

Rapid-Pulsing Artifact-Free Double-Quantum-Filtered Homonuclear Spectroscopy

The 2D-INADEQUATE Experiment Revisited

Maryse Bourdonneau* and Bernard Ancian†¹

*Unité Mixte de Recherche Bruker, CNRS, Université Louis Pasteur, UMR 50, 34, rue de l'Industrie, F-67166 Wissembourg Cedex, France; and

†Department of Chemistry, Université Paris 7-Denis Diderot, 2, Place Jussieu, F-75251 Paris Cedex 05, France; and

‡Bruker (UMR 50), Buroparc n° 1, 3, Avenue du Général de Gaulle, F-91090 Lisses, France

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Rapid pulsing artifacts are observed in the conventional phase-cycled carbon-13 2D INADEQUATE experiment. By using the product operator formalism, it is shown that they result from the effects of imperfect 90° and 180° excitation pulses on the most abundant molecules containing only one isolated carbon-13 nucleus. The labeled longitudinal magnetization remaining at the end of one scan is recycled by the subsequent acquisition, giving rise to multiple-quantum ($p = 0, \pm 1, \pm 2, \dots$) artifacts in the F_1 dimension. By considering pairs of scans instead of single scans, a new phase cycle is proposed. It is based on a scheme for compensating for imperfections in the excitation cluster by a proper combination of the pulse phases in two consecutive scans. Because the artifacts are 90° out of phase compared to the desired signal, a concomitant rearrangement of the receiver phase achieves suppression of all unwanted signals. Experiments are presented on menthol dissolved in CDCl_3 as a test compound. Improvements in spectrum quality as well as increased sensitivity are discussed.

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INTRODUCTION

Over the past decade, the COSY–DQF correlation and the 2D-INADEQUATE experiments have proven to be very powerful techniques for determining molecular structure (1, 2). By comparison of the proton topology and of the carbon–carbon connectivities obtained, these experiments allow us to trace out the organic skeleton.

As pointed out by Ernst, Bodenhausen, and Wokaun (3), multiple quantum spectroscopy may be considered as a generalization of 2D correlation, provided the preparation pulse in the COSY–DQF experiment is replaced by a more elaborate excitation sandwich sequence designed to create the

multiple-quantum coherence. Inherent to this class of spectroscopic techniques is the generation of spectral artifacts resulting from unwanted coherences which reach the receiver despite suitable phase cycling, multiple quantum filters, or tailored pulsed field gradients (3).

Moreover, these experiments suffer considerably from low sensitivity, particularly for the 2D-INADEQUATE where only 1 molecule in roughly 8100 gives the AX (or AB) system needed for the required double quantum coherence compared to the 180 times more intense undesirable single quantum responses. Of course, this problem is less severe in DQF–COSY, where the loss in sensitivity is only by a factor of 2 compared to the standard unfiltered COSY experiment. It is also less crucial in 2D double-quantum proton experiments, which have been shown to be more sensitive and more suitable than DQF–COSY for a complete analysis of the protein fingerprint region with water saturation² (4, 5). As a result, there is a constant temptation to speed up data collection by allowing only partial relaxation, since it is well known that the optimum sensitivity per unit time is expected with a recycle time of about $1.3 T_1$ when 90° pulses are used, assuming a complete transverse relaxation (6).

The major drawback of time averaging with such high repetition rates is the formation of a new class of artifacts which are related to incomplete longitudinal relaxation (7–9). Partial relaxation allows the longitudinal magnetization to be carried over from one transient to the next and to interfere with it, even if transverse relaxation is complete. Roughly speaking, since no more than 75% of the longitudinal magnetization will have recovered, the remaining 25% will be recycled and experience two evolution periods. This results in apparent multiple-quantum (MQ) coherences ($\Delta m = 0, \pm 2$) during t_1 which give after subsequent double Fou-

¹ To whom correspondence should be addressed at Bruker, Buroparc n° 1, 3, Avenue du Général de Gaulle, 91090 Lisses, France. Fax: (33) 01 60 86 34 90. E-mail: pad@bruker.fr.

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rier transformation a series of artifacts which we shall name MQ artifacts (7–9). As the ω_1 width is limited by the t_1 increment, these MQ artifacts are often aliased in the ω_1 dimension and their location depends on the type of detection scheme used for the ω_1 quadrature detection [TPPI (11), States–Haberhorn–Ruben (12), or States–TPPI (13)].

Although these artifacts are now well documented for the classical phase cycled DQF–COSY (7–9), we have shown recently (14, 15) that they can also give rise to serious problems for medium to large molecules in pulsed field gradient selected (PFGS) DQF–COSY: Their elimination requires not only a suitable gradient combination for magnitude spectra (14), but also more elaborate time-domain data processing for obtaining pure phase absorption spectra (15). To the best of our knowledge, no such artifacts have been reported for the 2D-INADEQUATE experiment. It is our experience, however, that the same class of rapid pulsing artifacts occur in this experiment, giving rise to $\Delta m = 0$, ± 2 MQ artifacts as well as $\Delta m = \pm 1$ single-quantum (SQ) artifact signals. The purpose of this paper is, first, to discuss the generation of these artifacts by analogy with DQF–COSY and by a careful comparison of many pulse phase cycles, and then to propose a new variant of the basic 2D-INADEQUATE phase cycle to remove them. The use of scrambling pulses as well as suitable gradient pulses along the coherence pathway will also be examined.

EXPERIMENTAL

All the phase-cycled carbon-13 spectra were recorded on a 4 M CDCl₃ solution of methanol at room temperature (21 ± 1°C) on a Bruker Avance 300-MHz spectrometer operating at 75.47 MHz and equipped with a 5-mm QNP probe. The carbon-13 pulse width was carefully calibrated on the menthol sample at the carrier frequency (90° pulse length = 7.7 μs). The spectral width was set to 4870 Hz in the F_2 dimension, according to the minimum spectral dispersion of carbon-13 chemical shifts. A preparation delay $\tau = 7.1$ ms was chosen in order to observe only one bond double quantum coherence ($^1J_{CC} \approx 35.2$ Hz). 4K complex points were acquired in t_2 under WALTZ-16 composite pulse proton decoupling for 128 t_1 increments; 64 dummy scans (only 32 in the phased mode) and 128 scans per t_1 increment were made. The relaxation delay between successive acquisitions was $D = 0.7$ s for the rapid pulsing experiments and $D = 4$ s for the fully relaxed ones which were run as check experiments. A pure cosine window was used for time-domain filtering in both dimensions prior to 2D Fourier transformation, and zero filling by a factor of 4 was performed in the F_1 dimension. Spectra were generally recorded in the magnitude mode and the complete pure absorption spectra were obtained according to the TPPI procedure (11). Carbon-13 longitudinal relaxation times were measured by the inversion recovery method within an accuracy of ±5%.

PFGS experiments were run at 100.63 MHz under the same experimental conditions as before on a Bruker Avance 400-MHz spectrometer equipped with an amplifier capable of generating shaped field-gradient pulses of 50 G/cm. A 5 mm QNP probe fitted with a self-shielded z-gradient coil and sine-shaped gradients of 1 ms length with a recovery time of 100 μs were used. The 90° carbon-13 pulse was 8.5 μs; 16 scans per increment t_1 were run with the following phase cycling: $\alpha = \beta = x, y, -x, -y, -x, y, x, -y$; $\gamma = 4x, 4y, 4(-x), 4(-y)$; $\phi = 2(x, -x), 2(-y, y), 2(-x, x), 2(y, -y)$. A read pulse of 11.3 μs (120°) was used for optimum detection of the N-type spectrum.

RESULTS

Figure 1 shows the magnitude mode 2D-INADEQUATE spectrum of menthol in CDCl₃ using the conventional pulse sequence obtained by (1) an eight-step sequence with 45° phase shift during the double-quantum (DQ) excitation period, followed by (2) a CYCLOPS incrementation of all the pulse phases and receiver reference phase and (3) an alternation of the 180° refocusing pulse giving a final cycle of 64 steps (16) (Fig. 2 and Table 1). The spectrum shown in Fig. 1a, obtained with a long repetition time (recycle time $D = 4$ s), does not exhibit any artifacts, whereas in the spectrum in Fig. 1b, recorded under the same experimental conditions, but with a rapid repetition rate (recycle time $D = 0.7$ s), a large number of artifacts along the lines $p = 0, \pm 1, \pm 2$ are clearly evident. For this sample, the longitudinal relaxation times range from 1 s (carbon C4) to 2 s (carbons C2 and C5) within an experimental accuracy of ±5%. Comparison of these two experiments suggests that artifacts arise from a frequency labeled residual longitudinal magnetization which is converted by the next sequence into apparent MQ coherences $p = 0, \pm 1, \pm 2, \dots$ during t_1 and then detected as a phase (or amplitude) modulation of the observed signal during t_2 . This can be summarized by the scheme shown in Fig. 3.

These artifacts may theoretically arise in several ways. We have performed a number of further experiments in order to glean more information concerning the exact source of these signals.

1. A strong pulsed field gradient G (sine-shaped with amplitude = 25 G/cm and time duration = 2 ms) was applied at the end of each acquisition i . This was shown to have no effect on the nature or the intensity of the artifacts in the 2D spectrum. This would suggest that they do not originate from some SQ or MQ coherences which could persist at the end of the acquisition.

2. Replacement of this gradient by two scrambling pulses made up of two perpendicular purging spin lock pulses (17); (2 ms along x and 1 ms along y , respectively, with the same RF high power as the other pulses) was also

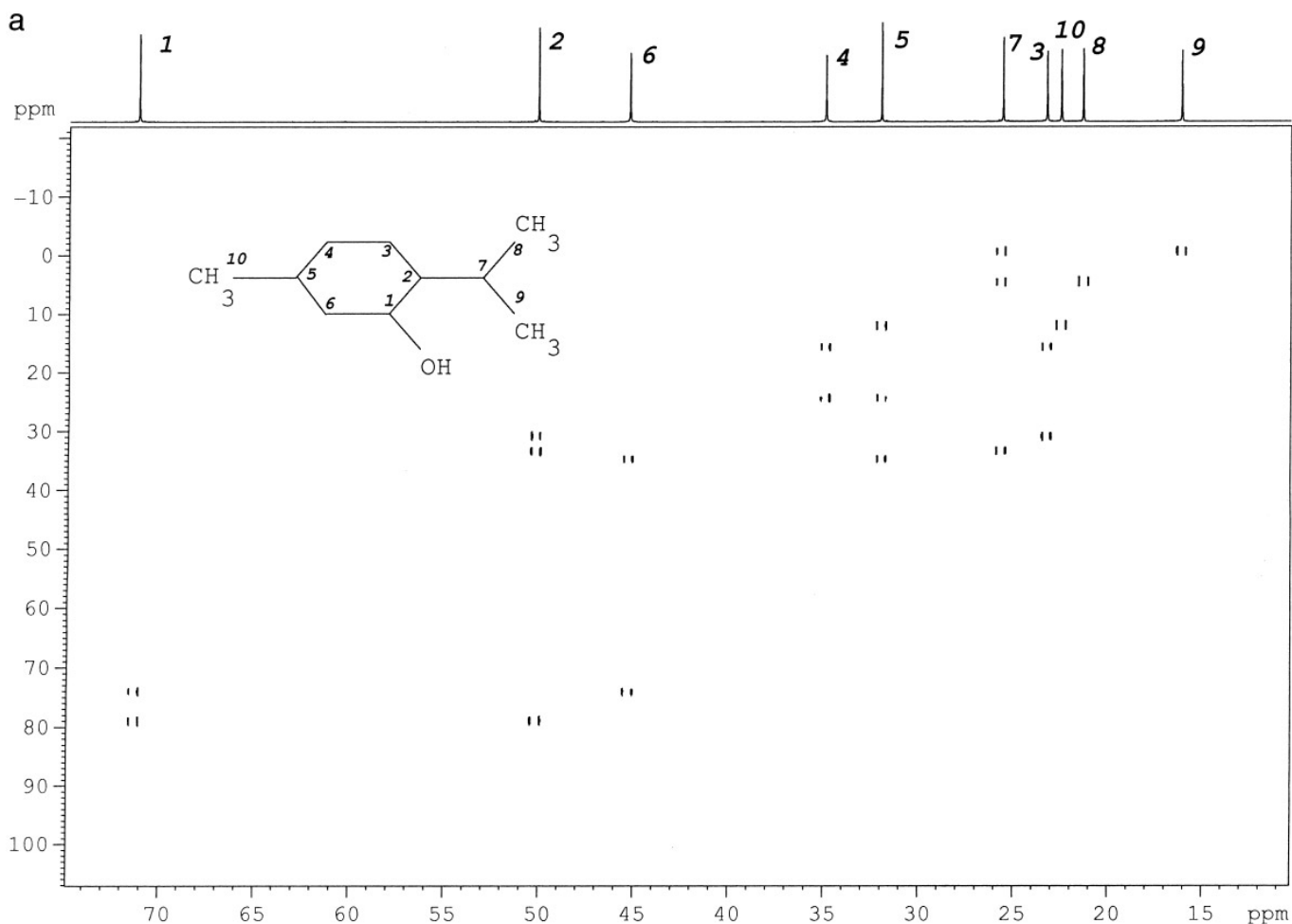


FIG. 1. Contour plots of the 2D-INADEQUATE spectra of menthol in CDCl_3 , recorded in magnitude mode with the classical phase cycling in Table 1. Carbon-13 chemical shift assignment is given on the 1D spectrum along the F_2 dimension. (a) Spectrum obtained with a long interscan delay $D = 4$ s. (b) Spectrum obtained under rapid-pulsing conditions ($D = 0.7$ s). The lines indicate the direction of the MQ spurious signals: ZQ ($p = 0$), SQ ($p = \pm 1$), and DQ ($p = \pm 2$) artifacts are clearly visible along the lines $F_1 = 0$, $F_1 = \pm F_2$, and $F_1 = \pm 2F_2$, respectively.

observed to afford only partial improvement in the spectral quality. There still remains zero-quantum (ZQ) artifacts along the line $F_1 = 0$. These pulses did succeed in significantly reducing $p = \pm 1$ SQ spurious signals compared to the conventional experiment shown in Fig. 1b and in particular resulted in good cancellation of double-quantum (DQ) artifacts in the 2D spectrum. This observation may be explained as follows: By defocusing SQ coherences and removing the frequency labeled residual longitudinal magnetization, they allow suppression of these apparent DQ cross peaks. In contrast, ZQ coherences are known to be completely insensitive to RF field inhomogeneity, so that they cannot be removed by the pair of scrambling pulses. Moreover, since they have longer relaxation times than other coherences (3), they may be present at the end of a given scan; as they are not affected by the 2D INADEQUATE excitation sequence $90^\circ_\alpha - \tau - 180^\circ_\beta - \tau - 90^\circ_\alpha$, where $\beta = \alpha$ or $\alpha + \pi$, these coherences pass directly through the subsequent excitation period

without any evolution during t_1 giving, after double Fourier transformation, spurious signals along the line $F_1 = 0$.

3. A "trim" pulse cluster was also tried as follows: At the end of acquisition and before the relaxation delay D , a first pulse gradient $G_1 = 30$ G/cm was applied during 2 ms, immediately followed by a nonselective 90° pulse and then by a second gradient $G_2 = 20$ G/cm, also during 2 ms. A very clean spectrum was thus obtained. This constitutes the first real evidence of the involvement of residual frequency-labeled longitudinal in the formation of these artifacts. The first gradient pulse removes residual transverse magnetization; in contrast, residual longitudinal magnetization is unaffected by this gradient pulse, but is eliminated by the 90° RF pulse and the second gradient pulse. This purge sequence has the added advantage of providing perfectly defined initial conditions before the next pulse sequence.

However, experiments 2 and 3 have major drawbacks. By

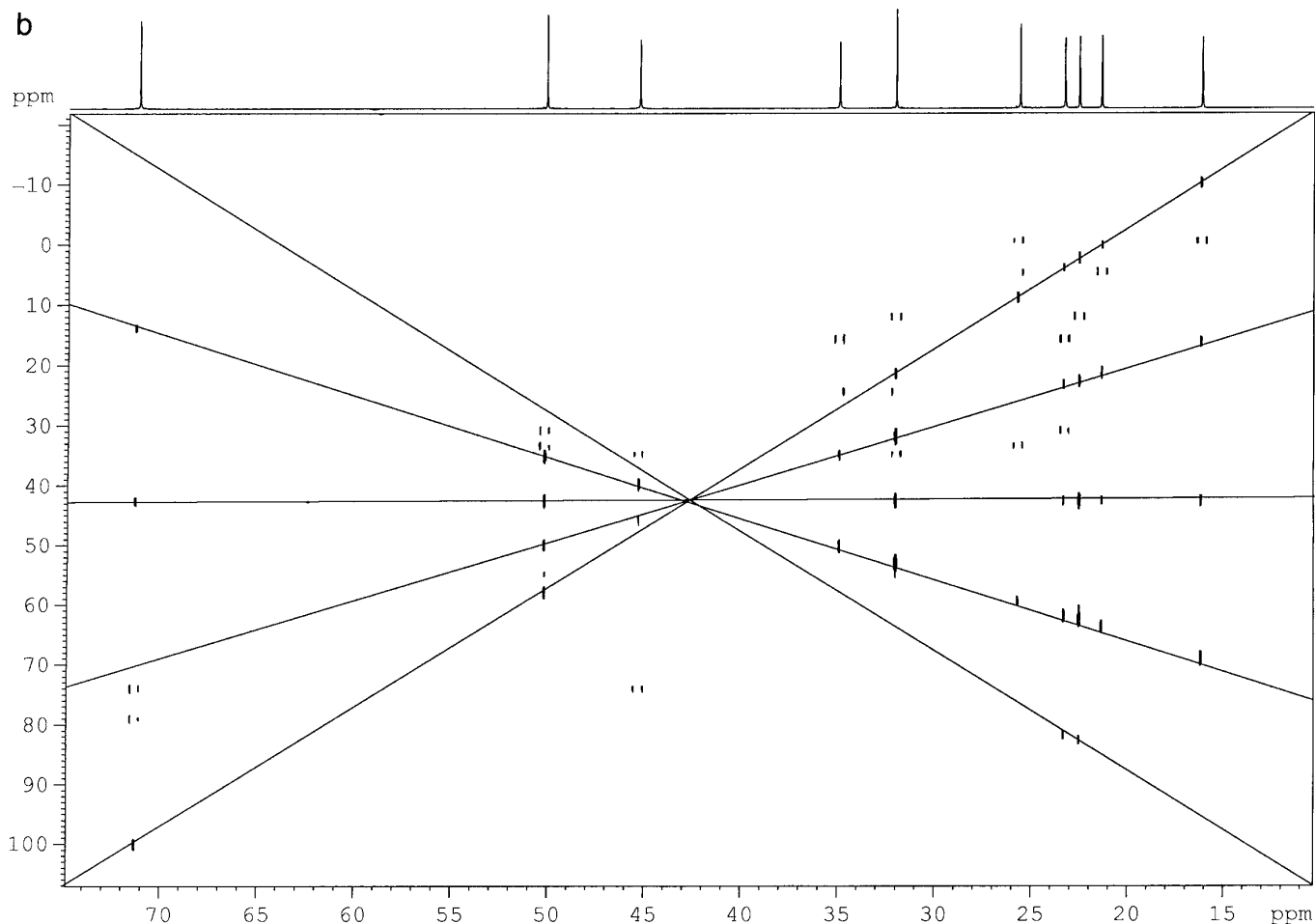


FIG. 1—Continued

destroying the magnetization recovery that occurs during the data acquisition period, they seriously reduce the sensitivity of the 2D-INADEQUATE experiment.

4. A PFGS-2D-INADEQUATE experiment (18) was performed with gradients $G_1 = 20$ G/cm during the evolution time and $G_2 = 40$ G/cm during the acquisition period, respectively (time duration = 1 ms), and with a read pulse of 120° for obtaining an optimum detection of the N-type spectrum. Although half of the intensity is lost in this PFGS experiment compared to the phase-cycled one, no MQ artifacts were observed because only the proper coherence transfer pathway is selected and thus unwanted magnetization is suppressed. In addition, inclusion of two identical gradients, symmetrically on each side of the 180° pulse in the excitation period, improves the spectrum cleanliness, but sensitivity is halved because only one coherence pathway is selected.

5. The problem which now remains is to clearly and accurately determine the origin of this undesirable labeled residual magnetization. In other terms, the question is whether it comes from molecules containing coupled ^{13}C - ^{13}C nuclei or only from the far more abundant molecules

with an isolated ^{13}C nucleus. To gain insight on this point, we have run a triple quantum (TQ) variant of the 2D-INADEQUATE experiment in which the second 90° pulse of the excitation period was phase shifted by 90° compared to the first one (3). To select TQ coherences, all the phases of the three excitation pulses were incremented by 30° giving, with CYCLOPS addition, an overall cycle of 48 steps. Because of the highly improbable creation of TQ coherence (only about 1 molecule in 10^6 can yield it), no signal at all should be observed in this experiment. This is in fact the case when using the same long relaxation delay ($D = 4$ s) as in the first conventional experiment described in Fig. 1a. In contrast, a large number of artifacts appear when the recording is performed under rapid pulsing conditions ($D = 0.7$ s). As even MQ coherences are filtered out (3), it is clear that these spurious signals cannot be generated either by a two spin order longitudinal magnetization or by any ZQ coherence and that they must result only from isolated noncoupled carbon-13 nuclei.

Although the subtraction procedure of these unwanted signals is adequate in the case of a long relaxation delay, it

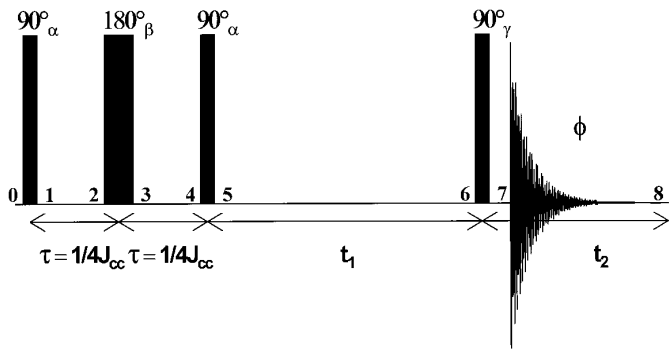


FIG. 2. Basic 2D-INADEQUATE sequence. Narrow and thick rectangles in the diagram represent 90° and 180° pulses, respectively. α and β , which are, respectively, the phases of the 90° and of the 180° pulses of the excitation period, are incremented by 45° in the experiment. The phases γ and ϕ of the read pulse and of the receiver, respectively, are incremented by 90° (Table 1). Numbers on the sequence timing are used in the text to define the various density operators.

may be not effective for only very partial longitudinal relaxation. It is thus clear that a revised phase cycling is needed in order to effectively remove these artifacts and one has to consider phase cycling in pairs of scans rather than in a single scan to get satisfaction, as will be discussed in more detail later.

THEORY

To facilitate evaluation of different phase cycles, it proves convenient to derive a general expression for the longitudinal magnetization generated on an isolated carbon-13 nucleus at the start of t_2 by the 2D-INADEQUATE sequence. The best way to do this is to use the popular product operator formalism (19–21) on a single spin $I = \frac{1}{2}$ with frequency

TABLE 1

Standard Phase Cycling for the 2D INADEQUATE Experiment in Magnitude Mode with Quadrature Detection in the ω_1 Dimension

α	(8)	0 1 2 3 4 5 6 7 2 3 4 5 6 7 0 1
		4 5 6 7 0 1 2 3 6 7 0 1 2 3 4 5
β	(8)	0 1 2 3 4 5 6 7 2 3 4 5 6 7 0 1
		4 5 6 7 0 1 2 3 6 7 0 1 2 3 4 5
		4 5 6 7 0 1 2 3 6 7 0 1 2 3 4 5
		0 1 2 3 4 5 6 7 2 3 4 5 6 7 0 1
γ		0 3 2 1 0 3 2 1 1 0 3 2 1 0 3 2
		2 1 0 3 2 1 0 3 3 2 1 0 3 2 1 0
ϕ		0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1
		2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3

Note. (8) indicates that the phase increment is equal to $45^\circ = 360^\circ/8$; all the phases are multiples of 45° . If no phase increment is indicated, the default value is 4 and all the phases are multiple of 90° . The α , γ , and ϕ phase cycles are repeated twice in order to complete the 64-step full cycle.

Acq i : Frequency-Labelled Longitudinal Magnetization

$$M_{zr}^i(\omega t_1, 0)$$



$$S_{zr}^{i+1}(\rho\omega t_1, \omega t_2), \rho = 0, \pm 1, \pm 2$$

Acq $i+1$: Spurious Phase (or Amplitude) Modulated Signal

FIG. 3. Sketch of the formation of MQ artifacts during the 2D-INADEQUATE experiment. The incompletely relaxed frequency-labeled longitudinal magnetization at scan i M_{zr}^i is recycled through the subsequent scan $i + 1$ in a frequency-labeled transverse magnetization S_{zr}^{i+1} .

offset ω , ignoring spin–spin coupling for the sake of clarity. We consider by way of example two consecutive acquisitions defined as 1 and 2. We assume that transverse magnetization cannot contribute to rapid pulsing artifacts because the overall repetition rate of the sequence ($\text{Acq} + D > 1.1$ s) is largely greater than the inhomogeneous broadening ($1/T_2^* \sim 200$ ms) (8, 9). We represent the RF phase shifts as α for the two sandwich 90° pulses since they should be the same in order to excite an even DQ coherence (3, 19) and as β and γ , respectively, for the remaining refocusing 180° pulse and 90° read pulse.

1. Ideal 90° and 180° Excitation Pulses

First, we assume ideal strong pulses, that is, we neglect the (unavoidable) B_1 inhomogeneity across the sample volume and off-resonance effects on the coherence transfer process. After some tedious algebra, it is readily shown that we are left at the end of the first acquisition (point 8 on the pulse sequence in Fig. 2) with a frequency-labeled longitudinal magnetization which can be written as (9, 14)

$$\sigma_8 = I_z \sin[2(\alpha_1 - \beta_1)] \sin(\omega t_1 + \alpha_1 - \gamma_1) \times \exp\left[-\left(\frac{t_1}{T_2^*}\right)\right] \exp\left[-\left(\frac{t_2 + D}{T_1}\right)\right]. \quad [1]$$

Here D is the relaxation delay between scans, the other symbols having their usual meaning. In principle, in the 2D-INADEQUATE experiment phase cycling, we always have $|\alpha - \beta| = k\pi/2$, $k = 0, 1, 2, \dots, p$, so that this term [1] should always be equal to zero, whatever the relaxation delay D . Indeed, the degree of cancellation of the sine function largely depends (1) on the phase instabilities (e.g., glitches), (2) on the inaccuracies in the phase shifter, and finally (3) on detector imperfections. Even with highly advanced digital electronics, phase stability and phase reproducibility better

than 0.05° cannot be expected. This means that the sine function always remains and varies in the range 0 to 3.5×10^{-3} . Although this amplitude looks very small, it is by no means completely negligible compared to the small amount of the doubly marked ^{13}C - ^{13}C isotopomers; in other words, because the molecules having only one carbon-13 are more abundant by two orders of magnitude, such a contribution cannot be neglected. In theory, these small phase errors should be compensated for by the CYCLOPS.

2. Imperfect 180° Pulse

Now, let us examine the B_1 inhomogeneity effects across the sample and the off-resonance effects on the coherence transfer pathway. Since the 180° pulse is never perfect and may be too weak to achieve proper inversion of carbon-13 spin at large offsets, transverse magnetization is created as well as possible MQ coherences in scalar coupled systems (3). In principle, phase alternation of the inversion 180° pulse by retaining the phase of the read pulse may remove most of these artifacts in the 2D-INADEQUATE experiment, providing their magnitudes remain constant from one scan to the other. Unfortunately, this is not the case for rapid pulsing experiments. One can also argue that addition of a full ‘‘Exorcycle’’ should be the proper phase cycling of the 180° pulse for removing all ‘‘ghost’’ and ‘‘phantom’’ signals (22). Indeed, we have observed that the spectra are of a worse quality with ‘‘Exorcycle’’ than without it: A large number of ZQ and SQ artifacts are generated by the ‘‘Exorcycle’’ for reasons which are discussed later. As before, a frequency-labeled residual magnetization persists at the end of a given acquisition; under the next pulse sequence, it leads to frequency-modulated signals during the subsequent evolution, giving in turn MQ artifacts after double Fourier transformation.

Let us suppose that such 180° pulse imperfections and offset effects can be accounted for to a first approximation by replacing the π pulse by a $\pi - \delta$ pulse; $\delta = \delta(\mathbf{r}, \omega)$ is a measurement of the 180° pulse imperfections both across the sample and along the carbon-13 chemical shift range. Although it is well known that precise chemical shift refocusing is precluded in such cases, deviations are nevertheless extremely small, even for large 180° pulse mis-settings (up to 20%, roughly) (3). The other important source of errors comes from the imperfect spin inversion of the coupling partners (22). This last effect is responsible for the MQ artifacts, as we now show.

We assume that, from the theoretical point of view, this 180° pulse imperfection needs a first-order correction to the density operator for an isolated carbon-13 that we denote $\Delta\sigma$; this is evaluated at point 4 in the pulse sequence, just before the second 90° excitation pulse (Fig. 2) as

$$\begin{aligned} \Delta\sigma_4 &= -I_z \sin \delta \cos(\omega\tau + \alpha_1 - \beta_1) \\ &\approx -I_z \sin \delta \cos(\alpha_1 - \beta_1). \end{aligned} \quad [2]$$

In the right-hand side (RHS) term of Eq. [2], we have neglected the small chemical shift dispersion due to the incomplete refocusing by the imperfect 180° pulse (see later discussion). This term gives, at the end of the evolution period 6, a frequency-labeled transverse magnetization

$$\begin{aligned} \Delta\sigma_6 &= -\sin \delta \cos(\alpha_1 - \beta_1) \\ &\quad \times [I_x \sin(\alpha_1 + \omega t_1) - I_y \cos(\alpha_1 + \omega t_1)], \end{aligned} \quad [3]$$

which is then converted by the 90° read pulse in [4] at the beginning of the detection period 7:

$$\begin{aligned} \Delta\sigma_7 &= I_z \sin \delta \cos(\alpha_1 - \beta_1) \cos(\omega t_1 + \alpha_1 - \gamma_1) \\ &\quad - [I_x \cos \gamma_1 + I_y \sin \gamma_1] \sin \delta \cos(\alpha_1 - \beta_1) \\ &\quad \times \sin(\omega t_1 + \alpha_1 - \gamma_1). \end{aligned} \quad [4]$$

The second RHS term in Eq. [4] should give SQ artifacts ($p = \pm 1$); in fact, this is very similar to the transverse magnetization resulting from an isolated single carbon-13 spin and therefore it is also eliminated during the acquisition by the addition–subtraction process according to the usual phase cycling. As a result, this spurious signal does not contribute to rapid pulsing artifacts.

In contrast, the first RHS term represents a frequency-labeled longitudinal magnetization which is recycled, but attenuated, by the factor $E_1 = \exp[-(t_2 + D)/T_1]$ by the next pulse sequence and thus gives rise to MQ artifacts ($p = 0, \pm 1, \pm 2, \dots$). This is shown by Eq. [5], which is the resulting density operator $\Delta\sigma_6^{(2)}$ at time 6 (Fig. 2) after the second scan:

$$\begin{aligned} \Delta\sigma_6^{(2)} &= I_z \cos[2(\beta_2 - \alpha_2)] \sin \delta \cos(\alpha_1 - \beta_1) \\ &\quad \times \cos[\omega t_1 + \alpha_1 - \gamma_1] \\ &\quad + [I_x \cos(\omega t_1 + \alpha_2) + I_y \sin(\omega t_1 + \alpha_2)] \sin \delta \\ &\quad \times \cos(\alpha_1 - \beta_1) \sin[2(\alpha_2 - \beta_2)] \\ &\quad \times \cos(\omega t_1 + \alpha_1 - \gamma_1). \end{aligned} \quad [5]$$

Equations [4] and [5] deserve further comment.

(i) Because of the $\cos(\alpha_1 - \beta_1)$ dependence of the signals, one can argue that shifting the phase of the 180° refocusing pulse by 90° ($\beta = \alpha \pm \pi/2$) compared to that of the 90° exciting pulses may remove the artifacts. We have observed that there is no significant improvement, but, on the contrary, SQ artifacts are even more abundant. In addition, two supplementary lines of SQ artifacts which are displaced from the main one by about ± 2000 Hz in the F_1 dimension seem to appear. Clearly, this corresponds to an overall frequency shift of all the carbon-13 resonances during t_1 , as will be explained later.

(ii) In order to compensate pulse imperfections for RF inhomogeneities, off-resonance effects and mis-set pulse lengths, composite pulses have been claimed to be one of the interesting alternatives to classical pulses. We have therefore investigated MLEV type pulses (23) $90_x^\circ 180_y^\circ 90_x^\circ$ as a refocusing and population inversion pulse instead of a single 180° pulse in the sequence, with very disappointing results. The variant cluster $90_x^\circ 240_y^\circ 90_x^\circ$ (24) also proves to be of no use. Even the most sophisticated composite pulse sequences which have been specially developed for the 1D-INADEQUATE experiment by Levitt and Ernst (25) for remedying imperfections in 90° excitation and read pulses, as well as in the 180° pulse, give unsatisfactory results. These observations are in agreement with previous conclusions by Schenker and Von Philipsborn (26), who have also remarked that composite pulses are not useful in 2D-INADEQUATE experiments.

Although the efficiency of composite pulses is easily understandable in 1D experiments where the addition–subtraction is fully operative, the situation in 2D spectroscopy is completely different. Because of the residual frequency-labeled longitudinal magnetization which is recycled from one scan to the other, analysis of MQ artifacts needs a careful consideration of pairs of two consecutive scans rather than individual scans. Specifically, all the phases of the 90° DQ excitation pulses, 180° refocusing and inversion pulses, and 90° read pulse should be examined as a whole in two successive scans. For example, we have observed that using the WALTZ sequence $90_x^\circ 180_x^\circ 270_x^\circ$ (27) as a refocusing and inversion composite pulse in place of a single 180° pulse results in a very significant improvement; this is because in the WALTZ cluster all the pulses have either identical or opposite phases, but never quadrature phases, so that the artifacts generated by a subsequent scan are blocked by the next scan (*vide infra*). Nevertheless, and as expected, composite pulses provide good results when long interscan delays are used.

3. Imperfect 90° and 180° Excitation Pulses

Finally, the tendency for the 2D-INADEQUATE experiment to exhibit rapid pulsing artifacts resulting from molecules containing only one carbon-13 should be investigated by a close examination of the relative phases of the cluster of the excitation pulses $90_\alpha^\circ - 180_\beta^\circ - 90_\alpha^\circ$. We represent each individual pulse by a rotation operator $R^{\mathbf{u}}(\theta) = \exp(-i\theta \mathbf{u} \cdot \mathbf{I})$, where \mathbf{u} is a unit vector in the direction of the \mathbf{B}_{eff} field in the rotating frame (3). We note as $U_{\alpha,\beta}^{\text{exc}}$ the propagator of the excitation cluster to act from the right on the density operator σ in order to retain chronological correspondence with the pulse sequence, in accordance to Ernst's convention (3, 19, 25):

$$\begin{aligned} U_{\alpha,\beta}^{\text{exc}} &= \exp(-i\alpha F_z) \exp[i(\pi/2 - \delta/2)F_x] \exp(i\alpha F_z) \\ &\times \exp(iH_0\tau) \exp(-i\beta F_z) \exp[i(\pi - \delta)F_x] \\ &\times \exp(i\beta F_z) \exp(iH_0\tau) \\ &\times \exp[i(\pi/2 - \delta/2)F_x] \exp(i\alpha F_z). \end{aligned} \quad [6]$$

Here $F_\nu = \sum_{i=1}^N I_\nu$ for $\nu = x, y, z$ and $H_0 = \sum_{i=1}^N \Delta\omega_{oi} I_{zi}$ for N carbon-13 isolated spins i , where $\Delta\omega_{oi}$ is the resonance offset of the spin i . Because $\delta = \delta(\mathbf{r}, \omega)$ remains very small along the whole range of the carbon-13 spectrum, we can assume that there is only, in a first-order approximation, a negligible imperfection of the magnetization refocusing at the end of the excitation period (point 4 in the pulse sequence in Fig. 2); we thus neglect chemical shift evolution during the excitation sequence which appears as a second-order correction. $U_{\alpha,\beta}^{\text{exc}}$ is then approximated as

$$U_{\alpha,\beta}^{\text{exc}} \approx \bar{R}_\alpha(\pi/2 - \delta/2) \bar{R}_\beta(\pi - \delta) \bar{R}_\alpha(\pi/2 - \delta/2), \quad [7]$$

where $\bar{R}_u(\theta)$ is the phase reversal, that is, the inverse of $R_u(\theta)$ [$R_u(\theta) \bar{R}_u(\theta) = 1$].

We first examine the case of a single 180° pulse in the middle of the excitation sequence. If all the phases are equal to zero, that is, they are all x -pulses, the propagator should read as

$$\begin{aligned} U_{x,x}^{\text{exc}}(i) &\approx \bar{R}_x(\pi/2 - \delta/2) \bar{R}_x(\pi - \delta) \\ &\times \bar{R}_x(\pi/2 - \delta/2) \approx \bar{R}_x(-2\delta). \end{aligned} \quad [8]$$

It thus generates a transverse magnetization $-I_y \sin 2\delta$ which is labeled during t_1 evolution and detected during t_2 acquisition at the scan i . Suppose that at the next scan $i + 1$ we invert the 180° pulse phase so that $\beta = \pi$ while α remains equal to zero. The propagator can then be concatenated according to

$$\begin{aligned} U_{x,-x}^{\text{exc}}(i + 1) &\approx \bar{R}_x(\pi/2 - \delta/2) \\ &\times R_x(\pi - \delta) \bar{R}_x(\pi/2 - \delta/2) \approx 1. \end{aligned} \quad [9]$$

No transverse magnetization will be created by this phase arrangement. As a result, the residual labeled longitudinal magnetization originating from the scan i is then blocked at the next scan $i + 1$; no additional labeling takes place and the addition–subtraction process can properly cancel all residual magnetization, whether it is labeled or not labeled, from any isolated carbon-13 nucleus.

Now, consider a 180° pulse whose phase is in quadrature compared to the 90° pulses, for example, $\alpha = 0, \beta = \pi/2$. According to the well-known identity [10] (28),

$$R_x(\pi)R_y(\delta) = R_y(-\delta)R_x(\pi), \quad [10]$$

the propagator is easily transformed as

$$\begin{aligned} U_{x,y}^{\text{exc}}(i) &= \bar{R}_x(\pi/2 - \delta/2)\bar{R}_{\pm y}(\pi - \delta)\bar{R}_x(\pi/2 - \delta/2) \\ &\approx \bar{R}_{\pm z}(\pi - \delta)\bar{R}_x(\pi). \end{aligned} \quad [11]$$

The neat effect of this propagator on an isolated spin is to induce a chemical shift precession by an angle $\pm(\pi - \delta)$ during 2τ . This is indeed what has been experimentally observed (see earlier discussion).

From the same kind of arguments, replacement of the 180° pulse by a MLEV composite pulse is of no use for minimizing artifacts. It is also straightforward to show that the quadrature phases of the two 90° pulses and of the 180° pulse, respectively, may lead to additive SQ artifacts along two lines which are parallel to the main one. In contrast, WALTZ composite pulses should be of help to improve the spectrum quality, providing that in two successive scans their phases are in opposition: Whereas WALTZ_x composite pulses may generate transverse magnetization, a WALTZ_{-x} composite pulse in the next scan blocks it so that artifacts are greatly reduced (*vide supra*). Notice also that the WALTZ sequence has a better offset compensation than the MLEV sequence with an additional advantage of being less sensitive to misadjustments of the relative RF phases (3, 27).

DISCUSSION

The preceding theory has demonstrated that a proper phase cycling sequence for the 2D-INADEQUATE experiment should incorporate error compensation schemes into two successive excitation periods in order to minimize rapid-pulsing artifacts. Although a careful calculation of propagators is helpful, it is not possible, however, to give an exact recipe for obtaining a completely clean spectrum. Nevertheless, some indications are now emerging:

(i) The different parts of the pulse sequence should be differentiated with respect to their function. Thus, in the 2D-INADEQUATE experiment, there is a clear distinction between the excitation pulses and the read pulse and they are well separated by the phase cycling procedure. As indicated earlier, the minimum cycle required should include 64 steps in order to perform quadrature detection in t_1 and in order to compensate for pulse errors in the DQ exciting period. The final CYCLOPS scheme is aimed at reducing quadrature detection misadjustments during the t_2 acquisition.

(ii) Since rapid pulsing artifacts mainly result from angle errors in 90° and 180° pulses in the exciting sequence during two consecutive scans, it is of prime importance to rearrange phases of a given pulse in pairs of scans. So far, a dramatic improvement is realized very simply by reversing the 180° pulse phases in two consecutive scans as shown in Eqs. [8]

and [9]. In order to avoid the effect of dummy scans (9), we have observed that the best implementation should begin with a 180° pulse with the phase π ($\beta_1 = \pi$). In addition, a concomitant phase inversion of the 90° pulses bracketing the excitation period, while retaining a constant 180° pulse phase in pairs of consecutive scans, leads to the most effective cancellation of MQ artifacts. ZQ and SQ artifacts are now barely detectable. Only DQ artifacts are clearly visible, mainly at the chemical shifts of the carbons numbered 9 (16.1 ppm), 8 (21.4 ppm), 10 (22.5 ppm), 3 (23.3 ppm), 5 (32 ppm), and 2 (50.1 ppm), which all have the longest longitudinal relaxation times (respectively, 1.6, 1.5, 1.5, 1.7, 2.0, and 2.0 s). Figure 4 shows the region of the DQ transitions between carbons 7 and 9 and between carbons 7 and 8, which is one of the parts most spoiled by MQ artifacts. The spectrum was recorded in the TPPI (11) phased pure absorption mode with positive and negative contours plotted without distinction (Fig. 4a). Figure 4b represents the corresponding horizontal cross-sections along the F_2 dimension from the DQ transition between carbons 7 and 9 (top) to that one between carbons 7 and 8 (bottom), through the rows of artifacts due to the residual labeled longitudinal magnetization of carbons 8, 10, and 3, from top to bottom, respectively. It is clearly evident that the MQ artifacts are in dispersion, whereas true signals are naturally antiphase doublets in this dimension.

(iii) Such a difference in phase during acquisition is indeed predictable from operator product formalism analysis. DQ coherence in t_1 is detected as an antiphase magnetization term ($2I_xS_z + 2S_xI_z$) in t_2 , while an artifact SQ coherence during t_1 is observed as a transverse magnetization $-I_y$ when all the pulse phases are along x ($\alpha = \beta = \gamma = 0$) (3, 19). Elimination of the spurious signals is therefore extremely simple. It is sufficient to rearrange the read pulse phases γ and the receiver phases φ in two consecutive scans so that their absolute relative phases $|\varphi - \gamma|$ are always in opposition, for example, $\gamma_i = \varphi_i = 0$ and $\gamma_{i+1} = 3\pi/2$, $\varphi_{i+1} = \pi/2$. With this combination, two consecutive artifacts have always their phases in opposition and are then canceled by addition while the desired double quantum coherences are correctly added (3, 29).

The new final phase cycles used for magnitude mode and for TPPI (11) phased spectra are presented in Table 2. Figure 5a shows the results for menthol in magnitude mode, while Fig. 5b is the excerpt of the same region as in Fig. 4a and plotted under exactly the same conditions, especially for the contour levels. Compared to Fig. 1, obtained with the classic phase cycling scheme shown in Table 1, spurious signals obscuring the interesting ones seem to be completely absent when this new phase cycle is used. The tremendous improvement in spectrum quality compares very well with that one obtained in PFGS spectroscopy, but without a loss in sensitivity by a factor of $\sqrt{2}$ for the same underlying resolution in F_1 and the same total experimental time for each experi-

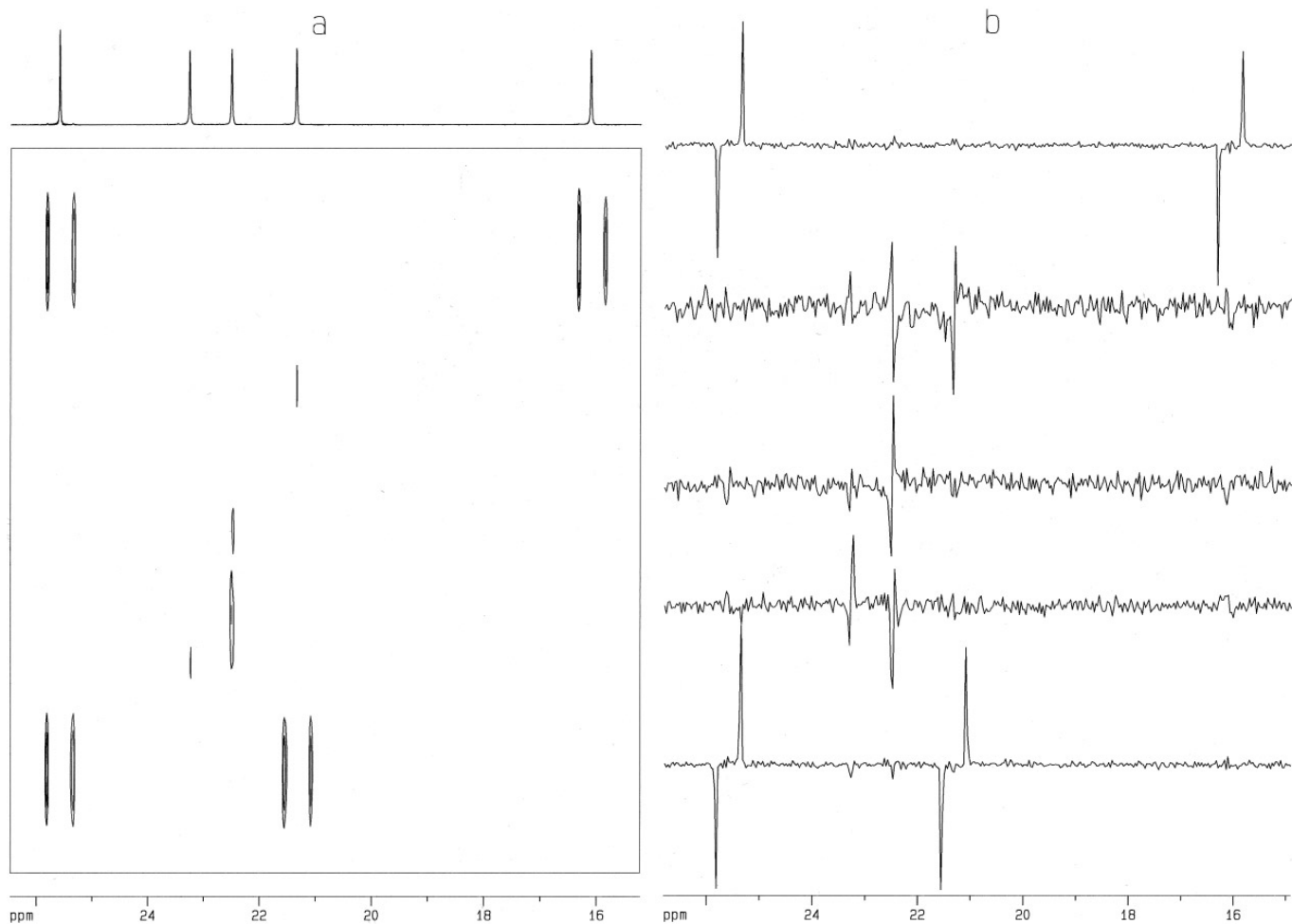


FIG. 4. Excerpt of the pure-phased 2D-INADEQUATE spectrum recorded with slight modifications of the conventional phase cycling (see text). The interscan delay $D = 0.7$ s. (a) Contour plot of the region around the DQ transitions between the carbons 7 and 9 and between the carbons 7 and 8. (b) Horizontal cross-sections along the DQ transition between the carbons 7 and 8, along the artifacts resulting from an incompletely relaxed longitudinal magnetization from the carbons 3, 10, and 8 and along the DQ transition between the carbons 7 and 9, from bottom to top, respectively. For the sake of clarity, the lines of artifacts were plotted with a distinct amplitude scale compared to the true signals.

ment. The suppression of all the unwanted MQ artifacts which have long dispersive tails along the F_1 dimension results in an important reduction in t_1 noise, making it easier to recognize correlations.

In addition, it also seems that, as a side effect, a significant enhancement in sensitivity is gained with this new phase cycling. To better understand this somewhat surprising feature, we have compared the signal-to-noise ratio obtained in each of the 10 cross-sections along the F_2 dimension using the classical phase cycling and the new rearrangement, respectively. An increase by a factor ranging from 1 to 2 is measured, depending on the row which is examined. For example, practically no improvement in sensitivity is observed for the DQ transition between the carbons C1 and C2 because no MQ artifacts appear along this line; in contrast, a important increase by a factor greater than 2 is measured along the DQ transition between the carbons C7 and C9

where the line reveals a numerous of DQ artifacts. Furthermore, we have compared integrations on two spectral regions, the first being almost completely free of artifacts and the second being one of the most affected, and this for each type of phase cycling. We have observed that the new phase cycling combination leads to an increase in sensitivity by about 30 to 40%.

Sensitivity loss due to resonance offset effects has long been noticed in the literature (25, 26, 30–32), and the application of composite pulses to compensate this phenomenon has been reported (25, 26, 30–32) in the case of long relaxation delays and providing special precautions are taken in choosing the phases of the clusters of phase-shifted RF pulses phases (32–34). Recently, Torres *et al.* (32) have found that the utilization of symmetric phase-alternating pulses which allow good compensation of imperfections in pulses and in delays is very efficient for recovering the lost

TABLE 2

Modified Phase Cycling for the 2D INADEQUATE Experiment with Quadrature Detection in the ω_1 Dimension

		Magnitude mode		Phase sensitive mode (TPPI)
α	(8)	0 0 4 4 0 0 4 4 1 1 5 5 1 1 5 5	(8)	0 0 4 4 0 0 4 4
		2 2 6 6 2 2 6 6 3 3 7 7 3 3 7 7		2 2 6 6 2 2 6 6
		4 4 0 0 4 4 0 0 5 5 1 1 5 5 1 1		4 4 0 0 4 4 0 0
		6 6 2 2 6 6 2 2 7 7 3 3 7 7 3 3		6 6 2 2 6 6 2 2
β	(8)	4 0 0 4 4 0 0 4 5 1 1 5 5 1 1 5	(8)	4 0 0 4 4 0 0 4
		6 2 2 6 6 2 2 6 7 3 3 7 7 3 3 7		6 2 2 6 6 2 2 6
		0 4 4 0 0 4 4 0 1 5 5 1 1 5 5 1		0 4 4 0 0 4 4 0
		2 6 6 2 2 6 6 2 3 7 7 3 3 7 7 3		2 6 6 2 2 6 6 2
γ		0 1 2 3 1 0 3 2 3 0 1 2 0 3 2 1		0 1 2 3 1 0 3 2
		2 3 0 1 3 2 1 0 1 2 3 0 2 1 0 3		2 3 0 1 3 2 1 0
ϕ		0 3 2 1 3 0 1 2		0 3 2 1 3 0 1 2

Note. (8) indicates that the phase increment is equal to $45^\circ = 360^\circ/8$; all the phases are multiples of 45° . If no phase increment is indicated, the default value is 4 and all the phases are multiples of 90° . The γ and ϕ phase cycles are repeated twice and eight times, respectively, in order to complete the 64-step full cycle for the magnitude mode; for phase-sensitive experiments, these phase cycles are repeated twice and four times, respectively.

signal. A sensitivity significantly higher than that of the conventional 2D-INADEQUATE experiment—by up a factor of 2 for some carbons—has been determined experimentally. Their analysis is based on a formal operator analogy between the DQ excitation sequence and the behavior of an inhomogeneous RF pulse as previously demonstrated by Barbara, Tycko, and Weitekamp (35) in studying the influence of a range of dipolar couplings in liquid crystal solvents. Their proposed compensation sequence is expected to give sensitivity enhancement by eliminating the offset-dependent phase shifts introduced by single pulses and by providing a more efficient coherence transformation during the sequence. Our present results are in full agreement with these observations: Alternation of pulse phases, as long as possible, in two consecutive scans is thus an extension of the ideas of the preceding authors concerning the compensation of RF inhomogeneities, but between a pair of successive sequences rather than in a single sequence. This neatly accounts for the observation that our new phase cycling gives not only an improvement in spectrum quality, but also a gain in sensitivity. Finally, one can also argue that the recommended phase combination improves the efficiency of the addition–subtraction process during the acquisition by reducing to a minimum the signal from the most numerous molecules containing a single carbon-13 nucleus and, consequently, allows better detection of the less abundant doubly enriched molecules.

CONCLUSION

In this paper, we have developed a formal analogy between rapid-pulsing MQ artifacts generated in the 2D-INADEQUATE spectroscopy and those generated in the COSY–DQF experiment. The prominent effects of the imperfections in the excitation pulses have been emphasized,

and it has been shown that a full rearrangement of all the pulse phases in two consecutive scans rather than in a single scan is very efficient in overcoming most of the difficulties. First, an improvement in spectrum quality is obtained so that it is easier to recognize correlations in cases of a poor signal-to-noise ratio. Second, it has been demonstrated that the proposed new combination also leads to a significant increase in the sensitivity. As the 2D-INADEQUATE experiment is typically a very time-consuming one, often lasting several days, even a small enhancement in the signal-to-noise can represent an important gain in experimental time. Finally, very recently, numerous publications have demonstrated a renewed interest in the 2D-INADEQUATE sequence, with particular effort being focused on improving the overall performance of this technique. Examples include computation of local and global symmetry (36–38), the use of cross-polarization (39) and inverse detection (40–44), and the development of well-designed complex pulse sequences for PFGS spectroscopy (45, 46). In our opinion, because the new proposed phase cycling is based on a very simple strategy, it appears as a prerequisite to these more elaborated current approaches.

The present paper is focused on the insensitive carbon-13 2D-INADEQUATE experiment where only direct connectivities are generally observed, so that it is usual to record magnitude spectra as an accepted custom. Nevertheless, the preceding conclusions also apply to all other 2D double-quantum experiments. 2D double-quantum proton spectroscopy which has been demonstrated to be an invaluable alternative to DQF-COSY for observing hidden correlations in protein in water may be an illustrative example of the improvement gained by the new phase cycling (see Footnote 2). When sensitivity is not a major problem, this new complete phase cycling can be reduced by a factor of 4 by omitting CYCLOPS on modern spectrometers equipped with

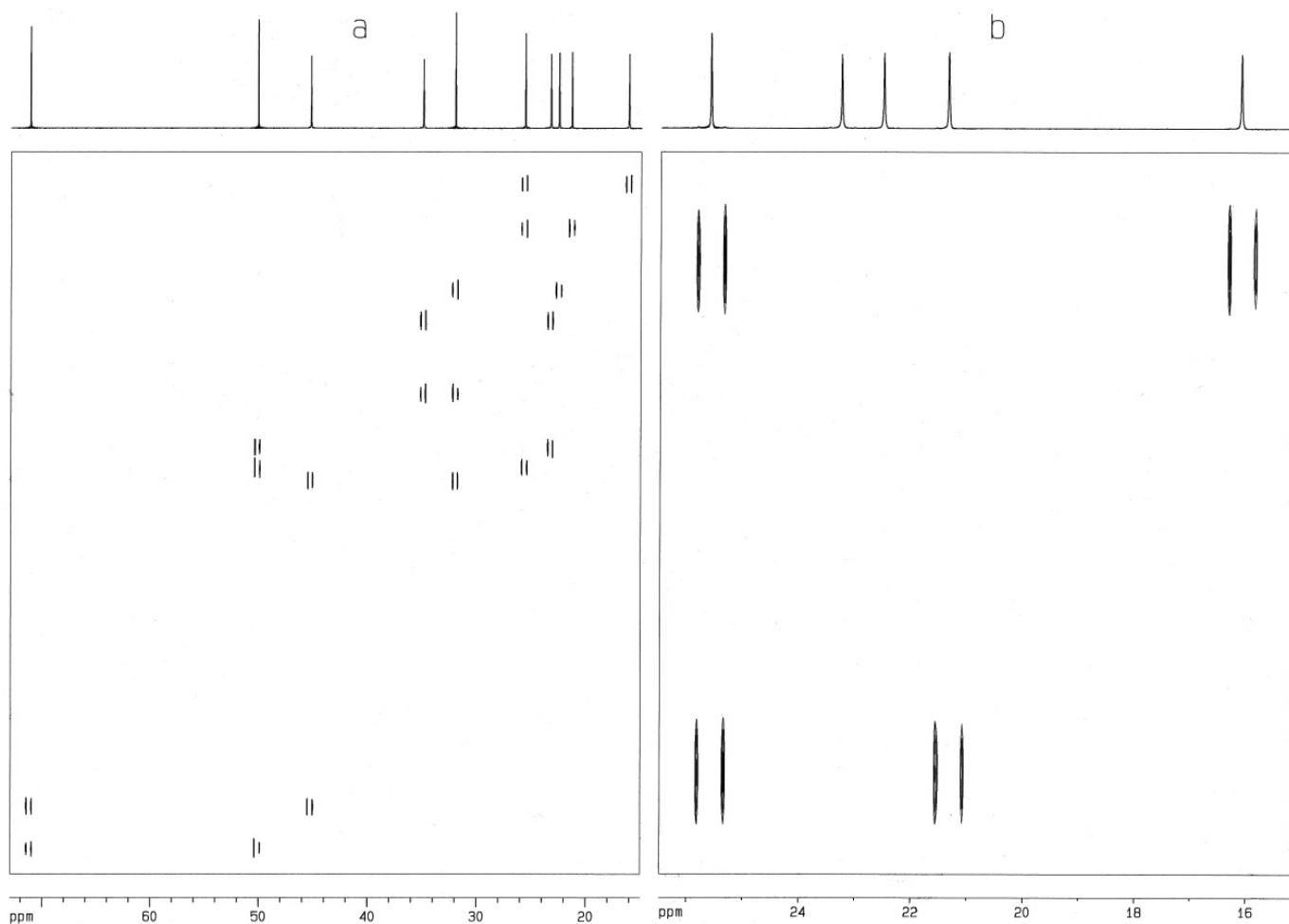


FIG. 5. 2D-INADEQUATE spectrum recorded with the new phase cycling in Table 2. The interscan delay is $D = 0.7$ s. (a) Contour plot in magnitude mode. (b) Excerpt of the pure phased spectrum showing the same region as in Fig. 3a and plotted with the same conditions.

digital detection quadrature, data oversampling, and digital filters. As pure absorption spectra should be preferred, since they have better sensitivity and better resolution than magnitude spectra and provide unique information on amino acid residues, degenerated resonances, and remote connectivities (4, 5), only an eight-step phase cycling which is easily derived from the one given in Table 2 may be used.

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