these experiments were of 40 ms.

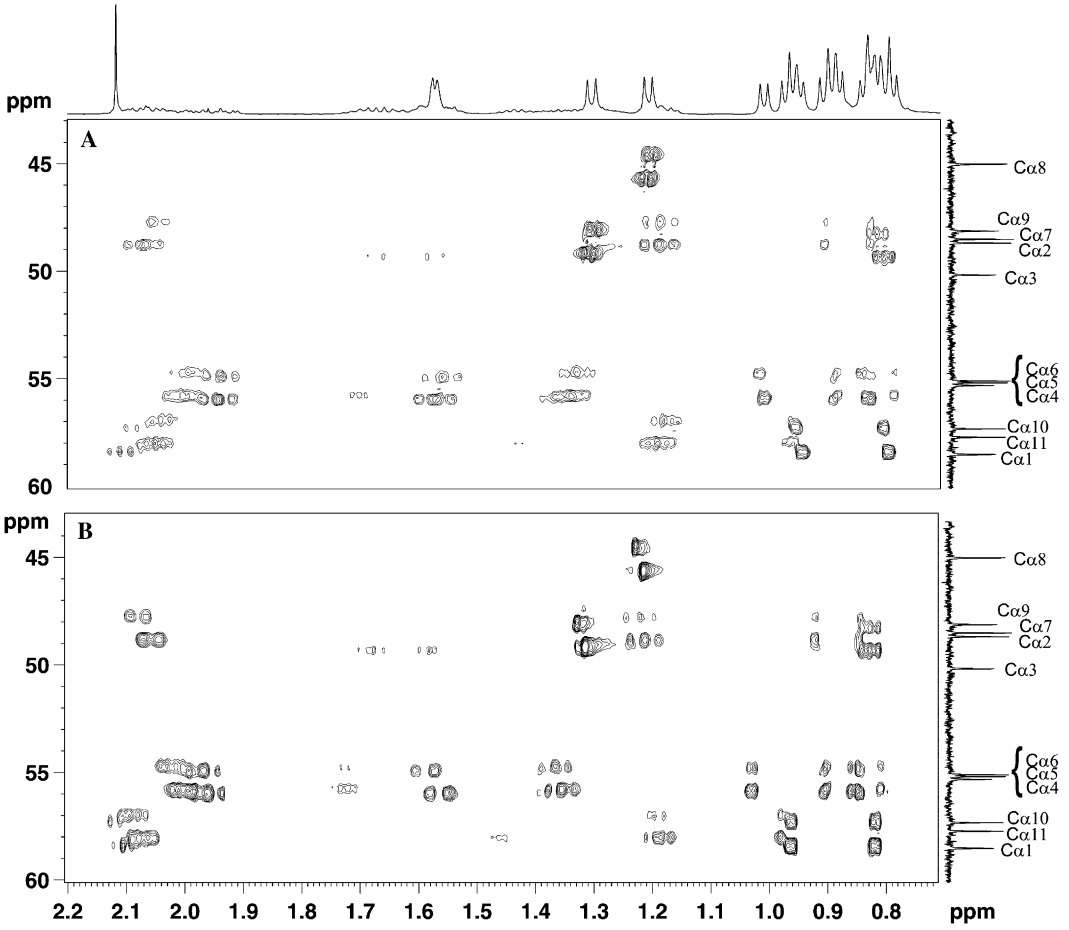

Fig. 8. Expansions plots corresponding to the aliphatic C^{α} region of the ^{1}H–^{13}C E.COSY HSQC–TOCSY (A) J(CH) and (B) J(HH) spectra of **2** acquired as described for Figs. 7A and B. From these spectra, the sign and the size of many different heteronuclear ($^{2}J(C^{\alpha}–H^{\beta})$, $^{3}J(C^{\alpha}–H^{\gamma})$ and $^{4}J(C^{\alpha}–H^{\delta})$) and homonuclear ($^{3}J(H^{\alpha}–H^{\beta})$ and $^{4}J(H^{\alpha}–H^{\gamma})$) coupling constants can be directly extracted with excellent accuracy.

4. Conclusions

In summary, a different number of long-range heteronuclear CH (and NH) and homonuclear HH coupling constants can be accurately measured from spin-edited sensitivity-enhanced HSQC–TOCSY spectra in molecules having a great number of IS spin systems, as demonstrated for peptides and carbohydrates. Their simplicity, the optimal sensitivity, the excellent high F1 dispersion and the extraction of both the magnitude and the sign of homonuclear and heteronuclear coupling constants values make of these methodologies interesting tools to be applied in a variety of conditions and increase the number of techniques currently available for their measurements. Whereas E.COSY-like spectra allow such measurements from a single spectrum, the separate acquisition of each one of these E.COSY multiplets offer improved resolution and dispersion features making this approach highly interesting where cross-peak overlapping can obscure such determinations.

We believe the proposed techniques can find widespread application in many different topics and, in particular, in J-based configurational analysis of acyclic compounds [25], natural products [26], and also for carbohydrates, as recently exemplified for the differentiation of aldohexopyranosyl residues from geminal ^{2}J(CH) values [27]. We are also interested at which extension the long-range J(NH) coupling constants could be included in the conformational analysis of nitrogen containing compounds and more studies on this topic is under investigation. In addition, what is especially beneficial in these experiments is that also the signs of coupling constants could be determined. This would be especially valuable for experiments in aligned media, where the signs of dipolar coupling constants are unpredictable. This particular application would however require a mixing sequence that would work on dipolar couplings. Because HSQC pulse timing is the basis of many multidimensional NMR experiments, the substitution of the traditional double-retro-INEPT PEP block by the proposed E.COSY and TROSY/anti-TROSY concepts affords a variety of possible general approaches to be applied for the measurement of scalar and residual dipolar couplings at natural

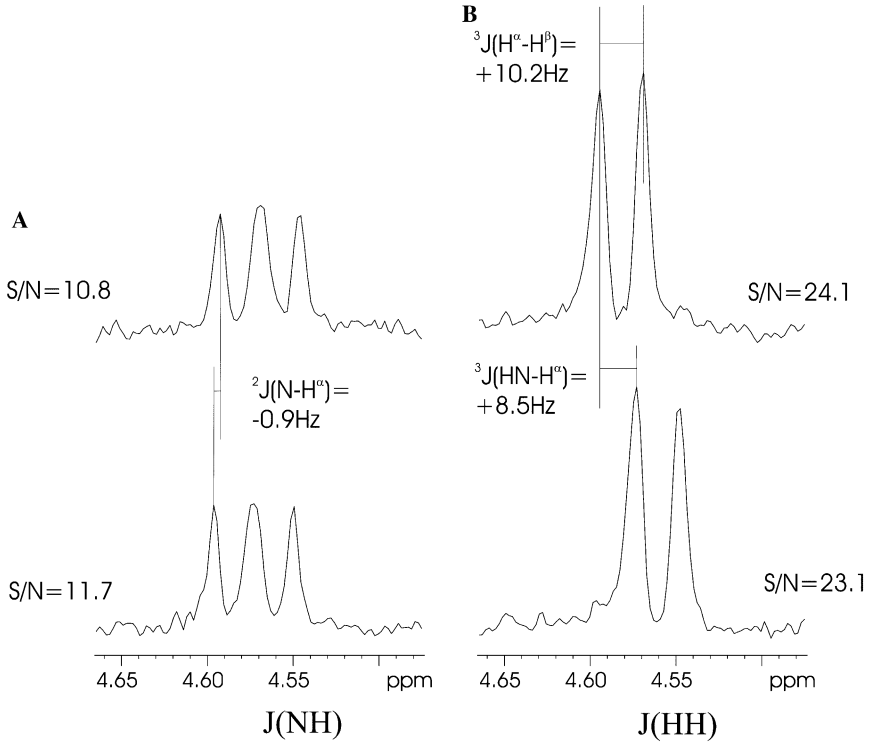

Fig. 9. 1D Slices extracted from Figs. 7C and D belonging to the N(5) amide nitrogen in **2**. It can be clearly observed that the last optional block in sequence 1A practically do not affect the overall sensitivity of the experiment. On the contrary, better relative sensitivity is obtained in the *J*(HH) version (slices B) because data is acquired under ^{15}N GARP decoupling and passive couplings can be also measured from the simplified multiplets. For each 1D row, the experimental signal-to-noise ratio is indicated.

abundance and also for labelled bio-molecules without sacrificing sensitivity and performance [28,29].

Acknowledgments

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