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Improved NMR methods for the direct ^{13}C-satellite-selective excitation in overlapped ^{1}H-NMR spectra

Pau Nolis,[a] Sergio Gil,[a] Juan Félix Espinosa[b] and Teodor Parella[a]*

Improved pulsed-field gradient echo methods are presented and discussed for the direct selective excitation of the ^{13}C-satellite lines in overcrowded ^{1}H NMR spectra of small molecules. Sensitivity enhancements in ^{13}C spin-state selection can be achieved by combining multiple-proton-frequency excitation and Hadamard phase encoding. Several satellite-selective (SATSEL) NMR experiments are proposed and exemplified by measuring the sign and the magnitude of small, long-range proton–carbon coupling constants for ^{1}H resonances showing several levels of signal overlapping.

Keywords: Selective excitation; satellite lines; Hadamard encoding; proton–carbon coupling constants; selective COSY; selective TOCSY; selective NOESY

Introduction

Selective excitation has become a common strategy in any NMR laboratory working on small and medium-sized molecules in order to obtain specific and more precise information in shorter acquisition times. Mainly, frequency-selective 1D version of the traditional COSY, TOCSY, NOESY and ROESY experiments are routinely used with high efficiency and reproducibility for both fast chemical shift assignment and complete structure elucidation.[1–2] The conventional ^{1}H NMR spectrum of an isolated CH spin system shows a strong signal centred to δ(H) with a relative intensity of ~98.9% representing its natural ^{1}H–^{12}C abundance. On the other hand, a tiny doublet with a separation corresponding to ^{1}J(CH) ≈120–160 Hz (each one with a relative intensity of ~0.55%) is always present centred around δ(H) (neglecting isotopic effects), that corresponds to the ~1.1% of ^{13}C magnetization at natural abundance. These small resonances are only visible under optimum sensitivity conditions (extended signal-to-noise averaging, high sample concentrations, use of high magnetic fields or highly sensitive probes) and they are usually omitted when analyzing and interpreting ^{1}H NMR spectra. However, the fundamentals of the inverse NMR spectroscopy rely on the selection of these small signals and important pulse sequences, such as HMQC, HSQC or HMBC, were specifically designed to observe them while removing the strong ^{1}H–^{12}C components with the aid of gradients or phase cycle selection.[3]

The selective perturbation on these satellite signals (hereafter referred to as SATSEL, SATelite SELective excitation) formed also the basics of fundamental concepts in the starting days of multiple-pulse NMR, such as the selective population inversion (SPI) or the selective population transfer (SPT) techniques, which were further developed in the actual heteronuclear polarization transfer experiments. Recently, some interesting SATSEL applications have been described such as the stereochemical and configurational assignment of symmetric compounds,[4] the measurement of the sign and the magnitude of small, long-range proton–carbon coupling constants,[5] and the determination of two-bond ^{13}C/^{12}C isotope effects on ^{1}H chemical shift.[6] These later methods were based on the separate selective excitation of each satellite line of a given ^{1}H resonance by means of a long-, low-power 180° selective pulse in the single pulsed-field-gradient echo (SPFGE) scheme followed by a conventional TOCSY mixing transfer. However, well-isolated satellite lines from other neighbour resonances are needed for unambiguous selectivity, making the method only suitable for simple cases. We propose here several and simple improvements of the SPFGE method to extend its application to more complex situations. First, we apply the concept of multiple-frequency excitation[7–8] to offer important sensitivity gains by appropriate linear data combination according to the established Hadamard phase encoding approach.[9–10] Secondly, several new schemes based on pulsed-field-gradient echoes are proposed to achieve clean SATSEL excitation in the overcrowded regions. These new NMR elements provide simple alternatives to the previously reported ^{13}C-selective 1D NMR methods[11] for achieving spin-state selection in a very straightforward way and on a variety of different overlapping cases. The SATSEL experiments can be used to measure proton–carbon coupling constants or NOEs in overlapped regions without the need to apply sophisticated pulse sequences. Experimental data is provided for different standard organic molecules as shown in scheme 1.

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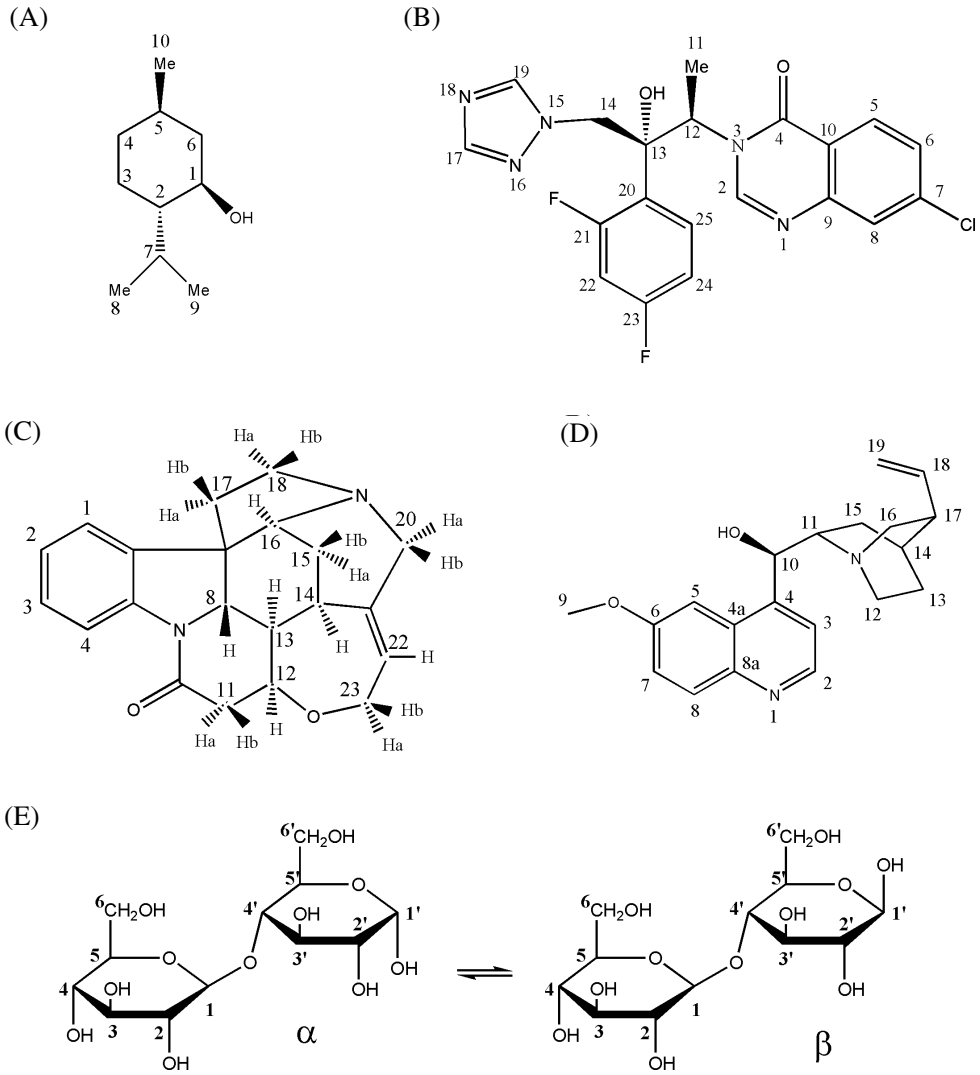

Scheme 1. Chemical structure and numbering of molecules used in this work. (A) Menthol, (B) Albacanazole, (C) Strychnine, (D) Quinine and (E) Lactose.

Results and Discussion

The probability to have resonance overlapping in the ^{1}H spectrum strongly depends on molecular complexity, proton density and signal dispersion of the NMR sample under study. Let us consider two different ^{1}H resonances, A and B, resonating at ν_A and ν_B, respectively, and their corresponding ^{13}C satellites α(A), β(A), α(B), and β(B) appearing at $\nu_A \pm J$(CH)/2 and $\nu_B \pm J$(CH)/2 (for simplicity, small ^{13}C/^{12}C isotope effects are neglected). Basically, several overlapping situations can arise depending on the ^{1}H chemical shift differences between A and B nuclei ($\Delta\nu_{A-B} = |\nu_A - \nu_B|$), as schematically represented in Fig. 1.

Case A: $|\nu_A - \nu_B| >> J$(CH)

The simplest situation for SATSEL excitation is a well-isolated CH spin system (Fig. 1, Case A) in which the central and the satellite lines appear well differentiated from each other and from other resonances. In this case, the original SPFGE pulse scheme (Fig. 2A) based on the conventional gradient-180°sel-gradient block[12–14] was chosen as SATSEL excitation method. The SPFGE block can be used for different purposes: selective excitation of the central ^{1}H–^{12}C signal (using a very selective pulse applied on resonance at ν_A), individual selective excitation on each ^{1}H–^{13}C component (using the same selective pulse but applied at $\nu_A + J/2$ or $\nu_A - J/2$), or simultaneous selection of both ^{1}H–^{12}C and ^{1}H–^{13}C magnetization components using a less selective pulse covering at least $\pm$ 100 Hz. Assuming a typical ^{1}J(CH) value range of 140 $\pm$ 20 Hz for aliphatic, olefinic and aromatic CH spin systems, the satellite lines are found around 70 $\pm$ 10 Hz, with respect to the central line at ν_A. Considering a proton multiplet width of $\pm$ 20 Hz, for instance, a 60 ms Gaussian-shaped pulse applied on resonance on each satellite line is enough to perform clean SATSEL line excitation without affecting the main ^{1}H–^{12}C peak in a 500 MHz spectrometer.[5] The SPFGE block is the initial element of the frequency-selective 1D experiments and therefore, any combination of the proposed SATSEL–SPFGE schemes presented in Fig. 2A–C with the conventional homonuclear mixing blocks (Fig. 2D–F) is feasible. These new experiments will be denoted as 1D SATSEL–COSY, SATSEL–TOCSY and SATSEL–NOESY. Other relevant mixing blocks such as multiple-step RELAY or ROESY (not shown in Fig. 2) would produce equivalent experiments.

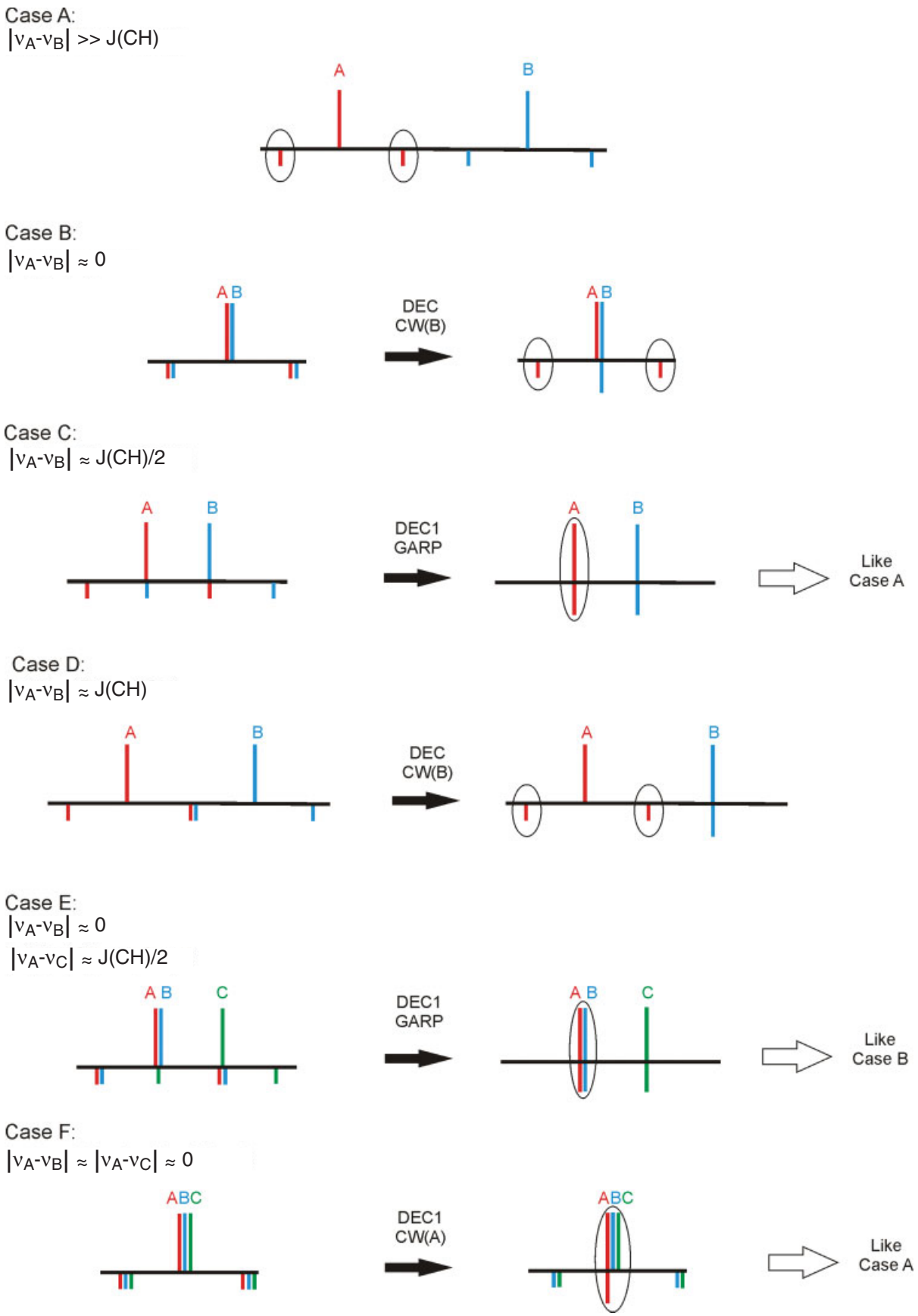

Figure 1. Possible overlapping problems when performing SATSEL excitation. For clarity, $^{1}H-^{12}C$ components are represented as intense positive lines, the satellites $^{1}H-^{13}C$ components as small negative lines and each proton is represented by a different colour. The lines excited by the selective ^{1}H pulse are encircled. In all the cases, satellite lines of spin A (red colour) are selectively excited with the SATSEL pulse schemes as described in Fig. 2.

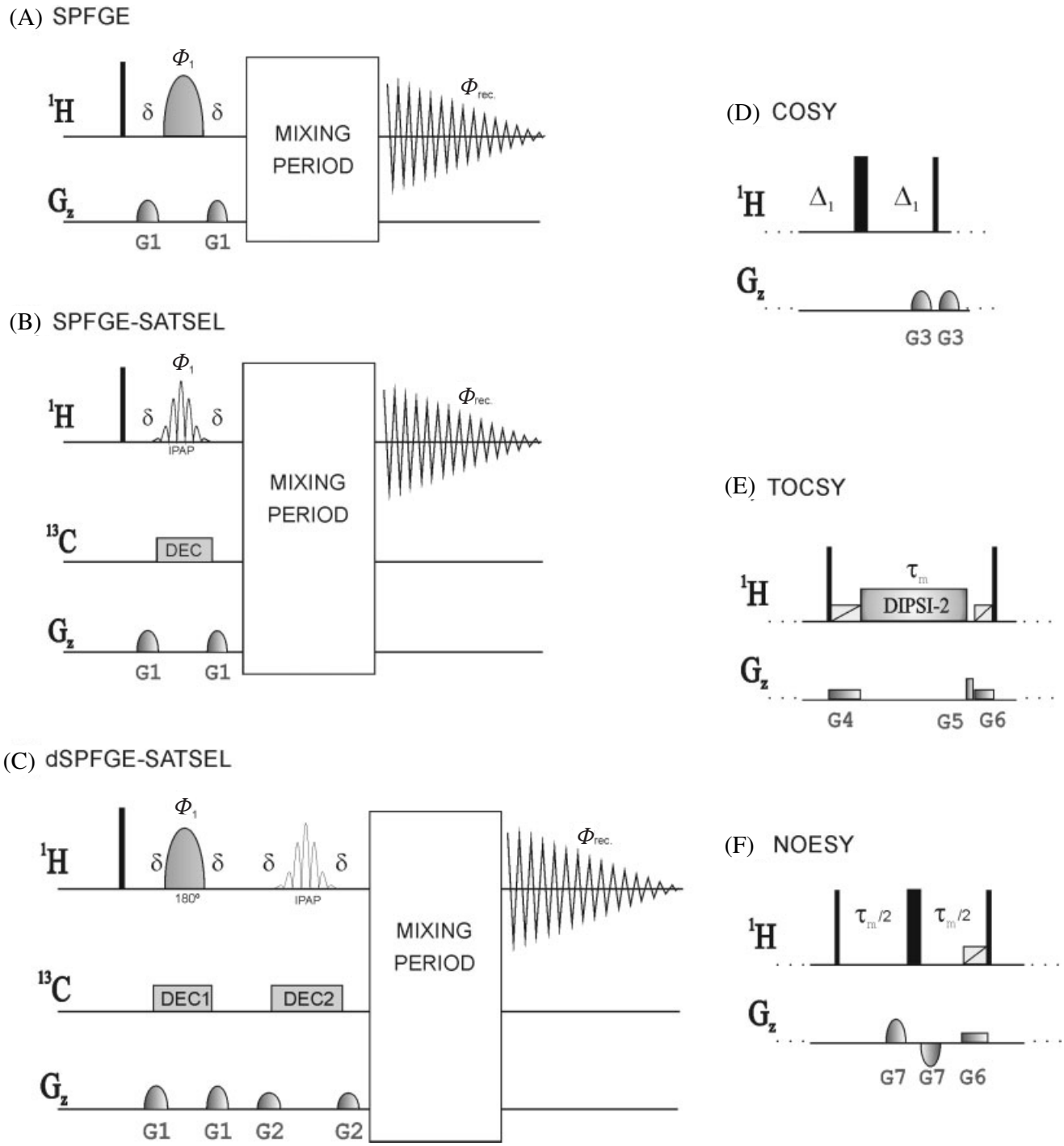

Figure 2. General pulse schemes to record SATSEL experiments. (A) Standard SPFGE block comprised of an initial hard 90° ^{1}H pulse to excite all the proton resonances followed by the (gradient - selective 180° ^{1}H pulse – gradient) sandwich that retained only the magnetization of the chosen proton. (B) Modified SPFGE–SATSEL sequence to use a dual-frequency selective 180° pulse applied on-resonance at ν_A and exciting off-resonance at $\nu_A \pm J/2$ to generate IP- or AP-SATSEL excitation. Optional CW on ^{13}C allows solving different types of satellite lines overlapping (Fig. 2, cases B and C). (C) Modified double-SPFGE (dSPFGE–SATSEL) sequence to perform satellite-selective excitation in high overlapped systems. In the first echo, a standard Gaussian-shaped pulse is applied on resonance at ν_A, whereas a dual-frequency pulse is used to achieve IP/AP excitation during the second echo. The pulse sequence can solve different overlapping cases depending on the application and type of ^{13}C decoupling (DEC1 and DEC2) during the selective pulses (Fig. 2, cases D–F). 1D SATSEL experiments can be designed by inserting a homonuclear mixing block [(D) COSY, (E) TOCSY or (F) NOESY] between any SPFGE block (A, B or C) and the proton detection period. A basic EXORCYCLE phase cycle step is implemented in all the experiments: $\phi_1 = x, y, -x, -y$ and $\phi_{rec} = x, -x$. More experimental details are described in the experimental section.

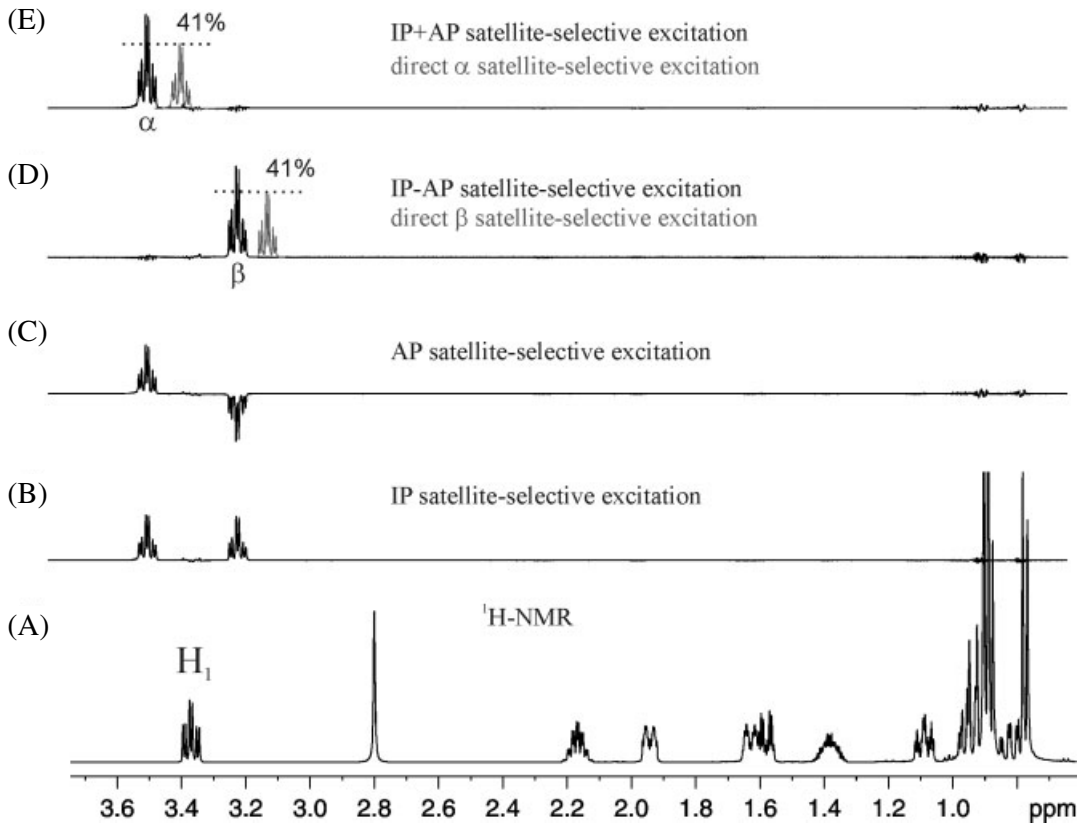

Figure 3. Case A example. (A) ^{1}H-NMR spectrum of menthol at 500 MHz. (B–C) IP- and AP-SATSEL selection of H_1 satellites using pulse scheme of Fig .2B without ^{13}C decoupling (8 transients each spectrum). (D–E) Resulting SATSEL spectra after proper combination of the FIDs corresponding to spectra B and C. (D) IP + AP and (E) IP – AP. This strategy affords a sensitivity gain per time unit of 41% when compared to the individual single-frequency direct excitation (red lines shifted 0.5 ppm right; 8 transients each one) using the conventional SPFGE sequence of Fig. 2A.

In terms of sensitivity, it should be more efficient to apply multiple-frequency pulses for simultaneous satellite excitation, combined with proper spin-state selection editing and Hadamard phase encoding strategies (SPFGE–SATSEL, Fig. 2B). In this way, two different data sets (referred as IP, in-phase, and AP, anti-phase modulation) are separately recorded with phase-modulated dual-frequency excitation simultaneously on both the satellite lines without affecting the strong ^{1}H–^{12}C central peak using a shifted laminar pulse.[8] Proper data combination (IP ± AP) affords the corresponding edited α (IP + AP) and β (IP – AP) satellite selection. As predicted theoretically, a sensitivity enhancement per time unit about 41% can be achieved individually for each satellite line excitation when compared with the individual satellite excitation in a two-site excitation. Experimental data performed at the isolated H_1 of menthol show an excellent agreement between theory and practice (Fig. 3). As a general trend and for automation mode, SATSEL excitation can be extended to *N*-different resonances (excitation of 2*N*-different sites) with a sensitivity enhancement proportional to sqr(2*N*). In the case of four-site SATSEL excitation applied on two different resonances (four different satellite line excitation) would produce a relative sensitivity gain around 100% (Fig. 4).

The sensitivity enhancements associated to multiple-frequency SATSEL excitation can be combined with the advantages to use any homonuclear mixing process just before acquisition. For instance, the accurate measurement of the sign and magnitude of long-range proton–carbon coupling constants, ^{n}J(CH), can be easily performed by measuring the relative frequency shift between the corresponding α and β in-phase multiplets in SATSEL–TOCSY spectra (Fig. 5F–G). In related SATSEL–COSY (Fig. 5B–C) and/or SATSEL–RELAY (Fig. 5D–E) spectra, simultaneous measurement of J(HH) (anti-phase pattern), ^{2}J(CH) and ^{3}J(CH) could be also performed. The same results can be extrapolated to a four-site SATSEL–TOCSY experiment, as shown in the case of the simultaneous satellite excitation of both anomeric resonances in lactose (Fig. 6).

Case B: $|\nu_A - \nu_B| \approx 0$

To resolve accidental ^{1}H–^{12}C overlapping of two resonances in SATSEL experiments (case B in Fig. 1), the application of selective ^{13}C decoupling during the 180° proton pulse in the SPFGE scheme is proposed to collapse the satellite lines of one of the involved resonances. While broadband ^{13}C decoupling leads to collapse of all proton satellite resonances of the spectrum, selective CW decoupling on a given ^{13}C resonance affords this for a single ^{1}H resonance, although prior knowledge of the corresponding ^{13}C frequency is required in this case. Therefore, a simple solution should be the application of the SATSEL–SPFGE pulse scheme with a two-frequency shaped pulse applied on proton A, while CW is applied on $^{13}C_B$ (Fig. 2B). By doing this, the satellite lines of B signal collapse to ν_B and satellite lines of proton A can be selectively excited. In order to demonstrate the efficiency of this strategy in fully overlapped signals, experimental verification has been done in albacanazole near 6.8 ppm at 600 MHz (Fig. 7). The

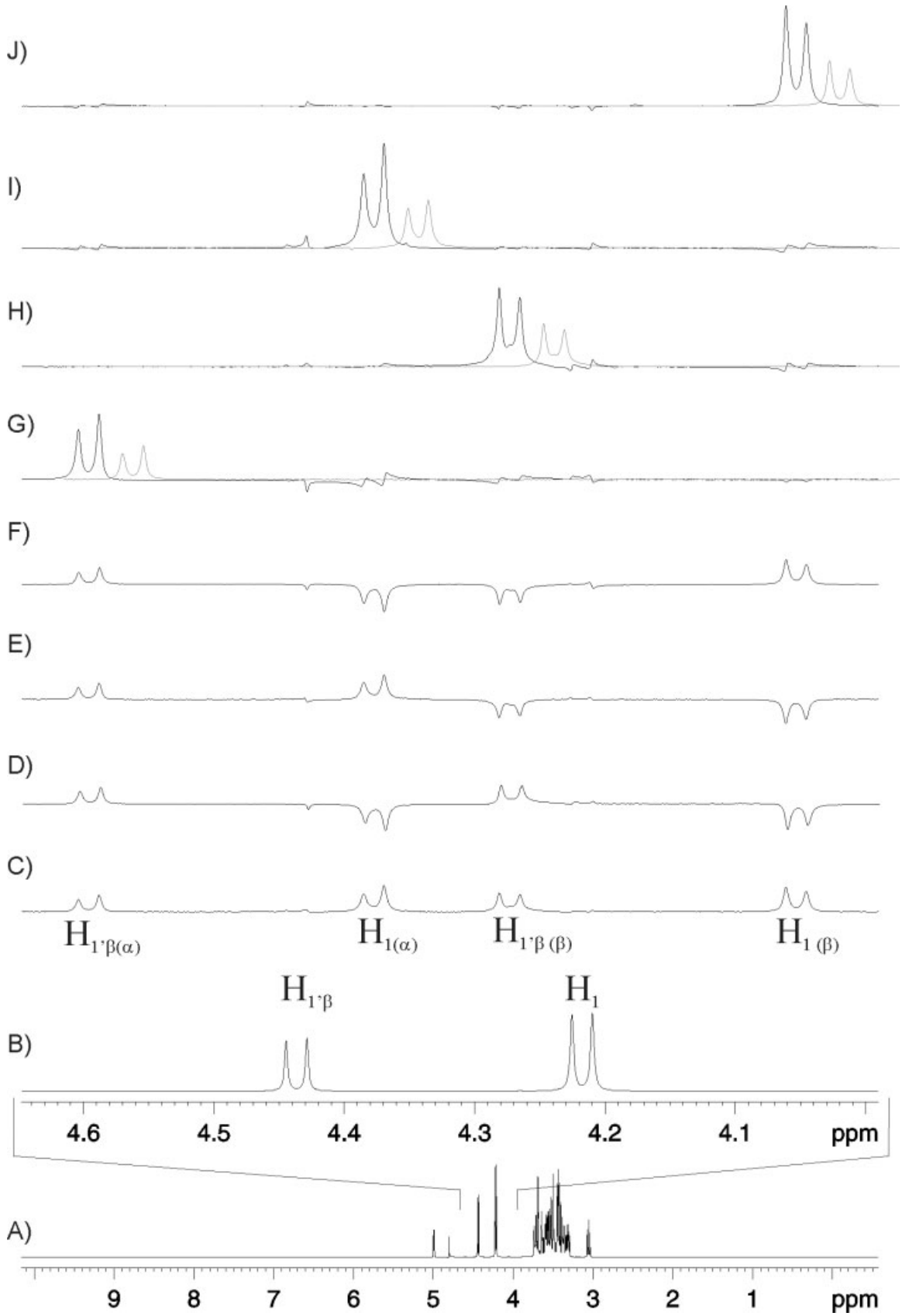

Figure 4. (A) ^{1}H-NMR spectrum of lactose in D_2O at 500 MHz. (B) Expanded area of the anomeric proton regions. (C–F) Example of a four-site SATSEL excitation (32 transients each spectrum) using Hadamard phase encoding. Individual SATSEL spectra can be obtained after proper linear combination: (G) C + D + E + F; (H) C + D – E – F; (I) C – D + E–F and J = C – D – E + F. These spectra are equivalent to a separate 128 transient acquisition. Thus, experimental sensitivity gains about 100% are achieved when compared to the conventional single-frequency excitation of 32 transients each (red lines shifted 0.25 ppm right).

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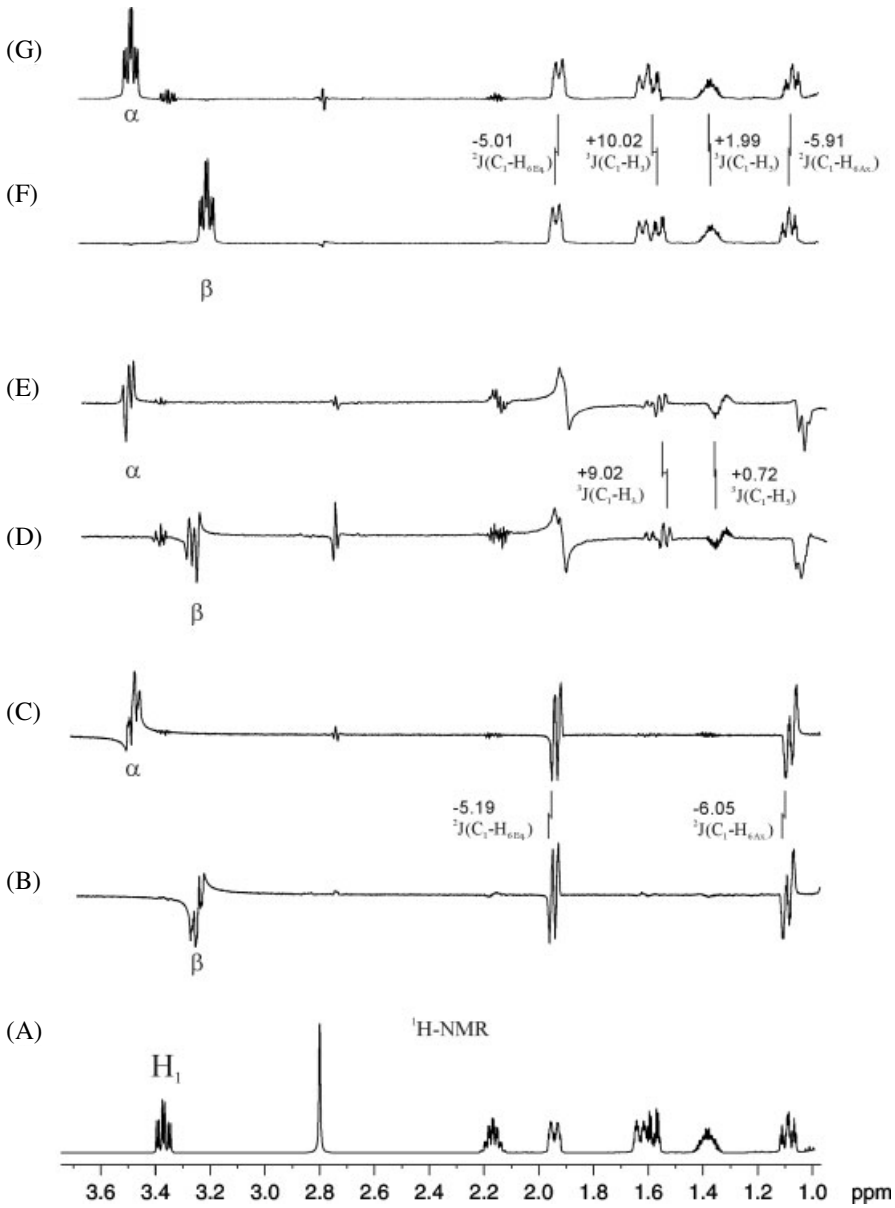

Figure 5. (A) Conventional ^{1}H-NMR spectrum of menthol. (B and C) SATSEL–COSY, (D and E) SATSEL–RELAY and (F and G) SATSEL–TOCSY spectra after SATSEL excitation of H_1 of menthol (at 500 MHz), as described in Fig. 3. A total number of 256 transients were accumulated (128 to obtain IP data and 128 to obtain AP data) and further processed conveniently. Both sign and magnitude of the corresponding long-range $^{n}J_{CH}$ values can be easily extracted by frequency displacement between α- and β-satellite data.

usefulness of the corresponding SATSEL–NOESY spectra for the unequivocally assignment of H22 and H24 proton resonances is also shown.

Case C: $|\nu_A - \nu_B| \approx J$ (CH)/2

In the case of overlapping of a strong ^{1}H–^{12}C signal and a ^{13}C-satellite of another signal (case C in Fig. 1), SATSEL excitation can be achieved by a two-step procedure following the double-SPFGE sequence of Fig. 2C (referred as dSPFGE–SATSEL experiment). Basically, the pulse sequence consists of two independent SPFGE blocks, one involving a conventional single-frequency selective 180° pulse and the other a 180° dual-frequency SATSEL pulse. During the first SPFGE block, a conventional Gaussian-shaped pulse is selectively applied on ν_A under broadband GARP ^{13}C decoupling. In this way, all satellite lines collapse with their strong ^{1}H–^{12}C component so that only resonances belonging to proton A are selectively excited. During the second echo, a two-frequency SATSEL pulse is applied on the satellite lines of spin A without ^{13}C decoupling (DEC = off) in order to distinguish between ^{1}H–^{12}C and ^{1}H–^{13}C components (similar as described in case A). An example is shown in Fig. 8C–F, in which the H20b(α) resonance overlaps with the main H8 resonance and H8(β) overlaps with H20b in strychnine (Fig. 8B).

Case D: $|\nu_A - \nu_B| \approx J$(CH)

When the difference between the chemical shifts of protons A and B is similar to the ^{1}J(CH) coupling value (Fig. 1, case D), the satellite line α(A) can partially or fully overlap with the satellite line

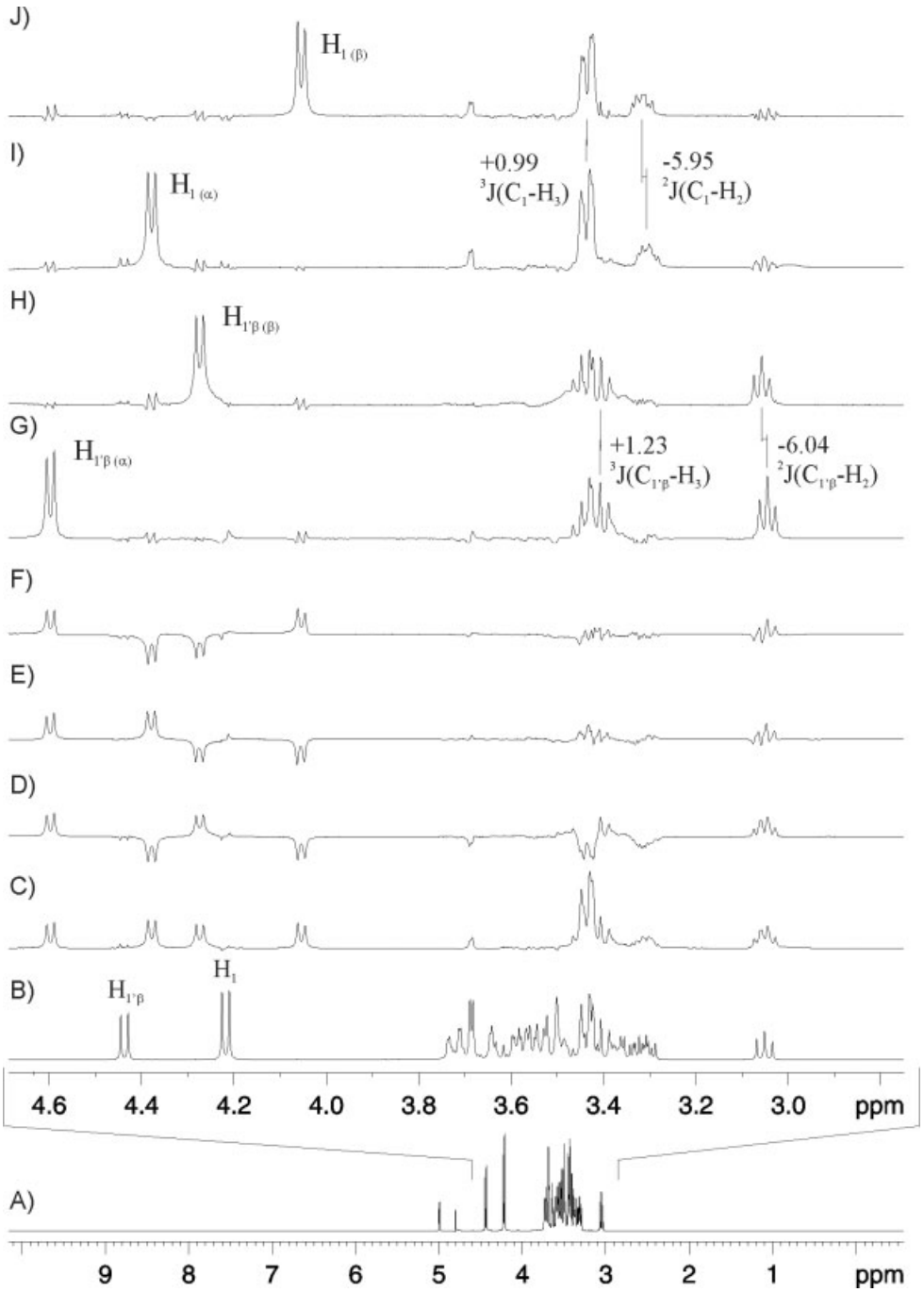

Figure 6. (A) ^{1}H-NMR spectrum of lactose at 500 MHz. (B) Expanded region of A and numbering of involved frequencies. (C–F) Example of four-site SATSEL–TOCSY experiment using Hadamard phase encoding in a sample of lactose (2048 transients each spectrum). The corresponding SATSEL–TOCSY spectra (G) C + D + E + F; (H) C + D – E – F, (I) C – D + E – F and (J) C – D – E + F allow the measurement of the sign and magnitude of long-range J_{CH} couplings.

β(B). In such situation, either of the SATSEL–SPFGE pulse schemes applied to cases B and C can resolve the overlapping. The major advantage of the dSPFGE method is that broadband rather than selective ^{13}C decoupling is used and knowledge of the chemical shift of $13C_B$ chemical shift is not a requirement. As an example of application, the accidental overlap between the α satellite of H6ax and the β satellite of H7 in menthol was overcome (Fig. 9). It is also demonstrated how the sign and the magnitude of ^nJ(CH) was extracted from the corresponding SATSEL–TOCSY spectra.

More complex situations involving three spins

More complex situations are encountered when additional protons are considered. In a three-spin systems as depicted in Fig. 1, case E ($|\nu_A - \nu_B| \approx 0$ and $|\nu_A - \nu_C| = J(CH)/2$) accidental overlapping between the ^{12}C-bonded proton A and proton B signals and the ^{13}C-satellite line of a third proton C occurs, which can be seen as a combination of cases B and C. In this case, SATSEL excitation can be achieved by following the dSPFGE approach. The first

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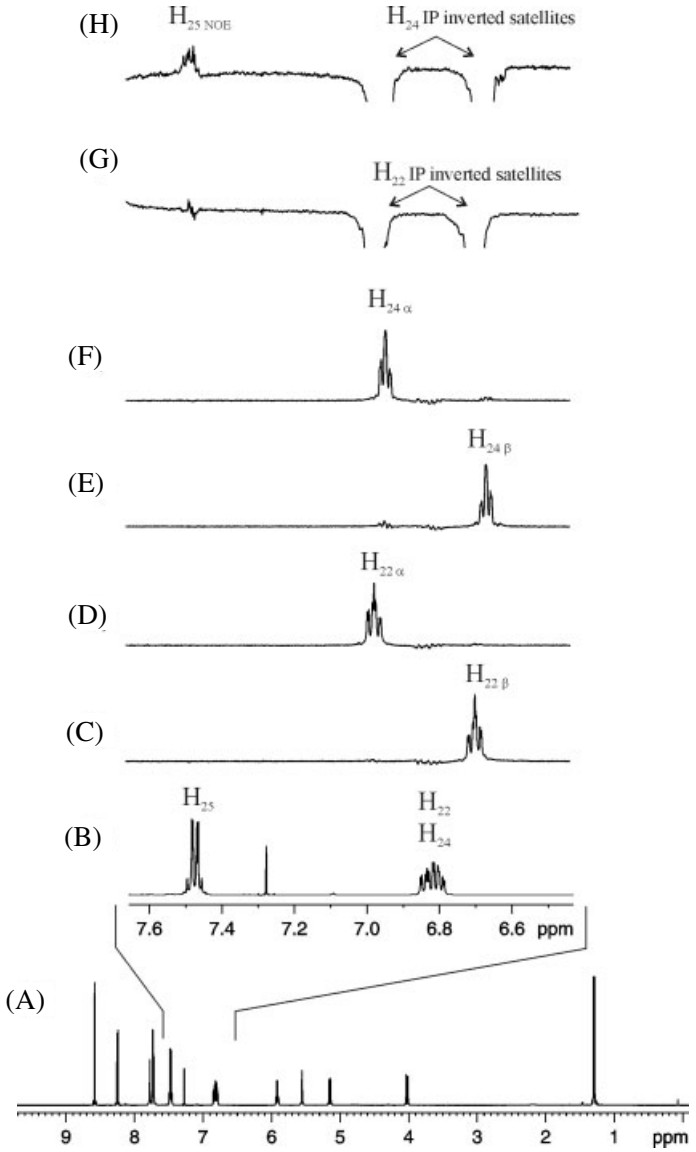

Figure 7. Case B example. (A) Full and (B) expanded ^{1}H NMR spectrum of albacanazole at 600 MHz ($CDCl_3$). β and α SATSEL excitation of (C–D) H_{22} and (E–F) H_{24} protons using the SATSEL–SPFGE scheme of Fig. 2B with selective ^{13}C CW decoupling on the non-selected proton. In total, 128 transients were recorded for each IP or AP data. (G–H) Corresponding SATSEL–NOESY spectra (2048 transients recorded for each one) after IP SATSEL excitation of the H_{22} and H_{24} protons, respectively.

echo is applied on ν_A under broadband decoupling to collapse all signal of the proton C at ν_C. Then, the satellites of spin A and B can be differentiated, as described in case B, with a two-frequency pulse applied on A with simultaneous CW on $\delta(^{13}C_B)$. An example is shown for quinine sample (Fig. 10). Finally, the same pulse scheme can be used when the three protons are completely overlapped in the ^{1}H NMR spectra (Fig. 1, case F; $|\nu_A - \nu_B| \approx 0$ and $|\nu_A - \nu_C| \approx 0$). During the first echo, selective excitation on ν_A is applied simultaneously to CW on the $\delta(^{13}C_A)$, allowing an initial selection of all ^{1}H–^{12}C magnetization components and only the ^{1}H–^{13}C magnetization of spin A. The second echo selects only the satellite lines as described in the case A. An example is shown for selecting H11 and H14a satellite lines of a strychnine sample (Fig. 8G–K).

Finally, there is a particular version of the dSPFGE–SATSEL experiment that can also work on many different situations. In the case that DEC1 = CW(A) and DEC2 = off, whereas both selective 180° pulses are applied on A can resolve overlapping situations except when CA and CB also overlaps in the corresponding ^{13}C spectrum. The only requirement of such an approach is the prior knowledge of $^{13}C_A$ chemical shift. Thus, with a pre-assigned ^{1}H and ^{13}C spectra of a given molecule, SATSEL experiments could be successfully recorded for multiple proton sites by including only their own ^{1}H and ^{13}C resonance frequency.

Conclusions

In summary, we have shown that the combination of multiple-proton-frequency excitation, ^{13}C decoupling and Hadamard phase encoding/editing strategy can be applied in simple gradient echo sequences for effective ^{13}C-satellite-selective excitation in a variety of overcrowded ^{1}H NMR regions. The proposed new

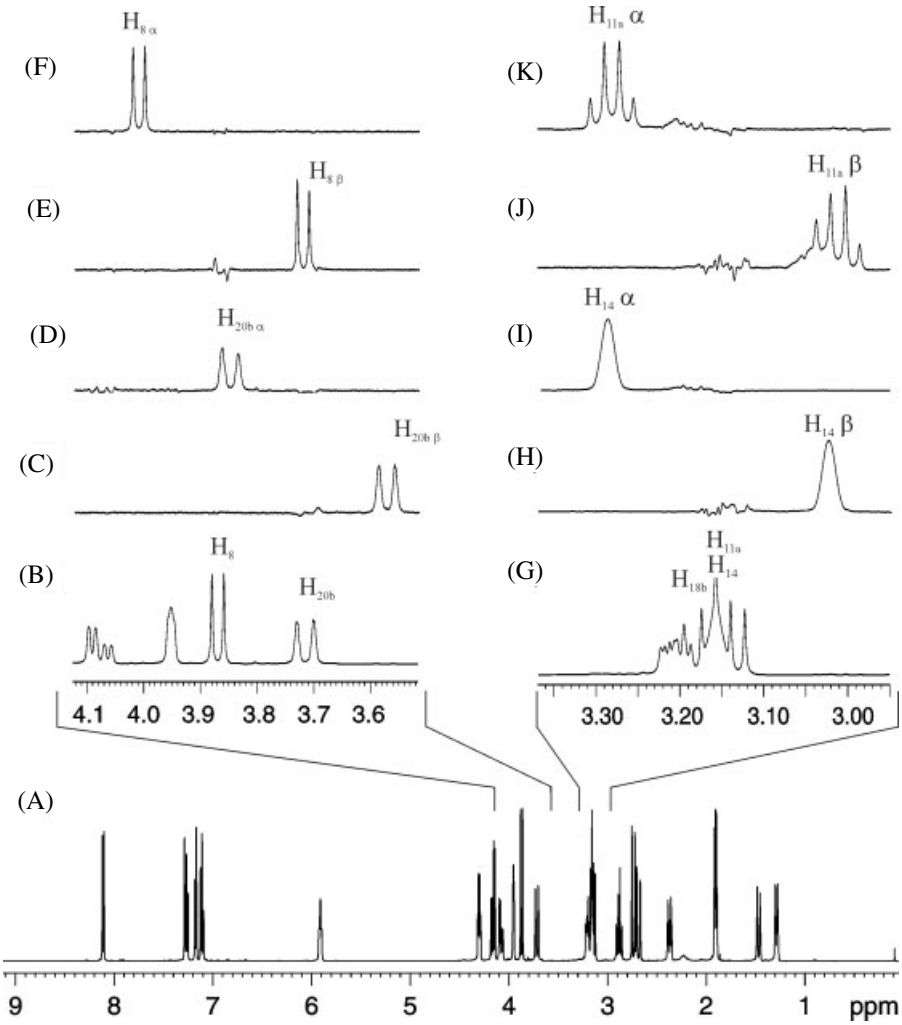

Figure 8. Case C and case F examples. (A) Full and B, (G) expanded ^{1}H-NMR spectrum of strychnine at 500 MHz ($CDCl_3$). β and α SATSEL excitation of (C–D) H_{20} and (E–F) H_8 protons using the SATSEL–dSPFGE sequence of Fig. 2C with ^{13}C GARP decoupling during the first echo. β and α SATSEL excitation of (H–I) H_{14} and (J–K) H_{11a} protons using the SATSEL–dSPFGE sequence of Fig. 2C with ^{13}C selective CW decoupling on the selected proton during the first echo. Eight transients were acquired for each individual IP or AP data. More experimental details can be found along the text and into the experimental section.

1D SATSEL pulse sequences keep the simplicity of the original experiments, are quite robust, of general use, and can be easily implemented for routine work. Not too many pulses and long delays for J-coupling evolution are involved and therefore, the methods present excellent tolerance to RF inhomogeneities, pulse miscalibrations, relaxation losses and J mismatching when compared with the traditional ^{13}C-frequency-selective HMQC- or HSQC-like experiments. In addition, no prior knowledge of specific ^{13}C frequencies or chemical shift assignments is required in some cases. The strategy promises to be useful in small-molecule NMR when heteronuclear spin-state selection can be of interest. We have shown the usefulness of the method by measuring the sign and the magnitude of small proton–carbon coupling constants.

Experimental Part

Chemicals shown in scheme 1 were purchased from a commercial source and used without further purification. The compounds (~50 mg) were dissolved in 0.6 ml of $CDCl_3$ (**A, B, C** and **D**) and D_2O (**E**) and transferred into an NMR tube. The NMR experiments for **A**, **C** and **E** were acquired on a 500 MHz Bruker spectrometer at 25 °C with a 3-channel 5-mm inverse cryoprobe and a *z*-gradient coil. The NMR experiments for **B** were acquired on a 600 MHz Bruker spectrometer at 25 °C with a 5-mm broadband probe head and a *z*-gradient coil and the NMR experiments for **D** were acquired on a 400 MHz Bruker spectrometer at 25 °C with a 5-mm broadband probe head and a *z*-gradient coil. The spectra were referenced to the residual solvent signal at 4.79 ppm (D_2O) or 7.26 ppm ($CDCl_3$).

In pulse sequences diagrams of Fig. 1, a rounded green shape indicates a 50 ms 1% truncated 180° Gaussian-shaped pulse, whereas a laminar shape indicates a 50 ms *n*-site excitation pulse ($n = 2$ or 4). This shape was created from a standard 1% truncated 180° Gaussian by applying the *Phase Modulation According to off set Frequency* option included in the stdisp tool of the Bruker TOPSPIN (v.2.1) software package. The alignment of the phase pulse is set to 0 at the middle of the shape. The reference frequency is set to the carrier frequency of the current data set. For $n = 2$, the irradiation frequencies are set to $+J/2$ and $-J/2$ from the carrier

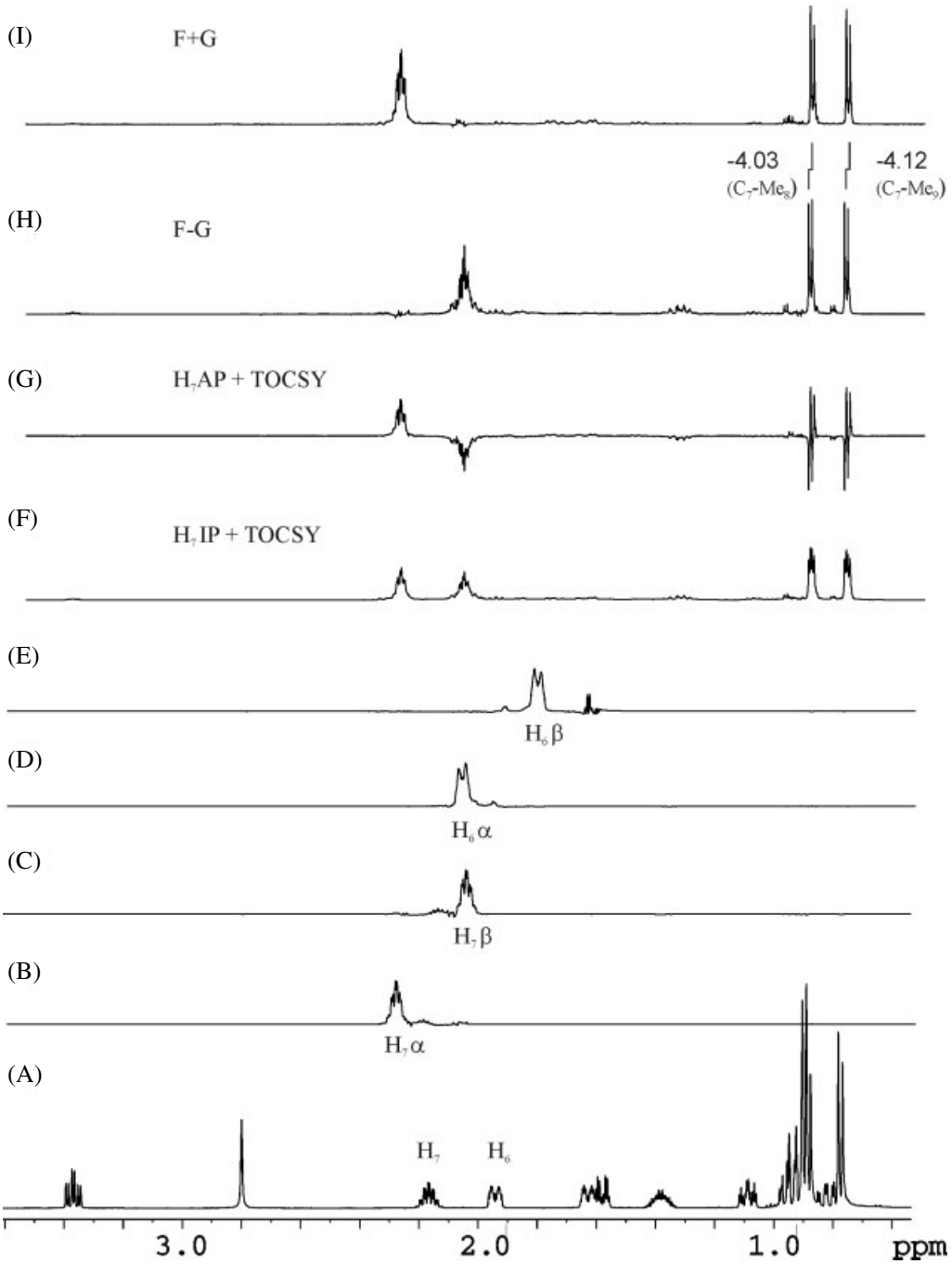

Figure 9. Case D example. (A) ^{1}H-NMR spectrum of menthol at 500 MHz. β and α SATSEL excitation of (B–C) H_7 and (D–E) H_6 protons using the SATSEL–dSPFGE pulse scheme of Fig. 2C with broadband ^{13}C decoupling during the first echo. Eight transients were acquired for each individual IP or AP data. (F–G) The corresponding IP- and AP-SATSEL–TOCSY spectra of H7 recorded with 128 transients each one. Data addition/subtraction combination affords the corresponding spin-state edited spectra (H and I) that allow the measurement of long-range proton–carbon coupling constants by comparing relative signal displacement.

frequency (usually set to $\pm$ 70 Hz, although it is recommended to introduce the values according to exact *J*). The complementary AP pulse is created in the same way but adding an additional phase correction of 90° between +J/2 and – J/2 irradiation. For $n = 4$, the irradiation frequencies are set from the carrier frequency of the current data set. For Hadamard encoding, four different laminar pulses has to be created with different additional phase setting as follows: 0°:0°:0°:0°, 0°:0°:90°:90°, 0°:90°:0°:90° and 0°:90°:90°:0°. In *n*-site excitation, *n*-different spectra are acquired separately and stored in different memory blocks. Data are further processed by adding/subtracting the corresponding FIDs which are Fourier transformed as usual.

Sine-shaped pulsed field *z*-gradients of strength G1 = 10%, G2 = 15%, G3 = 17% and G7 = 40% with a duration of 1 ms were used. For the removal of ZQ/DQ[15] effects during TOCSY and NOESY mixing times, square-shaped pulsed-field *z*-gradients with a strength of G4 = 3% and G6 = 3% are applied simultaneously to 180° adiabatic chirp pulses (represented as square shape) with

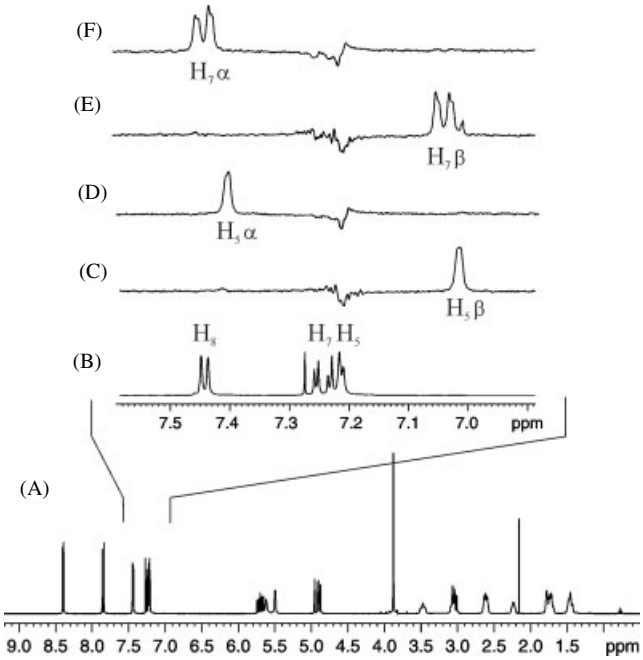

Figure 10. Case E example. (A) Full and (B) expanded area of the ^{1}H-NMR spectrum of quinine at 400 MHz. β and α SATSEL excitation of (C–D) H_5 and (E–F) H_7 protons using the SATSEL–dSPFGE pulse scheme of Fig. 2C with broadband ^{13}C decoupling during the first echo and selective ^{13}C CW decoupling on the non-selected proton during the second echo. In total, 128 transients were acquired for each individual IP or AP data.

a duration 50 and 30 ms, respectively. An additional 1 ms purge pulse with a strength of G5 = 28% is applied during the *z*-filtered DIPSI block. All *z*-gradient strengths are given in percentage, with respect to the maximum 53.5 G/cm.

The mixing period delays are set as the following. For COSY and RELAY transfers, Δ_1 was set to 30 ms. The isotropic homonuclear TOCSY mixing process consisted of a 7 kHz DIPSI-2 pulse train with a duration $\tau_m = 60$ ms in all the experiments and NOESY mixing time (τ_m) was set to 500 ms.

The power of the selective CW RF-fields decoupling was set to +40 dB and for broadband decoupling standard GARP pulse train conditions was used.

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