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A Three-Dimensional Heteronuclear Multiple-Quantum Coherence Homonuclear Hartmann–Hahn Experiment

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Since the introduction of two-dimensional NMR a further extension into three (or more) frequency dimensions, by the insertion of one or two additional evolution times, seemed only a logical extension of the method. In the area of the high-resolution NMR, three-dimensional experiments seemed impracticable for the same reasons that were at the heart of the slow introduction of 2D NMR: very large data matrices were generated and long measuring times were needed. Increased sensitivity of NMR spectrometers, ever increasing computer power, and speed of mass storage devices have alleviated these problems. Three-dimensional NMR can nowadays be done within a reasonable amount of time and without generating excessively large data matrices as judged by today's standards. This is borne out by a number of recently published 3D NMR experiments (*1–9*). The majority of these experiments entails the coupling of two 2D proton–proton correlation experiments, e.g., NOESY-COSY (*3*) and NOESY-HOHAHA (*5*). To limit the measuring time and the size of the data matrices in most cases the proton frequency domain was restricted by using selective excitation pulses, although also two 3D NOESY-HOHAHA experiments have been done in which the complete proton frequency domain was utilized (*6, 7*). In addition reports have appeared of 3D heteronuclear NMR experiments, where a 2D heteronucleus–proton correlation experiment is combined with a 2D proton–proton correlation experiment (*8, 9*). For uniformly ^{15}N- or ^{13}C-enriched samples, the sensitivity of such 3D heteronuclear NMR experiments is similar to that of 3D proton NMR experiments, since the heteronucleus is detected via the attached proton and thus with proton sensitivity (*10–12*). Therefore, in terms of measuring time and size of data matrices the same conditions apply to such 3D heteronuclear NMR experiments as to 3D proton NMR. The great promise of 3D NMR experiments lies in the increased spectral resolution with respect to 2D NMR experiments, which is of prime importance in the elucidation of the molecular structure by means of NMR of proteins as well as of nucleic acids and carbohydrates.

In this Communication we present a new 3D NMR experiment in which a ^1H-detected heteronuclear multiple-quantum coherence experiment (HMQC) (*10–12*)

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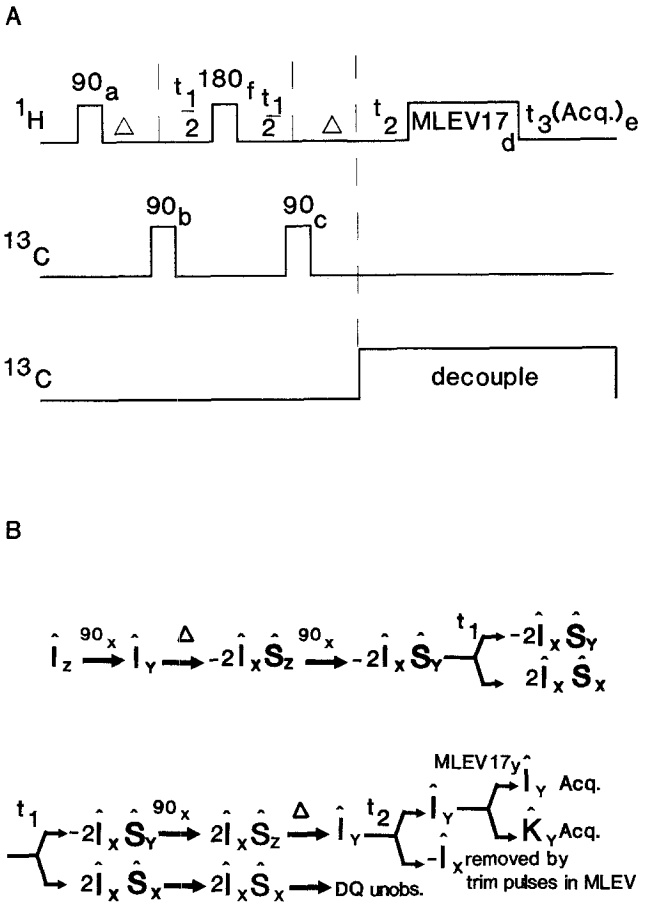
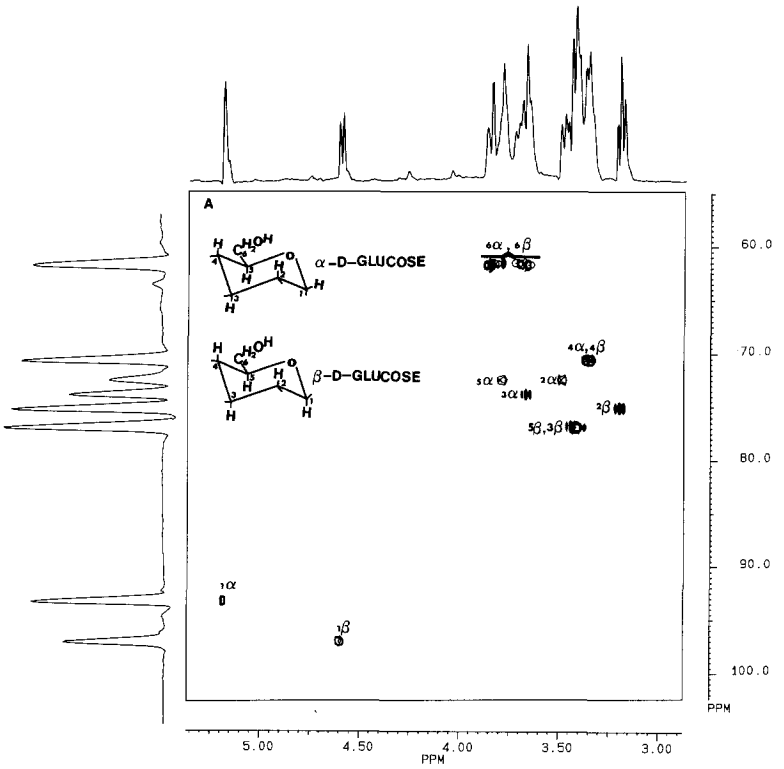
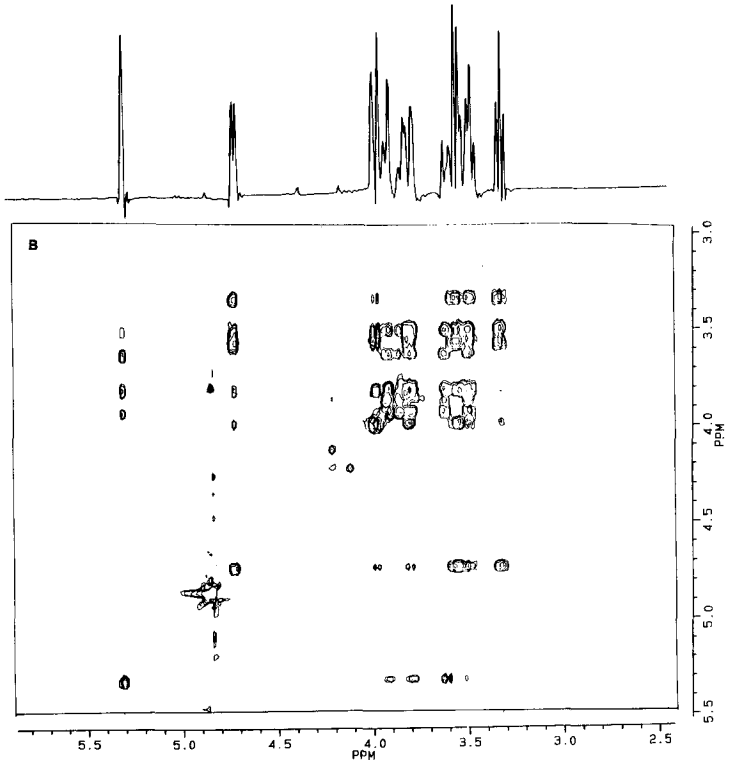


FIG. 1. (A) Pulse sequence for the 3D HMQC-HOHAHA experiment. The phase cycling used is as follows: a = x(+TPPI(t_2)); b = ($x, -x, x, -x$)(+TPPI(t_1)); f = x(+TPPI(t_2)); c = $x, x, -x, -x$; d = y; acquisition e = $x, -x, -x, x$ (TPPI(t_2) stands for TPPI in the t_2 direction). (B) The evolution and transfer of the most important coherences during the 3D HMQC-HOHAHA experiment.

is combined with a ^{1}H homonuclear TOCSY (HOHAHA) (*13–16*) experiment. A mixture of ^{13}C-enriched α- and β-D-glucose was used as a test sample. We want to demonstrate that this experiment can be done in a time comparable to that needed for a 2D experiment, and that it may help to alleviate the overlap of the resonances of nonanomeric protons of oligosaccharides. We shall discuss the potential usefulness of this and similar experiments for resonance assignment and structure determination of oligosaccharides and nucleic acids.

The pulse sequence of this 3D experiment is given in Fig. 1A; in Fig. 1B the fate of the most important coherences is shown. In the analysis we use the operator formalism and branching notation put forward by van de Ven and Hilbers (*17*). A branch going up leads to a cosine-modulated term and a branch going down to a sine-modulated term. In the first delay period, Δ, single-quantum antiphase coherence, $\hat{I}_x\hat{S}_z$, is


A
α-D-GLUCOSE
β-D-GLUCOSE
1α
1β
5.00
4.50
4.00
3.50
3.00
PPM
60.0
70.0
80.0
90.0
100.0
PPM


B
5.5
5.0
4.5
4.0
3.5
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3.0
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4.5
5.0
5.5
PPM

created, which is subsequently converted by the 90° ^{13}C pulse into zero- and double-quantum coherence, $\hat{I}_x\hat{S}_y$. Here I and S stand for ^1H and ^{13}C coherence, respectively. During t_1 the coherence, $\hat{I}_x\hat{S}_y$, evolves only with frequency ω_C as a result of the 180° ^1H pulse in the middle of the t_1 period. We note in passing that the influence of proton–proton coupling is neglected. At the end of t_1 only $\hat{I}_x\hat{S}_y$ coherence is converted back into $\hat{I}_x\hat{S}_z$, single-quantum antiphase coherence, which during the second delay, Δ, evolves into pure ^1H single-quantum coherence, $\hat{I}_y$. To enhance sensitivity and resolution the ^{13}C may be decoupled during the second evolution time (t_2) and during acquisition (t_3). After being labeled by the proton chemical shifts and couplings during t_2, the ^1H coherences are mixed by an MLEV17 sequence, sandwiched between trim pulses that remove magnetization not along the spin-lock axis (*14–16*). Finally, the magnetization is detected during the t_3 period.

Phase cycling has been kept to a minimum. Only the phases of the ^{13}C pulses are shifted in concert with that of the receiver to remove the signals from those protons not connected to the ^{13}C nucleus. It is our experience that for the inverse correlation experiment it is best not to cycle the proton pulses. Therefore, the spin-lock sequence, MLEV17, was not subjected to any phase cycling. TPPI was used to achieve quadrature detection in both t_1 and t_2 directions. In the 3D NMR spectrum absorptive lineshapes in all three dimensions can thus be obtained after 3D Fourier transformation, at least if, during t_1 and the delays Δ, evolution of proton magnetization under proton–proton coupling (J_{HH}) can be neglected, as is the case for direct ^{13}C–^1H couplings (*12*).

The 3D NMR experiment on a mixture of ^{13}C-enriched α- and β-D-glucose (see structure in Fig. 2A) was carried out on a Bruker AM 400 spectrometer equipped with a 5 mm QNP probe. Decoupling of the ^{13}C nucleus during t_2 and t_3 was done with an external WALTZ decoupler. The sweep widths were set to 1400 Hz in f_2 and f_3 to cover the spectral range of all sugar protons H1 to H6 and to 5000 Hz in f_1 to cover the ^{13}C spectral range (Fig. 2). For each of the 88 t_1 values, FIDs were measured for 128 different t_2 values. Each FID of 512 data points (t_3) was the accumulation of eight scans with each scan preceded by four dummy scans to establish steady state. To ensure sufficient relaying of coherences during the spin-lock period a relatively long mixing time of 100 ms was chosen. The complete 3D NMR data set of $88 \times 128 \times 512$ data points was obtained in 2 days. The data were Fourier transformed with the 3D NMR software written by C. Griesinger (running on an Aspect 3000). In the t_1 direction the data were zero-filled from 88 to 128 points and in the t_2 direction from 128 to 256 points. No zero-filling was done in the t_3 direction. In all directions the same window function was applied, i.e., a $\pi/4$-shifted quadratic cosine filter. Finally, after phasing to pure absorption, only the real points were retained, giving a 3D NMR spectrum of $64 \times 64 \times 256$ data points.

FIG. 2. (A) Phase sensitive ^{13}C HMQC spectrum of a mixture of ^{13}C-enriched α- and β-D-glucose in D_2O. Note that the J coupling between ^{13}C's of the sugar ring, which is of the order of 10 to 30 Hz, is not resolved because of the 24 Hz/point resolution in the ^{13}C direction. (B) Phase sensitive TOCSY(HOHAHA)-MLEV17 spectrum of the same sample.

For reasons of comparison a 2D $^{13}C-^{1}H$ correlation spectrum (HMQC) and a $^{1}H-^{1}H$ TOCSY(HOHAHA) spectrum were recorded (Figs. 2A and 2B). These spectra clearly demonstrate the overlap of resonances even for this simple molecule. In Fig. 3 a perspective overview is given of the complete 3D NMR spectrum. Most of the peaks cluster in the three-dimensional spectral window defined by the resonance frequencies of C2 to C6, and by the frequencies of H2 to H6, while the intensities originating from C1 and H1 lie relatively free. Some interesting features can be established if the planes through and/or projections of the 3D NMR cube are considered, especially when a comparison with 2D spectra is made. In the cross-diagonal plane, defined by $f_2 = f_3$, intensity accumulates that gives rise to a spectrum which corresponds with a 2D $^{13}C-^{1}H$ correlation spectrum; i.e., the intensity in this plane stems from magnetization that has not been transferred during the spin-lock period. This cross-diagonal plane is thus the direct analog of the diagonal in a homonuclear 2D spectrum, where on the diagonal intensity also accumulates from magnetization that is not transferred during the spin-lock period. Also, projecting the intensity accumulated in the 3D NMR spectrum onto the f_1, f_2 plane ($f_3 = 0$) generates a 2D $^{13}C-^{1}H$ type correlation spectrum, while projecting onto the f_2, f_3 plane ($f_1 = 0$) generates a 2D TOCSY(HOHAHA) spectrum, similar to 1D projections obtainable from 2D NMR spectra. The projection of the 3D spectrum onto the f_1, f_3 plane ($f_2 = 0$), shown in Fig. 3B, corresponds, on the other hand, with a new type of 2D heteronuclear NMR experiment in which during the t_1 period the ^{1}H coherences are labeled with the Larmor frequencies of the directly attached ^{13}C spins. Then coherence transfer takes place to *J*-coupled ^{1}H spins during the spin-lock period, after which acquisition takes place. Therefore, apart from the regular $^{13}C-^{1}H$ cross peaks, cross peaks also appear due to correlation between *J*-coupled protons. The cross sections taken perpendicular to the f_2 or f_3 axis may be viewed as edited $^{13}C-^{1}H$ correlation spectra, in which one semidiagonal cross peak is present at $f_1, f_2 = f_3$, representing ^{1}H coherence that has not been transferred during the spin-lock period. Additional cross peaks in these planes represent coherences that have been transferred via the spin lock either to (plane $\perp f_3$) or from the proton (plane $\perp f_2$), giving rise to the semidiagonal cross peak.

We consider the planes perpendicular to the f_1 axis (the ^{13}C axis) in somewhat more detail. These planes are shown in Fig. 4; each slice, taken at a ^{13}C resonance position, represents a ^{13}C-edited TOCSY(HOHAHA) spectrum. The cross peaks on the diagonal represent ^{1}H coherences that have not been transferred during the spin-lock period. In contrast, the off-diagonal cross peaks result from coherence transfer during the spin-lock period from the proton giving rise to the diagonal cross peak to its *J*-coupled neighbors.

The interpretation of the ^{1}H and ^{13}C spectra of a mixture of α- and β-D-glucose has been carried out before ((*18*); where the assignment of α-D-glucose and β-D-glucose has been interchanged). The assignments are indicated in Figs. 2 and 4. Examination of Fig. 4 shows that the measured 3D NMR spectrum yields a direct identification of the resonances of protons that belong to one sugar ring. In fact, the slice at the C1 frequency of the α as well as the β anomer shows all the ring protons, H1 to H6. The method does, however, not offer the most effective approach for the assignment of

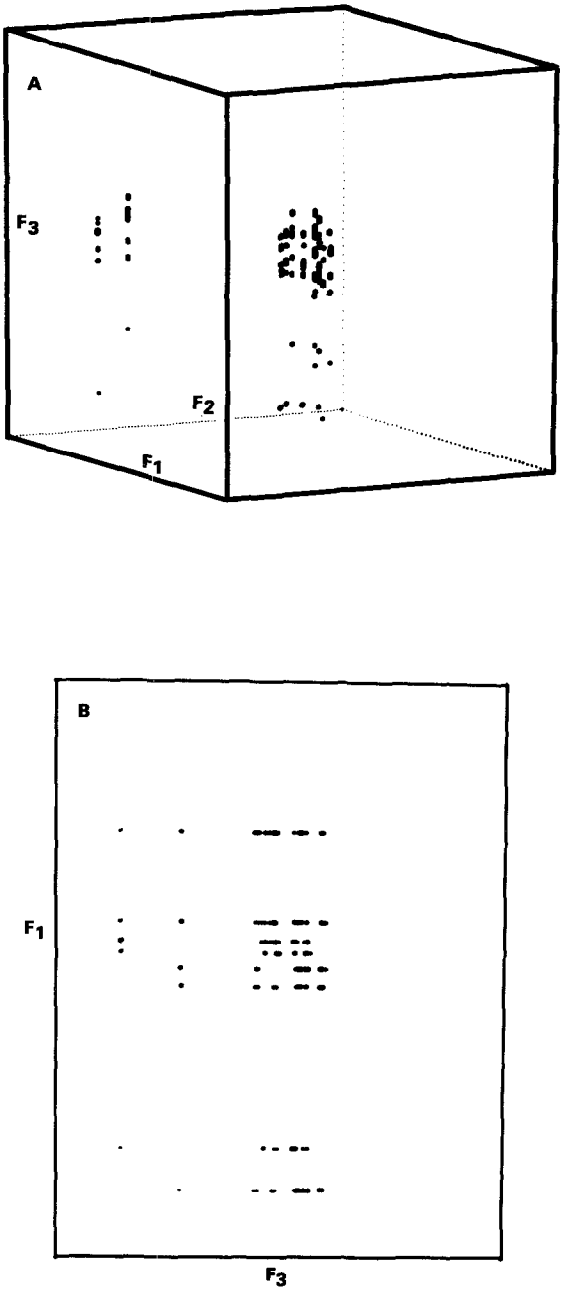

FIG. 3. (A) Perspective three-dimensional overview of the 3D ^{13}C-HMQC-HOHAHA spectrum of the mixture of ^{13}C-enriched α- and β-D-glucose. The peaks were found from peak picking and displayed on a SIGMEX graphics terminal using CHEM-X software. Each peak in the 3D spectrum shown here is represented in CHEM-X as an atom, which has a spatial position in the 3D cube determined by the spectral peak coordinates, a color corresponding to the peak intensity, and a width corresponding to the peak width. (B) Projection of the 3D NMR spectrum onto the F_1, F_3 plane.

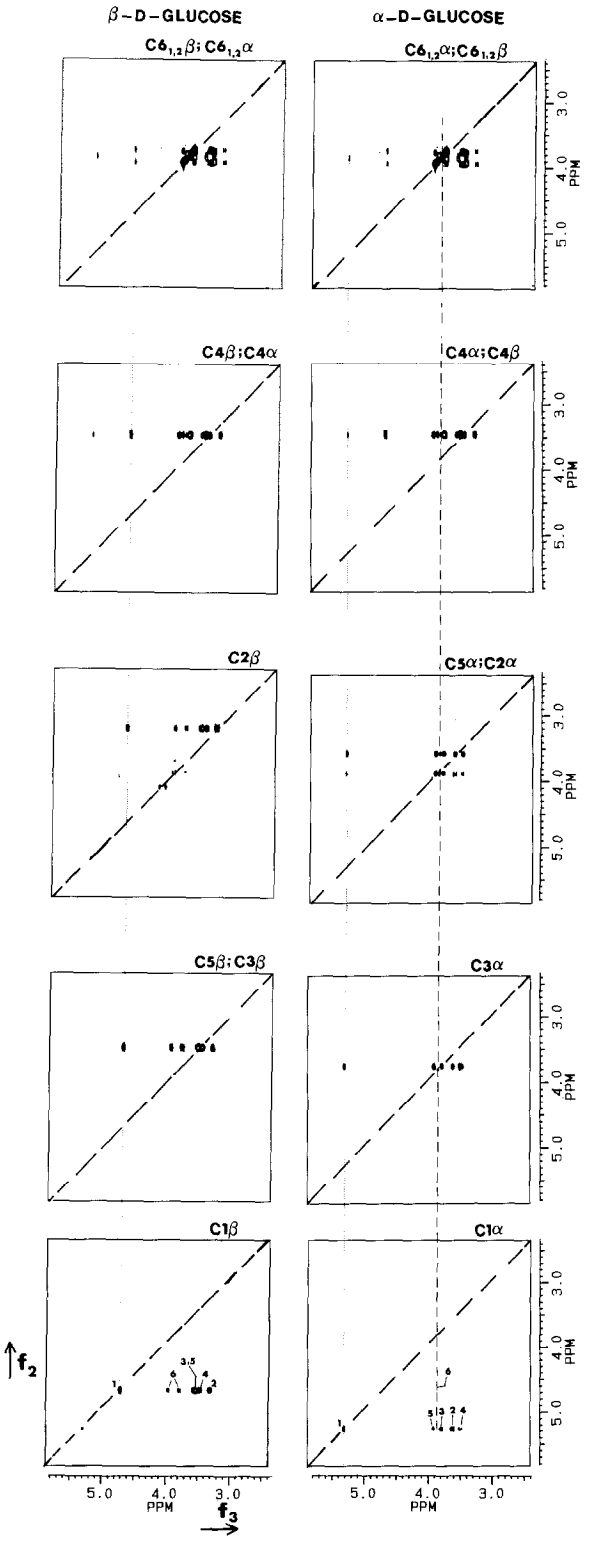

FIG. 4. Cross sections through the 3D spectrum taken perpendicular to the f_1 axis (^{13}C axis) at the frequencies corresponding to each of the ^{13}C signals of α- and β-D-glucose. The cross peaks on the dashed diagonal represent coherences that are not transferred during the spin-lock period. The dotted or dashed vertical lines indicate coherence transferred via the spin-lock from protons J-coupled to the proton giving rise to the cross peak on the dashed diagonal.

the individual protons within a sugar ring. One must rely on differences in intensity of the connectivities between the diagonal and its neighboring protons. During the preparation of the manuscript the 3D HMQC-COSY experiment of Fesik *et al.* (*9*) appeared, which represents a powerful technique for assigning resonances in individual rings. However, if in a 3D HMQC-COSY experiment two subsequent protons overlap it is hard if not impossible to distinguish between one ring and the other. Such problems of overlap are bound to occur in the case of larger molecules. Herein lies the advantage of the TOCSY(HOHAHA) experiment, where one of the protons, most likely the H1α, β, can be used as an anchor, so that the protons of one particular sugar ring can always be identified. This holds true as long as no complete overlap of all ^{1}H and ^{13}C resonances of two rings occurs. If in addition to this 3D ^{13}C HMQC-HOHAHA experiment a 3D ^{13}C HMQC-NOESY is performed, ambiguities in the individual assignments within each ring can be resolved because of the spatial proximity of certain protons within each set of ring protons. For example, the protons (H1, H3, H5)β are spatially close and so are (H2, H4)β. In addition, a 3D ^{13}C-HMQC-NOESY spectrum makes sequential assignment in polysaccharides possible and provides for spatial information. A reasoning similar to that used above for polysaccharides applies to nucleic acids.

Finally, we note that since the identification of the protons in one ring is done essentially from H1α, β, selective excitation would make the experimental measuring time even shorter. Since the H1 protons reside in a spectral region of approximately 1 ppm (=500 Hz) and $^{13}C_1$ in a region of 500 Hz only 10 t_1 increments are needed to obtain sufficient resolution in the ^{13}C direction. As a regular 2D HMQC experiment can be done in six hours on a natural abundance sample, with selective excitation this type of 3D experiment is, in principle, also feasible for natural abundance samples in a reasonable amount of time.

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REFERENCES

1. R. E. HOFFMAN AND D. B. DAVIES, *J. Magn. Reson.* **80,** 337 (1989).
2. G. W. VUISTER AND R. BOELENS, *J. Magn. Reson.* **73,** 328 (1987).
3. C. GRIESINGER, O. W. SORENSEN, AND R. R. ERNST, *J. Am. Chem. Soc.* **109,** 7227 (1987).
4. C. GRIESINGER, O. W. SORENSEN, AND R. R. ERNST, *J. Magn. Reson.* **73,** 574 (1987).
5. H. OSCHKINAT, C. GRIESINGER, P. J. KRAULIS, O. W. SORENSEN, R. R. ERNST, A. M. GRONENBORN, AND G. M. CLORE, *Nature (London)* **332,** 374 (1988).
6. G. W. VUISTER, R. BOELENS, AND R. KAPTEIN, *J. Magn. Reson.* **80,** 176 (1989).
7. G. W. VUISTER, P. DE WAARD, R. BOELENS, J. F. G. VLIEGENTHART, AND R. KAPTEIN, *J. Am. Chem. Soc.* **111,** 772 (1989).
8. S. W. FESIK AND E. R. P. ZUIDERWEG, *J. Magn. Reson.* **78,** 588 (1988).
9. S. W. FESIK, R. T. GAMPE, JR., and E. R. P. ZUIDERWEG, *J. Am. Chem. Soc.* **111,** 770 (1989).
10. L. MÜLLER, *J. Am. Chem. Soc.* **101,** 4481 (1979).
11. A. BAX, R. H. GRIFFEY, AND B. L. HAWKINS, *J. Magn. Reson.* **55,** 301 (1983).

12. M. F. SUMMERS, L. G. MARZILLI, AND A. BAX, *J. Am. Chem. Soc.* **108,** 4285 (1986).
13. L. BRAUNSCHWEILER AND R. R. ERNST, *J. Magn. Reson.* **53,** 521 (1983).
14. D. G. DAVIES AND A. BAX, *J. Am. Chem. Soc.* **107,** 2820 (1985).
15. D. G. DAVIES AND A. BAX, *J. Am. Chem. Soc.* **107,** 7197 (1985).
16. D. G. DAVIES AND A. BAX, *J. Magn. Reson.* **65,** 355 (1985).
17. F. J. M. VAN DE VEN AND C. W. HILBERS, *J. Magn. Reson.* **54,** 512 (1983).
18. R. R. ERNST, G. BODENHAUSEN, AND A. WOKAUN, "Principles of Nuclear Magnetic Resonance in One and Two Dimensions," p. 481, Clarendon, Oxford, 1987.