

Improved multiplicity-edited ADEQUATE experiments

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Received 18 June 2003; revised 19 September 2003

Abstract

A very simple strategy is proposed to extract carbon multiplicity information along with the classic knowledge of carbon–carbon connectivities in ADEQUATE experiments without affecting the sensitivity ratios of the original pulse schemes. These new multiplicity-edited ADEQUATE experiments prove to be highly helpful for complete ¹H and ¹³C resonance assignment and also for automated and easy spin system characterization of samples at natural abundance, using a single NMR experiment.

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Keywords: NMR; Adequate; Carbon multiplicity; 2D NMR

1. Introduction

The development of new NMR methodologies plays an important role in the successful use of NMR spectroscopy as a routine tool for structure elucidation of chemical and biochemical compounds. A given pulse sequence must satisfy some requirements to be of wide utility: (i) factors such as sensitivity and/or resolution must be enhanced, (ii) clean spectra with minimal artifacts must be obtained in acquisition times as short as possible, (iii) successful applicability on an extensive range of compounds, (iv) high accuracy and reproducibility of the obtained data, (v) good tolerance to miscalibrated parameters, and (vi) easy analysis and interpretation.

Conceptually, the INADEQUATE experiment [1,2] was proposed many years ago as a key and very important NMR experiment to achieve the complete structural elucidation of the carbon skeleton in organic molecules. However, the low probability of finding two consecutive ¹³C–¹³C spin nuclei in samples at natural abundance has limited its use to highly concentrated samples, and even so long acquisition times are often required. The first major improvement was the INEPT-INADEQUATE experiment [3] that offered two main

advantages: (i) sensitivity enhancement of the detected carbon signal thanks to the initial transfer polarization step via the INEPT pulse train, and (ii) faster repetition rate due to the shorter *T*₁ relaxation times of the initially excited proton nuclei. In recent years, with the advent of inverse spectroscopy based on proton detection and the use of pulsed field gradients for coherence selection [4], several new schemes usually based on the HSQC pulse train have been suggested to extract the same information under more favourable sensitivity conditions. Thus, several proton-detected HSQC-INADEQUATE experiments [5–9] were proposed, modelled on existing multidimensional HCCH experiments specifically designed for labelled nucleic acids and proteins, to trace out ¹³C–¹³C connectivity patterns of the skeleton atoms in natural-abundance compounds. These experiments were further developed with the so-called ADEQUATE experiments [10], which take profit of the combination of coherence selection using gradients with the echo–anti-echo approach and the PEP methodology, to obtain high-quality 2D correlation spectra with optimum sensitivity ratios. Comparison of the transfer efficiencies for different CH_{*n*} spin systems and of the performance of related pulse schemes have been made [11].

In its original form, the 2D 1,1-ADEQUATE spectrum correlates ¹H–¹³C–¹³C spin systems by displaying proton chemical shift in the F2 dimension and double-quantum (DQ) carbon frequencies in the indirect F1

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dimension. Alternatively, a ω_1 -refocused version was proposed [12] in which ^{13}C SQC also evolves during the last C–C refocusing period in order to display the most common carbon chemical shift value in the F1 dimension of the 2D correlation map. The resulting correlation spectrum looks like an HMBC spectrum but with the particular feature that only two-bond proton–carbon correlations are present due to the additive $^1J_{\text{CH}} + ^1J_{\text{CC}}$ pathways. Recently, improved versions have been reported for the ADEQUATE experiment that include the application of adiabatic 180° carbon pulses to improve the inversion/refocusing profile of conventional 180° rectangular pulses [13] or the use of accordion spectroscopy to compensate the fixed delay optimisation with respect to a single value of $^1J_{\text{CC}}$ [14]. In addition, other closely related versions have been also published for measuring one-bond and long-range carbon–carbon coupling constants at natural abundance [15–18].

Herein we propose a straightforward modification of the ADEQUATE experiments where carbon-multiplicity is additionally encoded in the phase of the resulting H–C–C cross-peaks. In fact, the idea was already implemented in the multiplicity-edited HSQC experiment [19], in which CH and CH_3 cross-peaks present opposite phase with respect to methylene CH_2 spin systems, and was also pointed out by us earlier for a different version of the ADEQUATE experiment [20]. Because the pro-

posed modifications are only based in the rearrangement of some inter-pulse delays, the overall length of the pulse sequence is not altered and, therefore, the full sensitivity and the performance of the original experiments are completely retained.

2. Results and discussion

For reference purposes, we have used the recently proposed ADEQUATE pulse schemes and experimental conditions described by Köck et al. [13], where all inversion and refocusing 180° carbon pulses in the ADEQUATE experiment were applied as adiabatic CHIRP pulses [21,22]. This feature extremely enhances the overall sensitivity and performance of ADEQUATE experiments, by reducing the undesired carbon offset effects, even using 400–500 MHz spectrometers.

Taking as a spin system model an H1–C1–C2 moiety, the original 1,1-ADEQUATE experiment (Fig. 1A without the insert box) can be understood as a H1[C1C2] experiment, in which H1 is detected in the F2 dimension and the sum of the C1 and C2 chemical shifts are obtained from the F1 dimension. As a very simple option, we propose to include carbon multiplicity encoding during the first spin-echo ^{13}C SQC period by the concatenated building block shown in Scheme 1B and without alteration of the overall duration of this period.

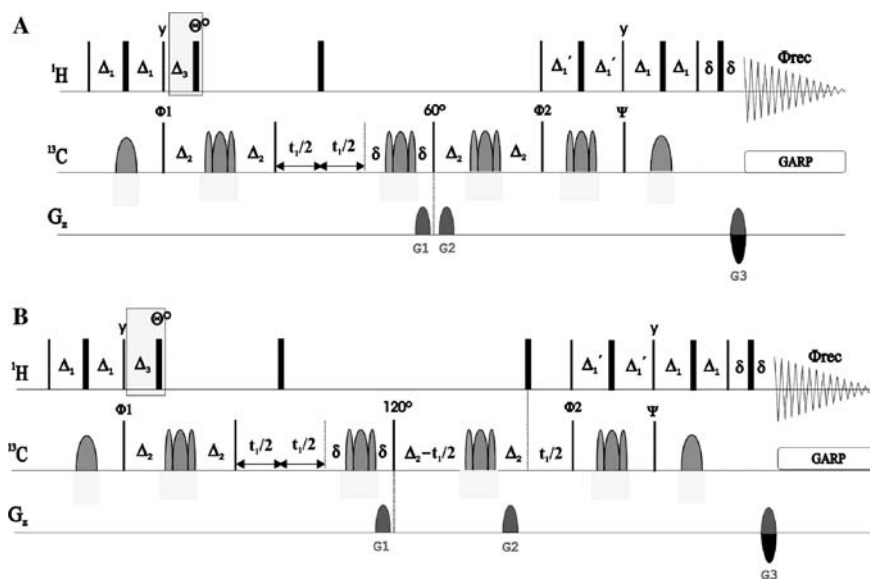


Fig. 1. Pulse schemes for: (A) multiplicity-edited 2D 1,1-ADEQUATE and (B) multiplicity-edited chemical-shift ω_1 -refocused 2D 1,1-ADEQUATE experiments. Θ° stands for the proton editing pulse, usually set to 180° (see text for details). The original unmodified ADEQUATE sequences are obtained by removing the $\Delta_3-\Theta^\circ$ (^1H) building block inserted in the marked box. Hard 90° and 180° pulses are indicated by vertical narrow and wide black bars whereas adiabatic 180° CHIRP carbon pulses are indicated by shaded pulses. All pulses are applied from the x-axis unless otherwise indicated. The inter-pulse delays, Δ_1 , Δ_1' , Δ_2 , and Δ_3 are adjusted to $1/(4 \cdot ^1J_{\text{CH}})$, $1/(6 \cdot ^1J_{\text{CH}})$, $1/(4 \cdot ^1J_{\text{CC}})$, and $1/(2 \cdot ^1J_{\text{CH}})$, respectively. Gradients of duration (δ) of 1 ms followed by a recovery time of $100\ \mu\text{s}$ are indicated by shaded shapes on the line G_z . The $G_1:G_2:G_3$ gradient ratios are set to $-78.4:-77.4:-59.0$ and $78.4:77.4:-59.0$ for (A) and (B) experiments, respectively. Basic four-step phase cycling: $\Phi_1 = x, -x$; $\Phi_2 = x, x, -x, -x$; $\Psi = y, y, -y, -y$; $\Phi_{\text{rec}} = x, -x, -x, x$. Both Ψ and G_3 gradient are inverted in alternate scans using the echo/anti-echo protocol. GARP decoupling was used for heteronuclear decoupling during acquisition.

The anti-phase carbon magnetization ($-2I_{1z}S_{1y}$) generated after the initial INEPT pulse train is allowed to evolve under the effects of the heteronuclear carbon–proton coupling constant during a $2\Delta_3$ period whereas homonuclear carbon–carbon coupling constant is not affected by this modification and it keeps evolving unaffected during the $2\Delta_2$ echo period:

X Chemical Shift Evolution : $\Delta_3 + (\Delta_2 - \Delta_3) - \Delta_2 = 0$

Heteronuclear $J(\text{CH})$ Evolution : $\Delta_3 - (\Delta_2 - \Delta_3) + \Delta_2 = 2\Delta_3$

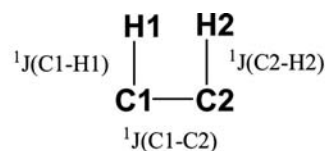
Homonuclear $J(\text{CC})$ Evolution : $\Delta_3 + (\Delta_2 - \Delta_3) + \Delta_2 = 2\Delta_2$

Optimising $\Delta_2 = 1/4 * {}^1J_{\text{CC}}$ as usual and $\Delta_3 = 1/2 * {}^1J_{\text{CH}}$ all signal intensities are modulated as a function of carbon multiplicities before conversion to double-quantum coherences:

$$-2I_{1z}S_{1y} \xrightarrow{\Delta_3 - \theta^\circ(t) - (\Delta_2 - \Delta_3) - 180^\circ(S) - \Delta_2} 4I_{1z}S_{1x}S_{2z} \cos^{n-1}(\theta) \xrightarrow{90^\circ(S)} -4I_{1z}S_{1x}S_{2y} \cos^{n-1}(\theta)$$

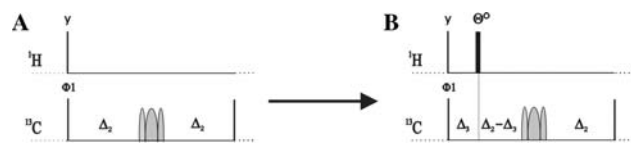
where θ is the angle of this additional proton pulse and n is the number of protons directly attached to the C1 carbon. When $\theta = 180^\circ$, the cross-peaks of protons belonging to CH and CH₃ groups will display opposite phase than those of CH₂ groups [19]. In this way, when the ¹³C spin-echo period is modified as proposed in Scheme 1, the resulting multiplicity-edited two-dimensional ADEQUATE experiment (Fig. 1A including the insert box) affords as an extra bonus the carbon-multiplicity information of the C1 target carbon by simple visual inspection of the cross-peak phase. In principle, by analysing the corresponding DQ rows it should be possible to distinguish between six different CH_{*n*}–CH_{*m*} spin system patterns (see Fig. 2). Alternatively, this editing block could be introduced in the second ¹³C SQC spin-echo period, just before the retro-INEPT block, achieving the same effects. In addition, if the θ pulse is set to 90° a CH-edited ADEQUATE spectrum should be obtained (data not shown).

The edited ADEQUATE spectrum of menthol (Fig. 3) is a very illustrative example of the usefulness of this modification. For instance, the proton signal resonating at 3.27 ppm presents two well-defined correlations at DQ frequencies of 115.5 and 120.5 ppm and, in addition, it can be confirmed that this proton belongs to a methine



Spin system	Pattern
CH-CH CH-CH ₃	$\begin{array}{cc} \oplus & -\oplus \\ \text{H1} & \text{H2} \end{array} \quad \text{C1+C2}$
CH-CH ₂ CH ₃ -CH ₂	$\begin{array}{cc} \oplus & -\ominus \\ \text{H1} & \text{H2} \end{array} \quad \text{C1+C2}$
CH ₂ -CH ₂	$\begin{array}{cc} \ominus & -\ominus \\ \text{H1} & \text{H2} \end{array} \quad \text{C1+C2}$
C-CH C-CH ₃	$\begin{array}{cc} \text{---} & \oplus \\ \text{H1} & \text{H2} \end{array} \quad \text{C1+C2}$
C-CH ₂	$\begin{array}{cc} \text{---} & \ominus \\ \text{H1} & \text{H2} \end{array} \quad \text{C1+C2}$
C-C	$\begin{array}{cc} \text{---} & \text{---} \\ \text{H1} & \text{H2} \end{array} \quad \text{C1+C2}$

Fig. 2. Typical pattern recognition for several CH_{*n*}–CH_{*m*} spin systems extracted from row analysis in multiplicity-edited 1,1-ADEQUATE experiments.



Scheme 1.

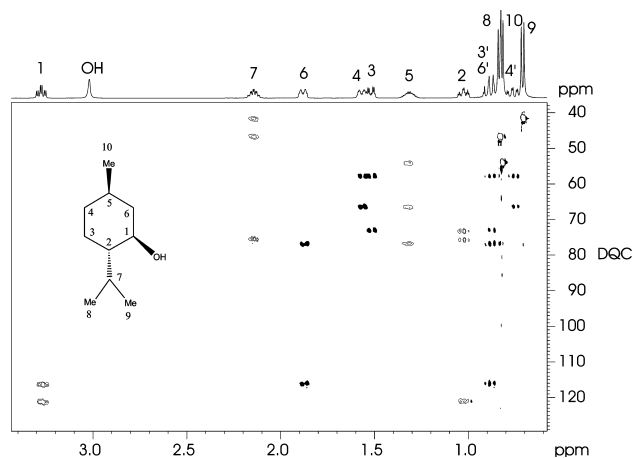


Fig. 3. 2D multiplicity-edited ADEQUATE spectrum of menthol acquired using pulse sequence Fig. 1A. For clarity, positive and negative peaks are plotted with empty and filled contours, respectively.

CH group because both peaks have positive intensity. Subsequent analysis of the rows for each one of these two correlations reveals separate connectivities to two negative peaks (characterised by two well separated diastereotopic protons at 1.9 and 0.9 ppm belonging to a methylene CH₂ group) and to a positive peak (resonating at 1.05 ppm, presumably a CH group), respectively. This simple analysis reveals the presence and full assignment of a three-carbon CH–CH–CH₂ spin system. Following the same procedure for other resonances, the full structure can be obtained quickly in a very straightforward way and without any prior knowledge about its chemical structure.

The multiplicity information can also be used to deduce the presence of functional groups (such as non-observable oxygen or nitrogen substituents) by counting the number of cross-peaks for a given proton. Thus, all positive methyl protons will always display a maximum of one correlation, making easy the differentiation with positive CH protons. In the example, three different methyl signals are clearly observed. Four different aliphatic CH protons are clearly distinguished by their positive signs in Fig. 3. Whereas the H1 proton only has two correlations, the remaining H7, H5, and H2 methine protons have three correlations each, evidencing the possible presence of a heteronucleus on the C1 carbon. In the case of aliphatic CH₂ methylene protons, two negative correlations must be present for each proton in the absence of directly bound heteronuclei.

Comparison of 1D traces of these 2D experiments with and without the editing block shows that the sensitivity of the original experiment is practically not affected (Fig. 4) and only improper refocusing during the $2\Delta_3$ delay due to unmatched $^1J_{\text{CH}}$ values would produce some minor sensitivity losses. For instance, the advantage of the editing encoding is observed in the C4 + C5 row at 76.2 ppm where a CH₂–CH spin system is auto-

matically detected and the corresponding proton chemical shifts are fully assigned.

Fig. 1B shows the new multiplicity-edited ω_1 -refocused 1,1-ADEQUATE experiment, where multiplicity editing is included in the first spin-echo CC, while the last spin-echo CC period is implemented as a variable constant-time period. This experiment can be understood as a H1(C1)C2 correlation experiment in which the phase of the proton signal determines the multiplicity of the non-observed C1 carbon resonance. Although the corresponding one-bond H1C1 cross-peak is not directly observed in this experiment, the C1 chemical shift is usually determined by analysing other cross-peaks as, for instance, the reverse H2(C2)C1 peak. As described previously, proper optimisation of Δ_2 and Δ_3 delays affords simple discrimination of signal intensities with respect to carbon multiplicity. The advantage of this refocused version is that the resulting spectra are displayed in a more amenable HMBC-like correlation format, in which carbon chemical shifts are directly represented in the F1 dimension instead of double quantum coherences (Fig. 5). Comparison of such spectra with regular HMBC spectra allows a clear distinction between two-bond and three-bond proton–carbon correlations [12].

Separate analyses of columns and rows give complementary information (Scheme 2). Analysis of specific columns affords the chemical shift of the selected proton, and the number, the multiplicities, and the chemical shifts of neighbouring carbons two bonds away from the selected proton. On the other hand, analysis of specific rows affords the chemical shift of the selected carbon, and the number, and multiplicities of the directly attached carbons as well as the proton chemical shift of the protons directly bound to the latter, i.e., two bonds

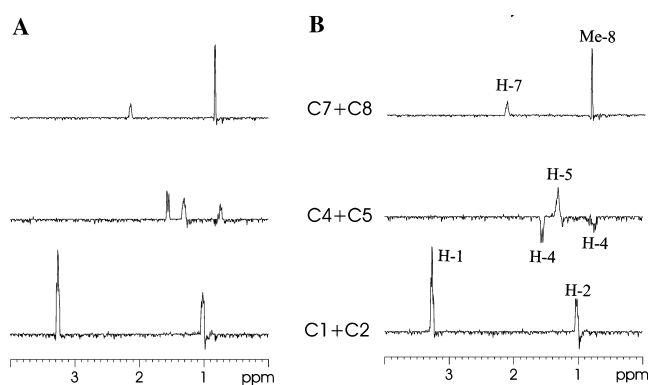


Fig. 4. Some 1D row traces taken from specific double-quantum frequencies: (A) conventional and (B) multiplicity-edited 1,1-ADEQUATE spectra (see Fig. 3) acquired with the sequence of Fig. 1A without and with the marked box, respectively.

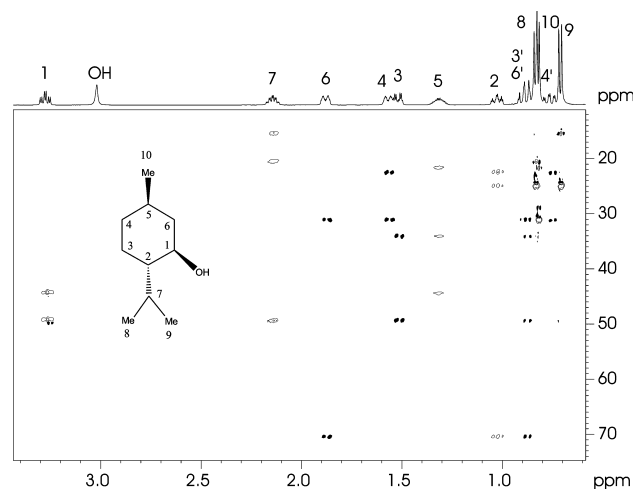
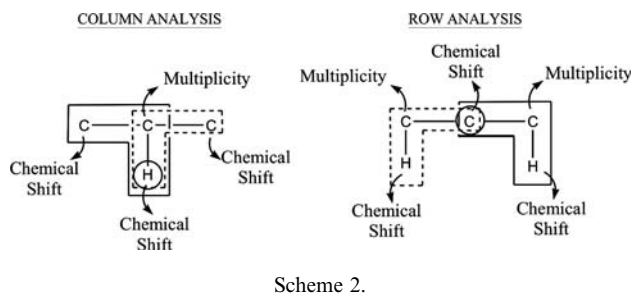


Fig. 5. 2D multiplicity-edited refocused-ADEQUATE spectrum of menthol acquired using the sequence of Fig. 1B. For clarity, positive and negative peaks are plotted with empty and filled contours, respectively.



away from the selected carbon. As discussed previously, the number of cross-peaks is a good evidence for the presence of non-observable heteroatoms.

Taking the column of the proton resonating at 3.27 ppm as an example (see Fig. 5), it can be stated that this proton belongs to a CH system that is only attached to two carbons resonating at 49.7 and 44.7 ppm. The advantage of the editing encoding is also clearly demonstrated from row cross-peak analysis (see Fig. 6). For instance, in the original ADEQUATE spectrum, the C2 carbon resonating at 49.7 displays four different two-bond correlations (Fig. 6A) but, in principle, these protons cannot be directly distinguished and assigned. From the edited spectrum (Fig 6B), it is clear that these four protons belong to a CH–C(CH)₂ spin system and, in addition, all four protons can be directly distinguished. In the same way, it can be stated that the C1 and C5 carbons belong to CH–C(X)–CH₂ and CH₂–C(CH₃)–CH₂ spin systems, respectively. Combined analysis of rows and columns for all other cross-peaks affords the full chemical structure. In practice, as discussed above, the sensitivity of the original experiment is minimally affected by the incorporation of the editing block (Fig. 6). However, large variations of the size of $^1J(\text{CH})$ and $^1J(\text{CC})$ coupling constants within the same molecule could cause undesired sensitivity losses by inefficient refocusing of these coupling constants. Fig. 7

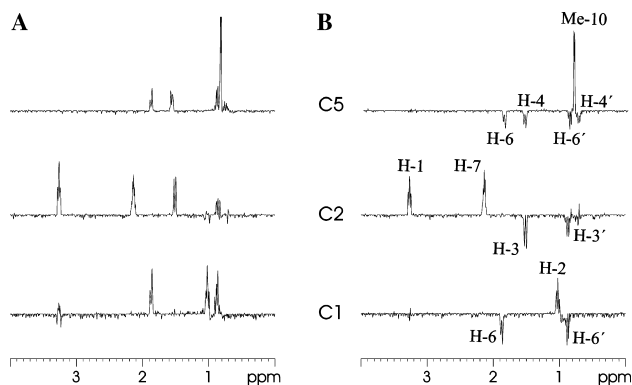


Fig. 6. Some 1D row traces taken at specific carbon chemical shifts: (A) conventional and (B) multiplicity-edited refocused-ADEQUATE spectra (see Fig. 5) acquired with the sequence of Fig. 1B without and with the marked box, respectively.

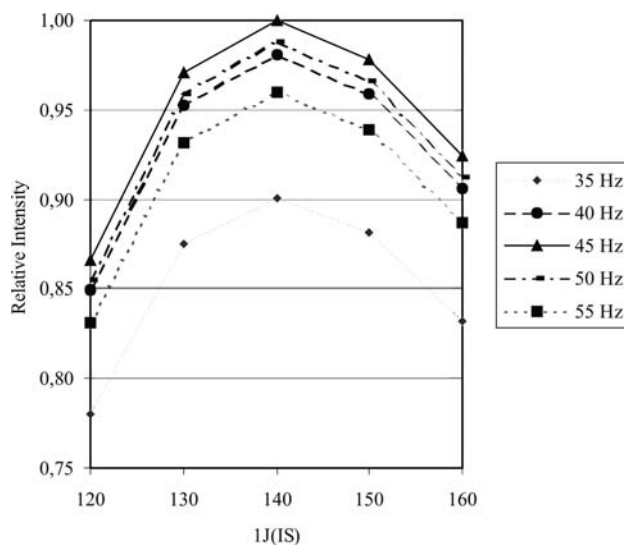


Fig. 7. Theoretical relative signal intensities for a $\text{H}_1\text{C}_1\text{--C}_2$ spin subsystem (assuming coupling constants values of $^1J(\text{H}_1\text{C}_1) = 140$ Hz and $^1J(\text{C}_1\text{C}_2) = 45$ Hz) in a multiplicity-edited ADEQUATE experiment optimised for a range of $^1J(\text{CH})$ and $^1J(\text{CC})$ values.

shows the theoretical sensitivity losses for different ranges of $^1J(\text{H}_1\text{C}_1)$ and $^1J(\text{C}_1\text{C}_2)$ values in a $\text{H}_1\text{C}_1\text{--C}_2$ moiety, whose signal presents a theoretical $\cos(2\pi J_{IS} \Delta_3) \sin(2\pi J_{S_1 S_2} \Delta_2)$ dependence. Variants to improve such refocusing efficiencies would improve the results as demonstrated for the ACCORD-ADEQUATE scheme [14], in which a decremented variable delay covering a wide range of $^1J(\text{CC})$ could be implemented to improve the results when compared with the normal-static scheme of Fig. 1B. On the other hand, the compensation of spin–spin coupling constants during adiabatic pulses has been also reported [23] and when a broad range of $^1J(\text{CH})$ coupling constants is present, approaches exploiting the rough linearity between the ^{13}C chemical shift and the magnitude of $^1J(\text{CH})$ could be advisable.

Unfortunately, the ADEQUATE experiment does not display cross-peaks when two consecutive quaternary carbons or heteroatoms are present. In this case, it should also be possible to include multiplicity editing in a less-sensitive $1,n$ -ADEQUATE experiment (data not shown), where the Δ_2 delay is optimised to a smaller $^nJ(\text{CC})$ value (about 5–10 Hz), and heteronuclear correlations at three and four bonds could be observed. Other minor drawbacks of the proposed edited experiments could be accidental signal cancellation due to resonance overlapping of signals with opposite phase or, as discussed earlier, the need for optimal adjustment of the evolution delays for heteronuclear proton–carbon and homonuclear carbon–carbon coupling constants.

It should be also possible to modify the proposed sequences in order to convert them into the less time-consuming 1D ADEQUATE experiments by removing the variable t_1 periods and introducing carbon-selective

pulses. This should be useful in cases where only specific information on a particular carbon is required. On the other hand, the experiments could also be modified to include band-selective carbon pulses in order to excite only a specific region of the carbon spectrum. This should be useful to record the same 2D experiments with a narrower spectral width in the indirect F1 dimension in order to achieve better spectral resolution when highly overlapping carbon regions are present.

3. Experimental

All experiments were performed on BRUKER DRX-500 spectrometer at 298 K equipped with a 5 mm inverse broadband probe head incorporating Z-gradients. All spectra were acquired and processed with XWINNMR v3.5. Menthol (80 mg dissolved in 0.7 ml of CDCl_3) was chosen as a model sample. Data matrices of 128×1024 points in t_1 and t_2 domain were acquired with spectral widths of 18,000 and 2000 Hz for the 1,1-ADEQUATE experiments and of 10,080 and 2000 Hz for the multiplicity-edited ADEQUATE experiments, respectively. The carbon offset was set to 50 ppm and number of scans for each t_1 increment was 64 for all experiments. The relaxation delay was set to 1 s, and the inter-pulse delays Δ_1 , Δ'_1 , Δ_2 , and Δ_3 were optimised to 1.78 ms (140 Hz), 1.19 ms (140 Hz), 5.5 ms (45 Hz), and 3.75 ms (133 Hz), respectively. Fourier transformation along both dimensions was performed by zero-filling to obtain data matrices of 1024×512 points in F1 and F2, respectively. Data were multiplied by a $\pi/2$ shifted squared sine-bell window function before Fourier transformation. Regular smoothed chirp (500 μs of duration) and composite smoothed chirp (2 ms of duration) shaped pulses were used as inversion and refocusing 180° carbon pulses, respectively. For more experimental details, please refer to [13].

4. Conclusions

In spite of their inherent low sensitivity compared to other NMR experiments, it has been demonstrated that the proposed multiplicity-edited ADEQUATE pulse sequences give a large amount of chemical information in a single experiment. Because sensitivity is minimally altered, the use of this version of the ADEQUATE experiment to analyse carbon-carbon connectivities is recommended. In this way, complete ^1H and ^{13}C chemical shift assignments and carbon-multiplicity information are simultaneously achieved in a straightforward step-by-step analysis through the different

cross-peaks. Although it is obvious that combinations of other most sensitive NMR experiments would give the same information, with the advent of enhanced sensitivity hardware for routine applications, such as higher magnetic fields or specific cryo- and micro-probes, the approach introduced herein will probably be considered an interesting tool for both chemical shift assignment and chemical structure determination protocols of organic molecules using a single experiment. Protocols for automated spectrum analysis are currently under study.

Acknowledgments

We thank the Servei de Ressonància Magnètica Nuclear, UAB, for allocating instrument time to this project.

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