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Molecular structure from a single NMR sequence (fast-PANACEA)

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ABSTRACT

The PANACEA experiment combines three standard NMR pulse sequences (INADEQUATE, HSQC and HMBC) into a single entity, and is designed for spectrometers with two or more receivers operating in parallel. For small molecules it offers a direct route to molecular structure. Often the INADEQUATE feature is the rate-determining step, being limited by the low natural abundance of directly coupled ^{13}C—^{13}C pairs. This new version, fast-PANACEA, speeds up this measurement by two alternative schemes. In the first, the individual ^{13}C sites are excited by selective radiofrequency pulses acting on double-quantum coherence, and encoded according to the rows of a Hadamard matrix. The columns of this matrix are used to decode the experimental data into separate F_2 spectra. This reduction in the number of required scans secures a faster result than the conventional stepwise exploration of the evolution dimension where the Nyquist condition and the resolution requirements must both be satisfied. The second scheme makes use of multiple aliasing in the evolution dimension. Significant speed improvements are achieved by either technique, illustrated by measurements made on samples of menthol and cholesterol. A new stabilization scheme (i-lock) is introduced. This is a software program that corrects the final NMR frequencies based on the observed frequency of a strong *X*-spin signal. It replaces the conventional deuterium lock, permitting measurements on neat liquids such as peanut oil and silicone oil, and offering advantages where deuterated solvents are undesirable.

1. Introduction

The recent development of NMR spectrometers with multiple receivers operating in parallel and tuned to different nuclei [1,2] has made it possible to combine certain standard pulse sequences (for example INADEQUATE, HSQC, and HMBC) into a single entity, allowing the structure of a small molecule to be determined in a single measurement. The duration of this 'PANACEA' experiment (Protons And Nitrogen And Carbon *Et Alia*) [3,4] is often set by the intrinsically poor sensitivity of the INADEQUATE feature which is handicapped by the low probability of finding a pair of directly-bound ^{13}C spins in natural abundance samples. In many practical situations this is the rate-determining step.

Several remedies have been suggested for improving the inherent sensitivity of INADEQUATE, ranging from a symmetrisation algorithm [5], single-spin operators [6], to relaxation agents such as oxygen dissolved under pressure and augmented by the addition of artificial blood [7]. The most significant improvement comes from the introduction of a cryogenically-cooled receiver coil [8] optimized for ^{13}C detection, which offers up to an order of magnitude gain in sensitivity. This article examines two alternative schemes for speeding up PANACEA by shortening the time required to record the INADEQUATE spectrum. The first method invokes selective radiofrequency excitation encoded according to the appropriate Hadamard matrix, and the second approach employs multiple aliasing in the evolution dimension.

2. Hadamard spectroscopy

Conventionally INADEQUATE spectra are made up of four-line AX (or occasionally AB) patterns in the acquisition dimension (F_2), spread out and separated in the evolution dimension (F_1) as a function of the relevant double-quantum frequencies. Each pattern is entirely independent of the rest and identifies a direct bond between two adjacent ^{13}C spins. The Hadamard scheme proposed here employs a new method of separation, replacing the conventional evolution period with selective excitation of the ^{13}C resonances. One of the parallel outputs of the PANACEA sequence is the conventional ^{13}C spectrum; this identifies the ^{13}C chemical shifts, which can then be used to set the frequencies of an array of soft radiofrequency pulses, one for each individual ^{13}C site. The phases of these pulses are encoded (0° or 180°) according to a pattern derived from the rows of the appropriate Hadamard matrix [9–12], typically H_4, H_8, H_{12}, H_{16}, H_{20}, H_{24}, H_{28} or H_{32}. The choice of matrix order N is determined by n, the number of ^{13}C sites

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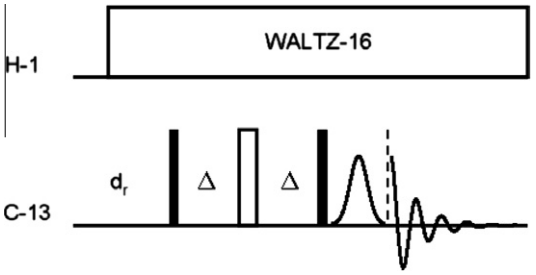

Fig. 1. Pulse sequence for generation of ^{13}C–^{13}C double-quantum coherence and subsequent conversion to observable magnetization by means of a set of selective radiofrequency pulses which replaces the conventional evolution period. The pulses are encoded (plus or minus) according to the rows of the appropriate Hadamard matrix.

in the molecule, where $N \geqslant n$. For example 14 chemical sites would require encoding with the 16 by 16 Hadamard matrix.

The PANACEA pulse sequence itself has been described in detail elsewhere [3,4]. The only modification is the new Hadamard-encoded pulse sequence set out in Fig. 1. This is the standard INADEQUATE experiment except that the double-quantum evolution period has been replaced by a set of n simultaneous selective radiofrequency pulses tuned to the pre-recorded ^{13}C chemical shift frequencies and encoded as indicated above. Once the double-quantum coherence has been excited, these selective ±90° (Gaussian) pulses convert it back into observable single-quantum coherence. In the product operator representation [13] a selective 90° radiofrequency pulse (I_X) applied to the source ^{13}C site converts double-quantum coherence into (encoded) antiphase magnetization at the target ^{13}C site (the S spins):

$$2I_XS_Y + 2I_YS_X \rightarrow 2I_XS_Y + 2I_ZS_X \quad (1)$$

The source site is defined by the irradiation frequency that was used, but the recorded signal is at the target frequency, identifying the two coupled ^{13}C spins unambiguously. There is negligible signal from the source site itself. Thus any one column of the Hadamard matrix provides the required C–C correlation information – the source radiofrequency and the target NMR response. During the

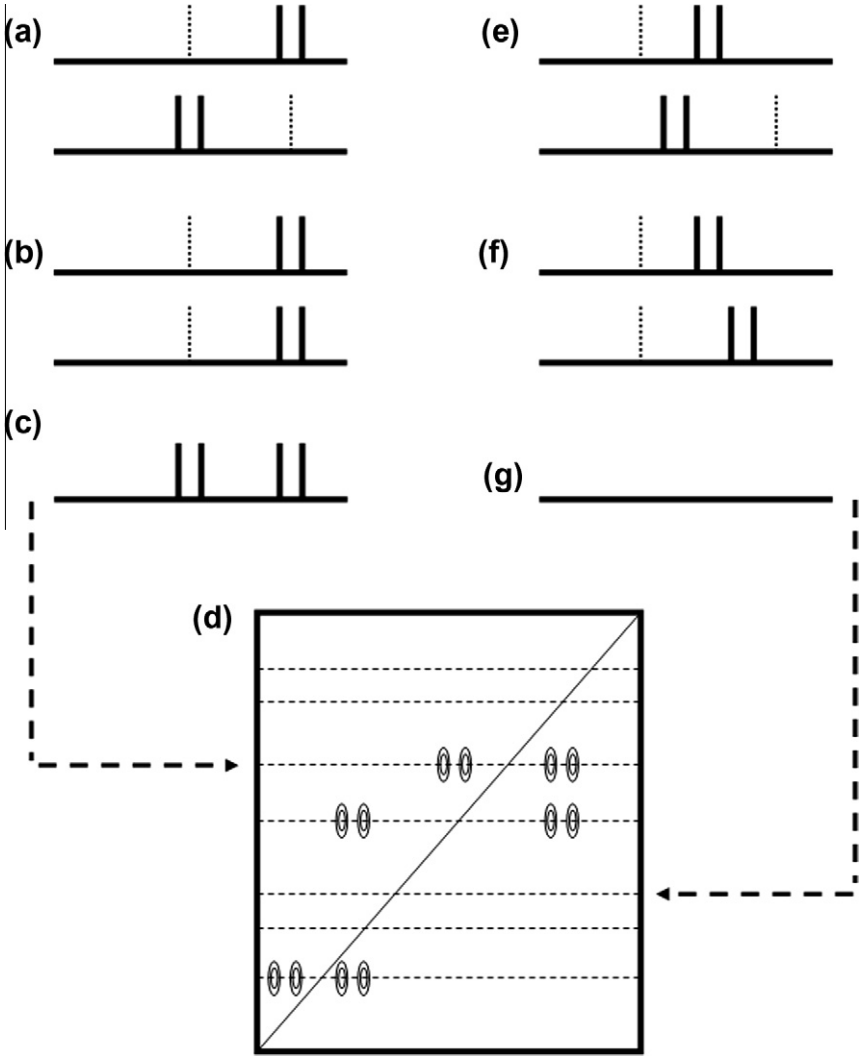

Fig. 2. Illustration of the reconstruction algorithm for converting the data from the columns of the Hadamard matrix into a more familiar format. The left column (a–c) represents genuine C–C correlations, whereas the right column (e–g) corresponds to uncorrelated C–C pairs. All possible combinations of pairs of traces are compared. The dashed lines indicate source frequencies (where the selective pulses are applied). In (b) and (f) one trace of each pair has been reflected in frequency and the two source frequencies brought into alignment. The lower-value algorithm acting on (b) retains the target signals, establishing a true correlation, but applied to (f) it suppresses these signals. Reversal of the reflection operations (c and g) generates symmetrical patterns that are used to reconstruct the INADEQUATE spectrum in its familiar format (d).

selective Gaussian pulse, J-evolution occurs under the $2I_ZS_Z$ operator, and the timing is adjusted to give in-phase magnetization at the detector:

$$2I_ZS_X \rightarrow S_Y \quad (2)$$

At the same time another column detects the reverse transfer, where an S_X pulse excites antiphase magnetization on the I spins:

$$2I_XS_Y + 2I_YS_X \rightarrow 2I_XS_Z + 2I_YS_X \quad (3)$$

During the Gaussian pulse this evolves under the $2I_ZS_Z$ operator:

$$2I_XS_Z \rightarrow I_Y \quad (4)$$

These manipulations occur in parallel but the results are separated by their different encoding patterns. The result is a set of traces that contain only signals from the target spins. Columns of the matrix that correspond to correlated ^{13}C spins generate two traces related by reflection symmetry – the target signal of one trace is aligned with the source frequency of the other, and vice versa (Fig. 2a). Conventional processing would generate a DQ-COSY type spectrum with essentially no diagonal responses, but the more familiar INADEQUATE format is to be preferred, and it is implemented by the following algorithm:

1. A Hadamard transform is applied in the F_1 dimension.
2. All possible combinations of the outputs of the columns of the Hadamard matrix are compared in pairs (Fig. 2a or e). If a pair of traces represents a true correlation, frequency reversal of one trace and realignment of the two source frequencies (represented by the dashed lines) brings the two target signals into exact alignment (Fig. 2b). In contrast, the same operation applied to a pair of traces from uncorrelated sites causes no such alignment of target signals (Fig. 2f).
3. The lower-value algorithm [14–16] is then applied to these pairs of (reflected) traces. The signals are retained for true correlations (Fig. 2b) but suppressed for uncorrelated sites (Fig. 2f).
4. Reversal of the reflection operation generates a symmetrised trace (Fig 2c or g).
5. These are used to form the familiar INADEQUATE spectrum (Fig. 2d) arrayed in the F_1 dimension according to the corresponding double-quantum frequencies calculated from the known chemical shifts.

For a Hadamard matrix of order N, a total of N scans must be performed. The time saving stems from the fact that N can be a much smaller than the number of required evolution steps in the conventional mode, determined by the Nyquist sampling condition and the desired resolution in the F_1 dimension. (A very small number of the available columns may carry no signal, owing to the slight discrepancy between the order of the matrix N and the number of different chemical sites n). This is why it is important to match N to n as closely as possible. The speed gain can be an order of magnitude or more.

A practical example is illustrated by the INADEQUATE spectrum of menthol examined as a 30% solution in $CDCl_3$. With ten carbon sites the highest possible speed advantage would be achieved with the Hadamard matrix H_{12}. In practice the H_{16} matrix was employed because it is almost as fast, and it was found that the power-of-two Hadamard matrices are more effective in suppressing instrumental artefacts not encoded by the radiofrequency modulation scheme. In Fig. 3 responses have been rearranged to match the format familiar to earlier INADEQUATE experiments, where each four-line spectrum is centred on a skew diagonal of slope two. All the expected C–C connectivities are obtained in a measurement lasting only 56 s.

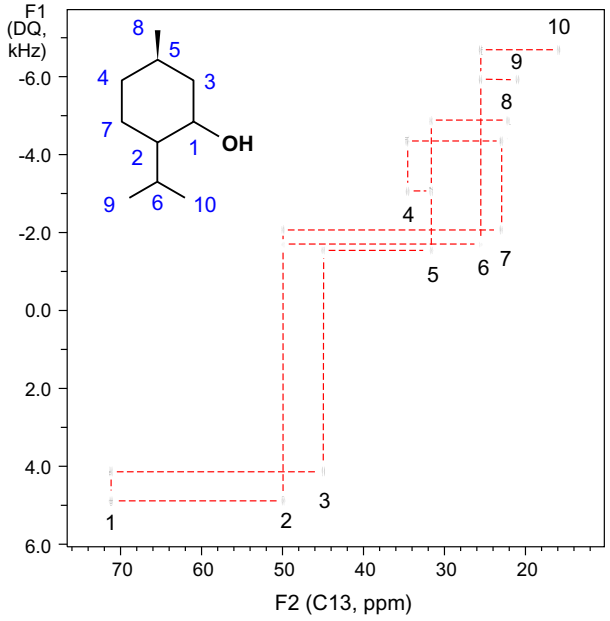

Fig. 3. The INADEQUATE spectrum of menthol (30% in $CDCl_3$) obtained by Hadamard spectroscopy. It was recorded in a 500 MHz spectrometer with a cold probe optimized for ^{13}C detection, as part of a PANACEA experiment with an overall duration of only 56 s.

3. An alternative field/frequency regulation scheme: i-lock

It sometimes happens that the addition of a deuterated solvent for field/frequency lock is inconvenient or indeed impossible. Examples include the situation where it is important to avoid deuterium exchange with labile protons, or where the high-resolution deuterium spectrum itself is to be investigated. Note that the conventional deuterium lock, acting on the magnetic field, may sometimes introduce undesirable t_1 noise in the recorded spectrum. At very high magnet fields the molecules of $CDCl_3$ are partially oriented, and the signal may acquire some doublet character, rendering it unsuitable as an error signal. Another problem can arise if the frequency of the lock signal is sensitive to temperature (a particular difficulty with D_2O), since this broadens or shifts the observed resonances. In order to avoid dilution, some samples are best examined as neat liquids rather than solutions in a deuterated solvent.

For such cases we propose an alternative frequency stabilization technique called i-lock. The effects of slow drifts of the main magnetic field are compensated by reference to an accurately determined frequency of a strong ^{13}C (or X) resonance, measured by a line-fitting technique. The detected frequency deviations are then used to introduce compensating shifts of the NMR frequencies in the observed free induction decays *post mortem*. Any resonance with adequate signal-to-noise ratio can be used; indeed a computer routine could be written to choose the strongest isolated resonance automatically. This alternative stabilization technique works well in two-dimensional experiments where the frequency corrections are applied at each evolution step. Fig. 4a shows the instabilities in an evolving ^{13}C response when the usual deuterium lock and temperature regulation have been deliberately disabled. When the conventional stabilization is replaced by the i-lock device the same signal becomes rock-steady (Fig. 4b). A practical application is provided by the PANACEA–HMBC spectrum of 1 M sucrose in D_2O is recorded without deuterium lock or temperature control. The picture (Fig. 4c) is garbled by instabilities, but with the new i-lock scheme (Fig. 4d) normality is restored.

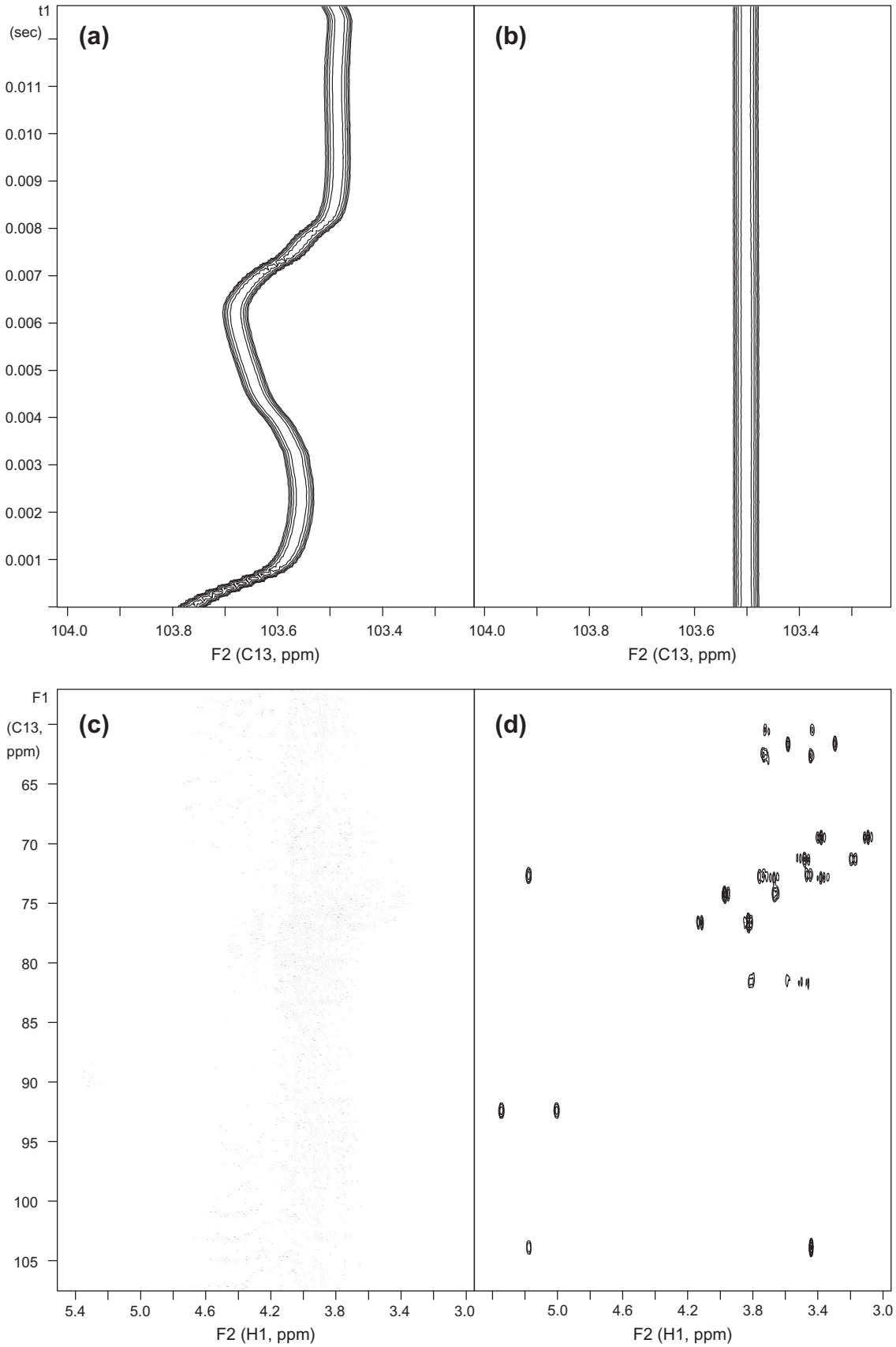

Fig. 4. (a) Instabilities recorded on an evolving ^{13}C signal. The deuterium lock and temperature regulation have been deliberately disabled. (b) The same signal with i-lock. (c) The garbled PANACEA-HMBC spectrum of 1 M sucrose in D_2O with no deuterium lock or temperature regulation. (d) The same spectrum obtained with i-lock. Recorded on a 500 MHz spectrometer with sweep width = 8 kHz with 4 k data points in the direct dimension; sweep width (2) = 10 kHz with 128 evolution increments and four scans per increment. The second F_1F_3 plane was recorded with t_2 = 18.75 ms.

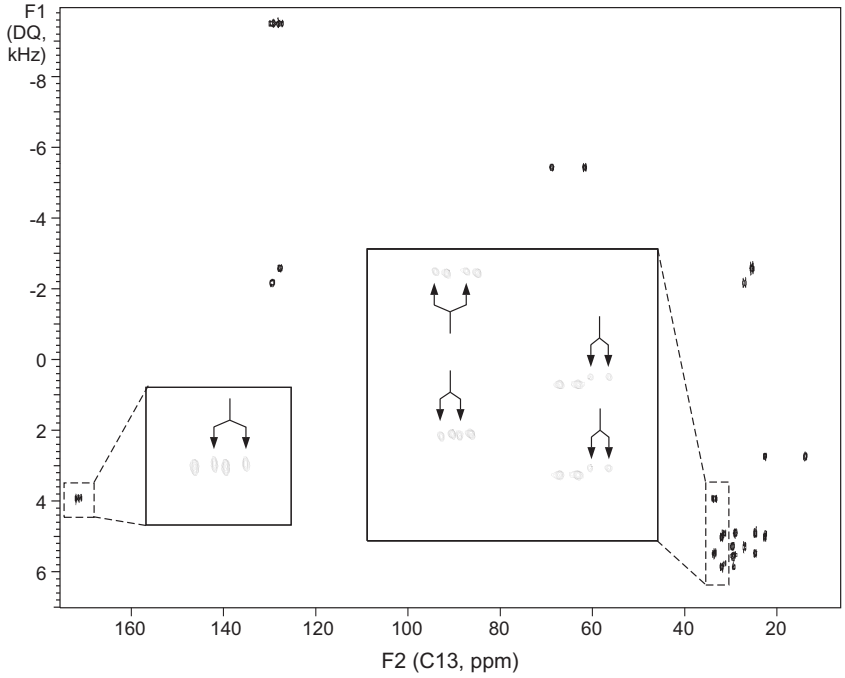

Fig. 5. The PANACEA–INADEQUATE spectrum of neat peanut oil recorded on a 500 MHz spectrometer with a cold probe optimized for ^{13}C detection. No deuterated solvent was added; i-lock was used for radiofrequency stabilization. Signals from the minor component (linoleic acid) are indicated by arrows in the expanded insets. Sweep width = 25 kHz with 10 k data points; sweep width (1) = 20 kHz; 256 increments in t_1 and eight increments in t_2, with four scans per increment. Total duration 15.5 h.

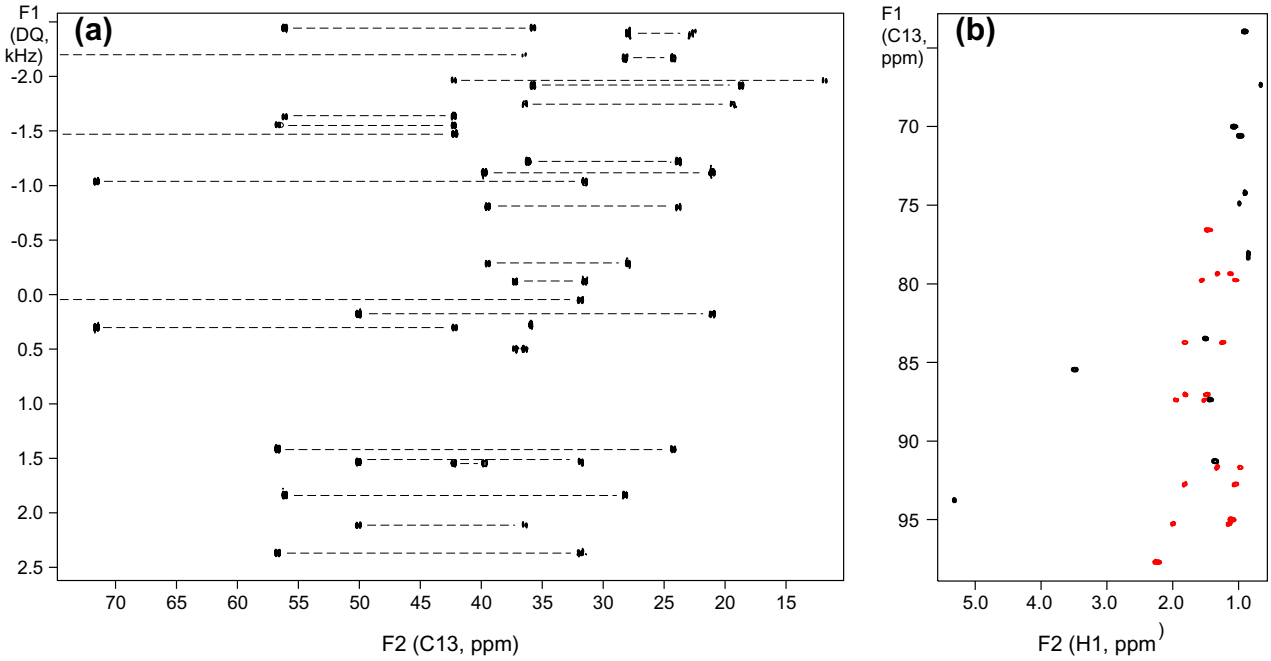

Fig. 6. A PANACEA experiment showing (a) INADEQUATE and (b) multiplicity-edited HSQC spectra of 1 M cholesterol in $CDCl_3$ recorded in parallel in a 23-min measurement. Three responses in (a) are in fact correlated with signals that lie well outside this spectral window. Red peaks in (b) are negative and belong to CH_2 groups; the black peaks represent CH and CH_3 groups. Four-fold aliasing was used to reduce the experimental duration. Sweep width = 20 kHz; sweep width (1) = 5.2 kHz with 4 k data points and two scans per increment.

Another practical application shows the PANACEA–INADEQUATE spectrum (Fig. 5) of neat peanut oil, which comprises oleic acid (56.6%) and linoleic acid (26.7%) as major components. The C–C correlation patterns of both these molecules are clearly visible and separable. No deuterated solvent was added and frequency regulation was achieved with the i-lock scheme based on an intense ^{13}C signal. This i-lock principle could well prove useful in many other experiments where the use or presence of a deuterated solvent is undesirable.

4. Multiple aliasing in the evolution dimension

A second way to accelerate a two-dimensional experiment is by multiple aliasing in the evolution dimension [17]. The term *k*-fold

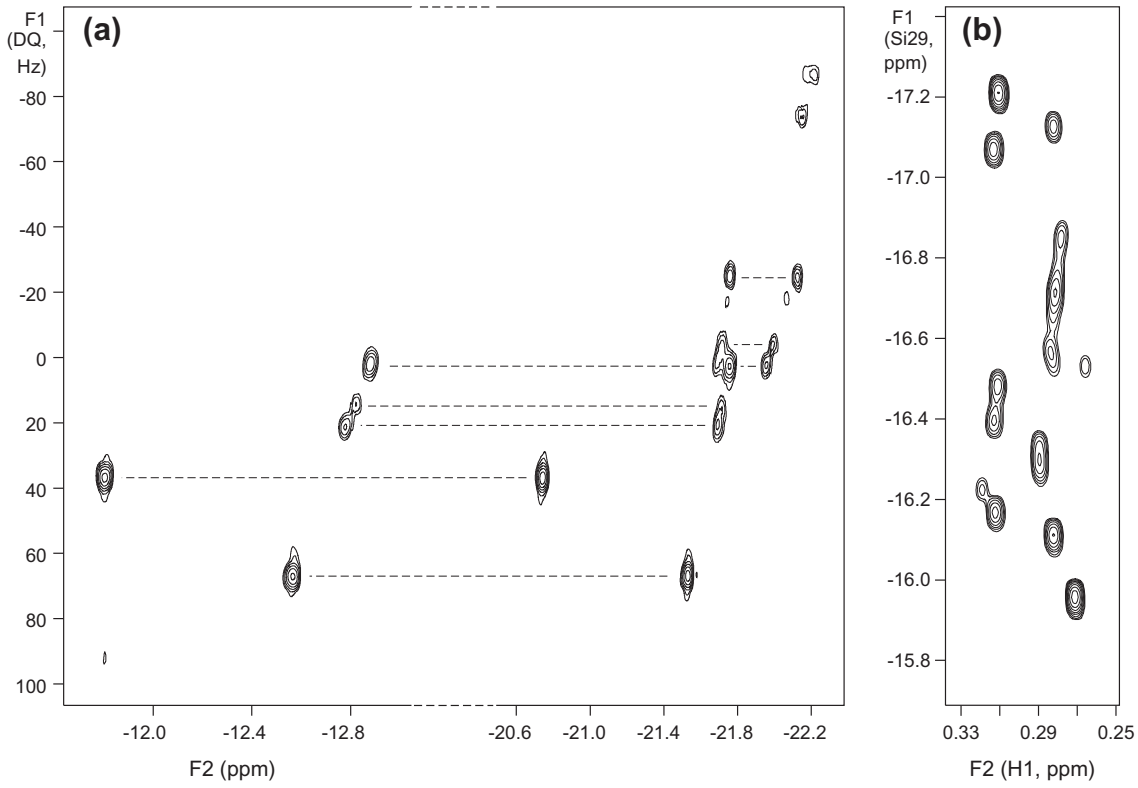

Fig. 7. ^{29}Si-PANACEA spectra of neat silicone oil showing (a) ^{29}Si–O–^{29}Si INADEQUATE correlations recorded with 16-fold aliasing in the double-quantum dimension, and (b) the HMBC spectrum with eightfold aliasing in the F_1 dimension, reducing the total measurement time by a factor of about eight. These measurements employed the i-lock scheme operating on a strong ^{29}Si resonance. The sweep widths were 2000 Hz (512 complex data points) and 256 Hz (32 complex data points). With a repetition time of 1 s, two scans per increment, and an acquisition time of 0.256 s, the overall experimental duration was 8 min.

aliasing is here taken to mean that the spectral width is reduced *k*-fold (rather than by *k* cumulative aliasing operations). For the HSQC and HMBC elements of PANACEA, aliasing in the F_1 dimension could be optimized 'on the fly' under computer control, using ^{13}C information recorded in the first traces. This is not feasible for the INADEQUATE experiment, but the required separation of the different F_2 spectra is readily achieved, and even exact overlap can be accommodated without jeopardizing the determination of the connectivities.

This strategy is illustrated by the INADEQUATE and multiplicity-edited HSQC spectra of 1 M cholesterol in $CDCl_3$ recorded in parallel on a 500 MHz spectrometer. With four-fold aliasing in the evolution dimension the 'compressed' INADEQUATE spectrum shown in Fig. 6a is obtained. Although this spectrum has not been rearranged in the familiar format, there is no uncertainty about the correlations. The direct ^{13}C–^{1}H correlations (Fig. 6b) were also determined, along with their multiplicities. The two experiments ran in parallel and were completed in 23 min.

Not all INADEQUATE experiments need be limited to ^{13}C–^{13}C correlations. Another example of deliberate aliasing is provided by the ^{29}Si–O–^{29}Si INADEQUATE spectrum of silicone oil with no added deuterated solvent, exploiting the i-lock scheme based on a strong ^{29}Si signal. The ^{29}Si nucleus has a natural abundance of 4.7%, offering a higher intrinsic sensitivity than the corresponding ^{13}C–^{13}C correlation experiment, but handicapped by the smaller ^{29}Si–O–^{29}Si coupling constants (~2 Hz). For this experiment a new INEPT-PANACEA pulse sequence [18] was employed to improve sensitivity through an initial magnetization transfer from the protons. In this sample all the ^{29}Si sites are coupled to protons so there is no danger that the INEPT element will 'lose' particular ^{29}Si resonances. Fig. 7 shows ^{29}Si-PANACEA spectra of neat silicone oil showing (a) ^{29}Si–O–^{29}Si INADEQUATE correlations recorded with 16-fold aliasing in the double-quantum dimension, and (b) the HMBC spectrum with eightfold aliasing in the F_1 dimension. The total measurement time was only 8 min.

5. Conclusions

Several of the proposed techniques for speeding up PANACEA by shortening the duration of the INADEQUATE element can be combined [5–8]. Two alternative schemes described here offer further significant improvements in speed (particularly the Hadamard method), rendering PANACEA a more practical solution for structure determinations of small molecules. For example, the menthol ^{13}C–^{13}C correlation spectrum required only 56 s of instrument time.

Note that because PANACEA delivers several different types of NMR information during a single run, two innovations outlined above could be thought of as the first step towards a regime in which the NMR spectrometer itself makes choices about operating parameters, based on measurements that it is currently recording. One example describes the establishment of an array of soft radio-frequency pulses based on chemical shifts derived from the one-dimensional ^{13}C spectrum. The new i-lock scheme for compensating drifts of the magnet field is another example of the use of real-time measurements to control NMR results. More sophisticated applications can be imagined. If generalized, this concept could prove a popular mode of operation where unsupervised NMR investigations are carried out based on artificial intelligence. A machine that thinks for itself?

References

[1] E. Kupče, R. Freeman, B.K. John, Parallel acquisition of two-dimensional NMR spectra of several nuclear species, J. Am. Chem. Soc. 128 (2006) 9606–9607.

[2] E. Kupče, S. Cheatham, R. Freeman, Two-dimensional spectroscopy with parallel acquisition of ^{1}H–X and ^{19}F–X correlations, Magn. Reson. Chem. 45 (2007) 378–380.
[3] E. Kupče, R. Freeman, Molecular structure from a single NMR experiment, J. Am. Chem. Soc. 130 (2008) 10788–10792.
[4] E. Kupče, R. Freeman, High-resolution NMR correlation experiments in a single measurement (HR-PANACEA), Magn. Reson. Chem. 48 (2010) 333–336.
[5] T. Nakazawa, H. Sengstschmid, R. Freeman, Enhancement of INADEQUATE spectra according to symmetry criteria, J. Magn. Reson. A 120 (1996) 269–273.
[6] A. Meissner, O.W. S∅rensen, Exercise in modern NMR pulse design: INADEQUATE CR, Concepts Magn. Reson. 14 (2002) 141–154.
[7] D.L. Mattiello, R. Freeman, Blood, sweat and tears. Towards a rehabilitation of the INADEQUATE experiment, J. Magn. Reson. 135 (1998) 514–521.
[8] P. Styles, N.F. Soffe, C.A. Scott, D.A. Cragg, F. Row, D.J. White, P.C. White, J. Magn. Reson. 60 (1984) 397.
[9] J. Hadamard, Résolution d'une question relative aux déterminants, Bull. Sci. Math. 17 (1893) 240–248.
[10] E. Kupče, R. Freeman, Two-dimensional Hadamard spectroscopy, J. Magn. Reson. 162 (2003) 300–310.
[11] E. Kupče, R. Freeman, Fast multidimensional Hadamard spectroscopy, J. Magn. Reson. 163 (2003) 56–63.
[12] E. Kupče, T. Nishida, R. Freeman, Hadamard NMR spectroscopy, Prog. NMR Spectrosc. 42 (2003) 95–122.
[13] O.W. S∅rensen, G.W. Eich, M.H. Levitt, G. Bodenhausen, R.R. Ernst, Prog. NMR Spectrosc. 16 (1983) 163.
[14] R. Baumann, G. Wider, R.R. Enst, K. Wüthrich, Improvement of 2D NOE and 2D correlated spectra by symmetrisation, J. Magn. Reson. 44 (1981) 402.
[15] L. McIntyre, X.-L. Wu, R. Freeman, Fine structure of cross-peaks in truncated COSY experiments, J. Magn. Reson. 87 (1990) 194–201.
[16] E. Kupče, R. Freeman, Fast reconstruction of four-dimensional NMR spectra from plane projections, J. Biomol. NMR 28 (2004) 391–395.
[17] D. Jeannerat, High resolution in heteronuclear ^{1}H–^{13}C NMR experiments by optimizing spectral aliasing with one-dimensional carbon data, Magn. Reson. Chem. 41 (2003) 3–17.
[18] E. Kupče, B. Wrackmeyer, Multiple receiver experiments for NMR spectroscopy of organosilicon compounds, Appl. Organomet. Chem., in press.