Journal of Biomolecular NMR, **25:** 349–354, 2003.
KLUWER/ESCOM

Fast multi-dimensional NMR of proteins

Ēriks Kupče[a] & Ray Freeman[b,*]
[a]*Varian, Inc, Eynsham, Oxford, U.K.;* [b]*Jesus College, Cambridge, U.K.*

Received 5 December 2002; Accepted 7 January 2003

Key words: agitoxin, Hadamard, HNCA, HNCO, proteins, three-dimensional spectra

Abstract

Three-dimensional HNCO and HNCA subspectra from a small protein (agitoxin, 4 kDa, enriched in carbon-13 and nitrogen-15), have been obtained by direct frequency-domain excitation of selected carbon and nitrogen sites. This new technique applies an array of several simultaneous soft radiofrequency spin-inversion pulses, encoded (on or off) according to nested Hadamard matrices, and the resulting responses are extracted by reference to the same matrices. This not only simplifies the spectra but, by avoiding extensive sampling in the traditional evolution dimensions, affords a speed advantage of more than two orders of magnitude.

Multidimensional NMR spectroscopy has many useful applications, particularly for structural studies on biological macromolecules such as proteins. This powerful technique would surely be applied to a much wider range of problems were it not for the protracted nature of the data-gathering process, which grows inexorably as the number of dimensions is increased. This severely limits the application of multidimensional spectroscopy to protein binding studies, where the spectra may need to be recorded many times. There is a practical limit on how long an expensive NMR spectrometer can be tied down by a single investigation. Compromises must be made – notably by sparse sampling in the evolution dimensions, minimal phase cycling, and deliberate aliasing where this can be tolerated (van de Ven, 1995).

Complexity, particularly in the proton spectra, can also be a stumbling block. This problem is often addressed by non-specific enrichment in carbon-13, nitrogen-15 or both, thus separating the proton subspectra according to these heteronuclear frequencies. However, in an ideal world, enrichment at a few carefully chosen sites would be even more desirable for this would provide further simplification. This paper demonstrates how the equivalent result can be achieved by a new Hadamard transform procedure (Hadamard, 1893) working with globally enriched proteins. Attention may then be concentrated on resonances near the active site of the protein. At the same time the measurement is speeded up by a very large factor compared with the traditional multidimensional Fourier transform method, with no loss of sensitivity per unit time.

The aim of this work is to derive simple subspectra of proteins. The innovation is to replace the conventional time-domain evolution periods by direct irradiation in the frequency domain (Kupče and Freeman, 2003a, b, c), using prior knowledge of the relevant chemical shifts. Hadamard spectroscopy labels a given set of NMR responses according to a binary code, so that although they are all lumped together by a multiplexing scheme, the individual responses can be disentangled later by reference to the original modulation code. Multiplexing involves the generation of a set of selective radiofrequency pulses at the appropriate frequencies, applied simultaneously and suitably encoded so that the corresponding NMR responses can be separated at the processing stage. Modern NMR spectrometers are well-equipped for multiplex excitation at several different frequencies (Geen et al., 1989; Kupče and Freeman, 1993a, b, c, 1994). Earlier experiments (Kupče and Freeman, 2003a) have demonstrated that as many as 2048 simultaneous se-

*To whom correspondence should be addressed. E-mail: rf110@cus.cam.ac.uk

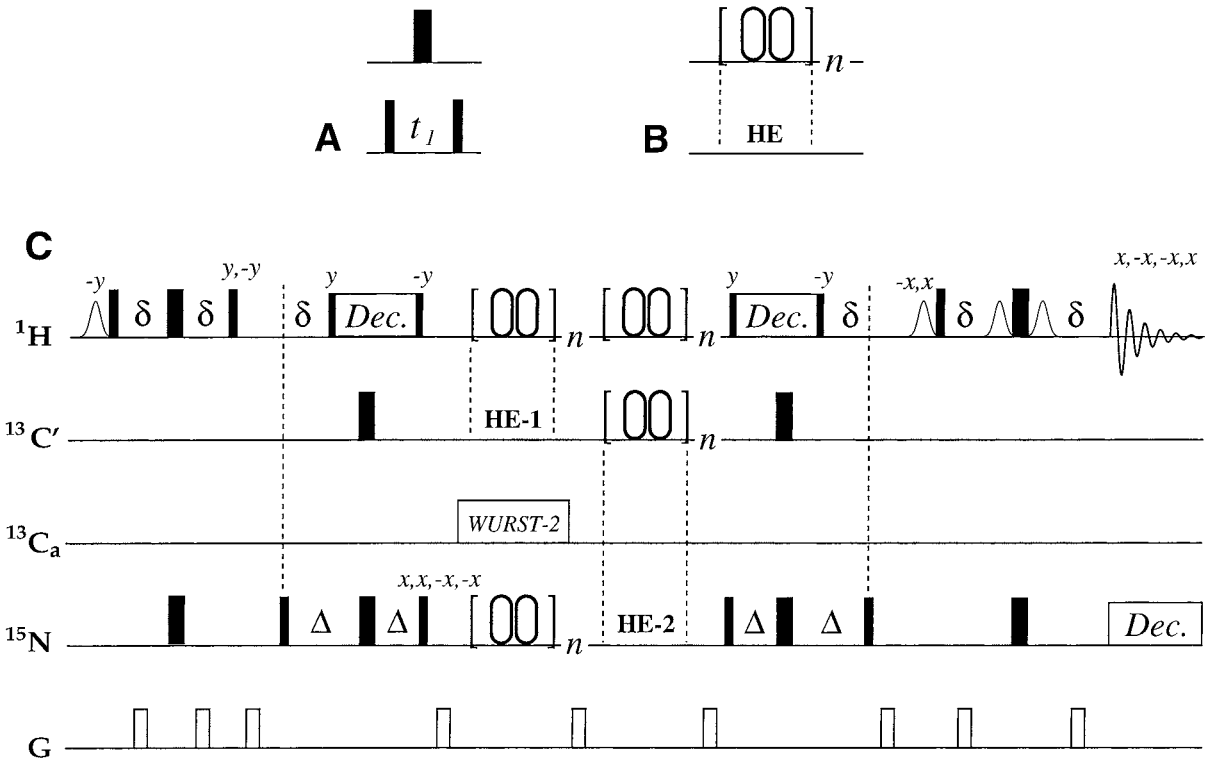

Figure 1. The Hadamard experiments were derived from the standard three-dimensional HNCO sequence simply by replacing the usual evolution periods (A) by Hadamard-encoded arrays of simultaneous soft radiofrequency pulses (B). These are represented by 'HE' and employ simultaneous adiabatic decoupling, represented by $[00]_n$. The HNCA version is similar but has the two carbon-13 channels interchanged. These are the only modifications to the standard HNCO and HNCA sequences described in the literature (Kay et al., 1990, 1994; Yamazaki et al., 1994), where details of the water flip-back pulses and field-gradient pulses are given.

lective radiofrequency pulses can be generated without undue power dissipation.

The selectivity in the frequency domain is determined by the length of the direct excitation sequence – typically 20 milliseconds in these experiments. There is minimal 'cross-talk' between frequency channels because Hadamard decoding makes a clean separation of responses from adjacent channels. The individual radiofrequencies are used for spin inversion; they are modulated (on or off) according to the signs in the appropriate row of a Hadamard matrix of order N. This modulation pattern is changed for each new scan until a total of N scans has been completed. In a three-dimensional experiment two levels of encoding are used, requiring a total of MN scans, where M and N are the orders of the Hadamard matrices. It is this product MN that determines the overall duration of the measurement.

The Hadamard technique involves only minor (but crucial) modifications of existing standard multi-dimensional pulse sequences. Consider the case of a three-dimensional heteronuclear 'triple-resonance' experiment where the proton information is traditionally separated into new dimensions by reference to the carbon-13 and nitrogen-15 chemical shift frequencies. The Hadamard method dispenses with free precession of transverse carbon and nitrogen magnetization; instead it labels the information from the various sites by means of an array of selective spin inversion pulses, *coded on or off* in a pattern defined by the rows of the Hadamard matrix. At each heteronuclear site the sign of the product operator term $2I_ZS_Z$ is either reversed or left unchanged, thereby reversing (or not) the sign of the final proton response. (For heteronuclear multiple-quantum correlation experiments the relevant product operator term would be $2I_XS_Z$ or $2I_YS_Z$.) No time is wasted on free evolution of heteronuclear spins, and spin-spin relaxation losses are minimal; the duration of the selective 180° pulses is comparable with conventional evolution times. The traditional phase encoding according to precession frequencies is re-

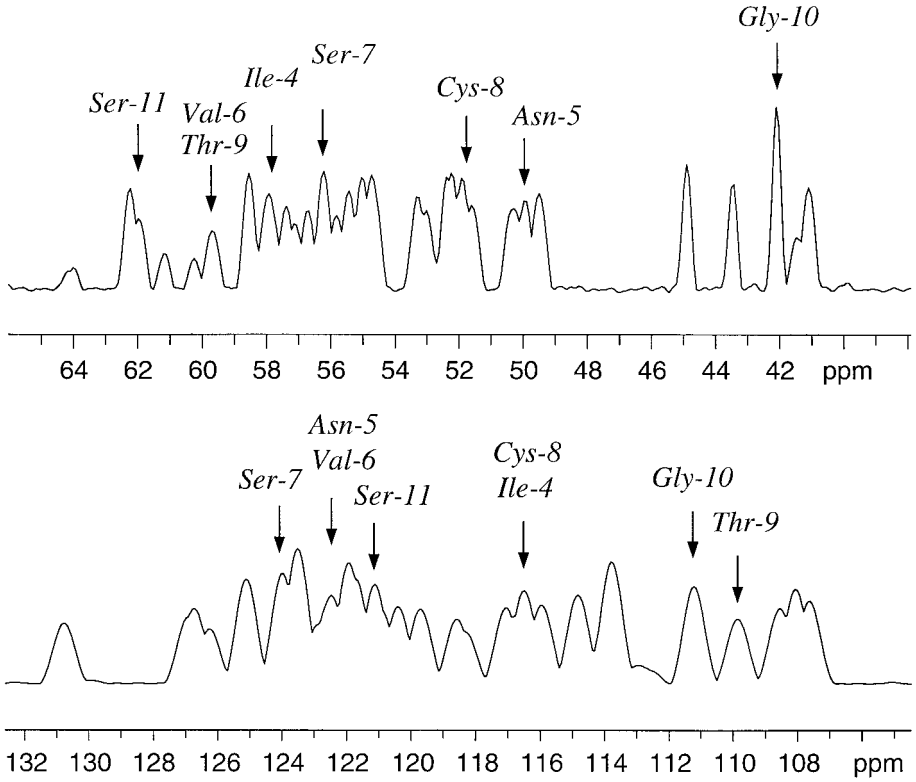

Figure 2. The carbon-13 and nitrogen-15 irradiation frequencies required to set up the Hadamard technique were selected from projections of a conventional three-dimensional HNCA spectrum of agitoxin. Top: the carbon-13 spectrum, where the resonances of Val-6 and Thre-9 lie within the effective bandwidth (45 Hz) of the inversion pulses. Bottom: the nitrogen-15 spectrum, where Asn-5 and Val-6 lie within the 45 Hz bandwidth, as do Ile-4 and Cys-8.

placed by a binary Hadamard code, essentially a Walsh function (Marshall and Comisarow, 1978).

Each scan acquires information from all the selected sites in the form of a composite free induction decay, which can be finely digitized without any significant time penalty. The succession of different composite free induction decays is separated into 'pure' components by combining them according to the plus or minus signs in the columns of the appropriate Hadamard matrix – essentially a Hadamard transformation (Sloane, 1982). For three-dimensional spectroscopy, two successive layers of encoding and decoding are required – a two-dimensional Hadamard transform. High-definition frequency-domain traces are derived by conventional Fourier transformation of the decoded free induction decays. These traces contain all the necessary information. Like constant-time experiments, they have no *intrinsic* linewidths in the carbon-13 and nitrogen-15 dimensions, although broadening functions can be imposed if necessary. Standard routines convert these traces into a three-dimensional spectrum or into selected plane sections (Kupče and Freeman, 2003c).

The time saving arises from three different sources. The most important is the replacement of the extensive sampling in the traditional evolution dimensions by a limited number of direct excitations at selected carbon-13 or nitrogen-15 sites in the Hadamard mode. Suppose that the sampling in the t_1 and t_2 time domains of a conventional three-dimensional experiment requires A and B increments to cover the spectral widths and achieve the desired resolution, whereas the direct excitation mode selects only a and b sites, where a and b are far smaller numbers. The orders of the Hadamard matrices (M and N) must be chosen to equal or exceed a and b respectively. Hadamard matrices exist for all orders $4n$ where n is an integer (at least up to $4n = 264$); typical values for the present type of experiment might be $4n = 8$, 12, 16 or 20. The speed advantage is therefore determined by the ratio AB/MN which is only slightly smaller than AB/ab. Clearly the more sites selected for direct excitation, the lower the

relative advantage of the Hadamard experiment, but the speed factor only becomes comparable with the conventional Fourier method for the completely unrealistic case that the number of selected sites equals the number of evolution increments used by the Fourier mode. Furthermore, the Hadamard technique can normally dispense with the usual phase cycling and quadrature detection in the evolution dimensions, saving even more time. As more frequency dimensions are introduced, correspondingly larger time factors can be anticipated.

Multiplex frequency-domain spectroscopy detects all the chosen signals in every scan, thereby achieving a sensitivity per unit time at least as high as conventional Fourier transform NMR (Kupče and Freeman, 1993c; Blechta and Freeman, 1993; Blechta et al.,1994; Nishida et al., 1995, 1996; Anderson, 1996; Schraml et al., 1997; Krishnamurthy, 2001). Indeed it is expected to enjoy an advantage of $\sqrt{2}$, because the full signal is detected rather than a rotating component (Kaiser, 1974). Consequently, the Hadamard mode is preferable whenever an adequate signal-to-noise ratio can be achieved in the shorter accumulation time.

For multidimensional spectroscopy of relatively small molecules, the resonance frequencies required to set up a Hadamard experiment are readily obtainable from one-dimensional spectra recorded in a short time. In contrast, protein spectra are usually too complex to be attacked directly by Hadamard spectroscopy. The resolution and sensitivity are too low to find the requisite carbon-13 and nitrogen-15 irradiation frequencies unless three-dimensional spectra are recorded. The Hadamard frequency-domain excitation technique really comes into its own for fast recording of subspectra of proteins where the carbon-13 and nitrogen-15 frequencies and their assignments are already known. The advantage is that it permits these subspectra to be targeted on the really important sites, and the measurements can be repeated very rapidly indeed, allowing repetitive studies of protein binding or protein folding.

Spectra were recorded on a Varian Inova 700 spectrometer. Agitoxin (Krezel et al., 1995), a small 39-residue protein labelled with carbon-13 and nitrogen-15, was studied as a 0.3 millimolar aqueous solution containing 10% heavy water. The simplification and the faster speed of the Hadamard mode were illustrated by reference to two widely-used three-dimensional experiments, HNCO and HNCA (Kay et al., 1990). Figure 1 shows the pulse sequence for the HNCO experiment (Kay et al., 1994). The HNCA version is very similar (Yamazaki et al., 1994), with the two carbon-13 channels interchanged. The first stage is the usual direct polarization transfer from protons.

The requisite carbon-13 and nitrogen-15 frequencies were obtained from the appropriate projections of three-dimensional HNCA spectra (Figure 2). For the Hadamard experiment the standard three-dimensional HNCO and HNCA sequences were modified by replacing the conventional evolution periods (Figure 1A) with arrays of Hadamard-encoded selective radiofrequency inversion pulses (Figure 1B) applied directly in the carbon-13 and nitrogen-15 frequency dimensions. During these soft pulse sequences, simultaneous broadband adiabatic decoupling was applied to protons and nitrogen-15, or to protons and carbon-13, making sure that the decoupling sequence corresponded to a whole number of 360° rotations. The C-N decoupling used two 10 ms WURST-2 pulses (Kupče and Freeman, 1996), and the C-H and N-H decoupling employed 1.25 ms WURST-2 pulses nested within an MLEV-16 phase cycle (Levitt et al., 1982). All the other experimental parameters of the HNCO and HNCA sequences were standard settings.

Figure 3A shows strip plots extracted from the three-dimensional HNCO spectrum of agitoxin, targeting seven residues chosen at random in the carbon-13 and nitrogen-15 dimensions. The selective Gaussian inversion pulses had a 45 Hz effective bandwidth in the nitrogen and carbon frequency dimensions, and were gated on and off according to the signs in the rows of a Hadamard matrix of order 8, changing the coding pattern in each successive scan:

$$
\begin{array}{c}
+\,+\,+\,+\,+\,+\,+\,+ \\
+\,+\,+\,+\,-\,-\,-\,- \\
+\,+\,-\,-\,+\,+\,-\,- \\
+\,+\,-\,-\,-\,-\,+\,+ \\
+\,-\,+\,-\,+\,-\,+\,- \\
+\,-\,+\,-\,-\,+\,-\,+ \\
+\,-\,-\,+\,+\,-\,-\,+ \\
+\,-\,-\,+\,-\,+\,+\,-
\end{array}
$$

The 'all plus' column, which is more sensitive to instrumental shortcomings, was not used. Two layers of encoding were employed, one on nitrogen-15, the other on carbon-13, requiring a total of 64 scans; there was no further multiscan averaging. With a recycle

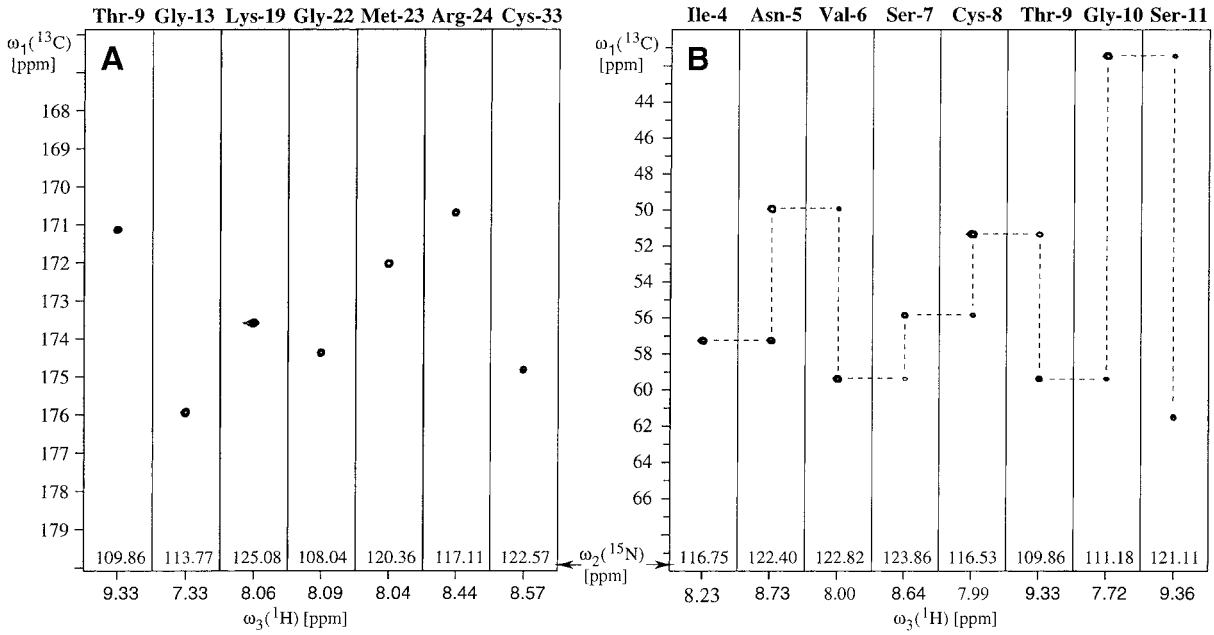

Figure 3. (A) Strip plots taken from the three-dimensional HNCO Hadamard experiment on 0.3 millimolar agitoxin simplified by random selection of seven peaks in the carbon-13 and nitrogen-15 dimensions. Experiment duration: 1 min 39 s. (B) The equivalent HNCA Hadamard experiment, showing the two-bond correlations. Experiment duration: 6 min 4 s.

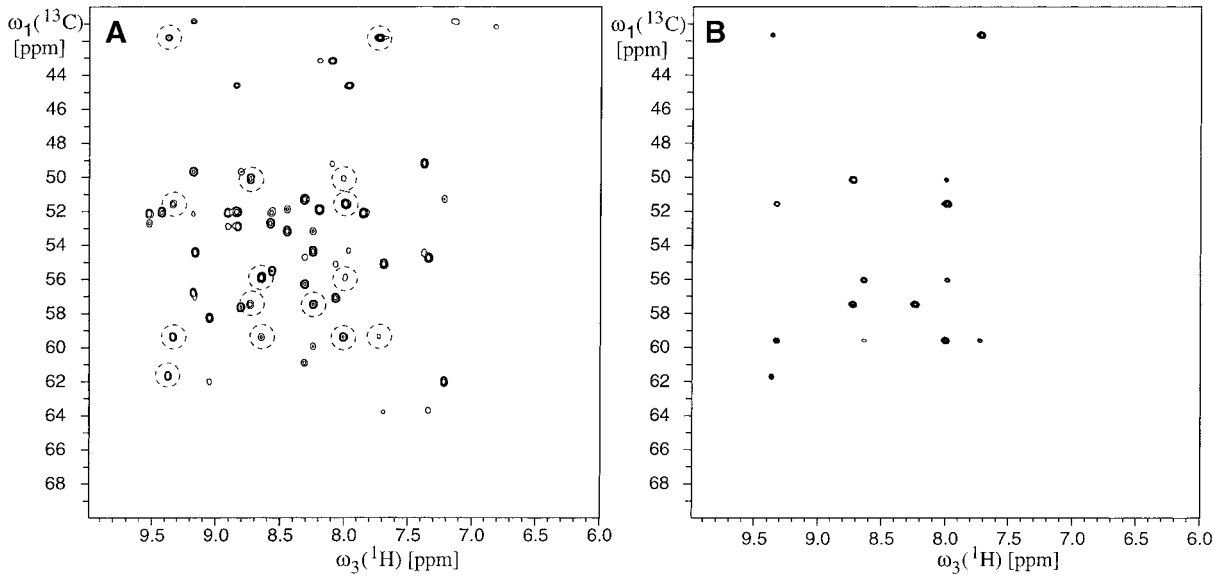

Figure 4. Demonstration of the simplification achieved in Hadamard spectroscopy by comparing projections of the three-dimensional HNCA spectra of agitoxin onto the $\omega_1\omega_3$ plane. (A) The conventional Fourier transform mode. (B) The Hadamard mode, showing responses from residues 4 through 11, identified by the dotted circles in (A).

time of 1.2 s, the overall duration of the measurement was only 1 min 39 s.

Figure 3B illustrates how the Hadamard HNCA technique can be used to focus on a particular backbone sequence – the residues 4 through 11, a chain terminated by the Pro-3 and Pro-12 residues. The Hadamard subspectrum was recorded as an 8 × 8 × 1024 data matrix with 512 complex points in the Fourier dimension. After the mandatory 64 scans (two nested Hadamard matrices of order 8) the sensitivity was not quite high enough to detect the two-bond correlations, so the sequence was repeated four times for time-averaging purposes. This entailed a total measurement time of 6 min 4 sec. This is far shorter than a conventional three-dimensional Fourier transform experiment with comparable resolution in the indirect dimensions.

The simplification achieved by the Hadamard technique is demonstrated in Figure 4 for three-dimensional HNCA spectra of agitoxin projected onto the $\omega_1\omega_3$ plane, a mode of display that does not discriminate with respect to the nitrogen-15 frequencies. The Hadamard spectrum (Figure 4B) is significantly simpler because it focuses on one specific part of the molecule, namely the residues 4 through 11, indicated by dotted circles in Figure 4A. The full conventional three-dimensional spectrum used to derive the projection in Figure 4A was recorded in 20 h 43 min with two scans per increment, whereas the corresponding Hadamard spectrum required only 6 min. This is a time saving factor of over 200.

There is every reason to believe that this Hadamard frequency-domain excitation technique will be applicable to much larger proteins, and that the consequent time saving for screening experiments will prove invaluable. Initial measurements on a 19 kDa protein appear to bear out this contention. In many investigations of protein function the prime interest is on the structure and behavior of the region around the active site, so simplified versions of the three-dimensional spectra are well-suited to this type of investigation.

Acknowledgement

We thank Professor Gerhard Wagner of the Harvard Medical School, for the sample of agitoxin.

References

Anderson, W.A. (1996) In *Encyclopedia of Nuclear Magnetic Resonance*, Vol 1: *Historical Perspectives*, Grant D.M. and Harris, R.K. (Eds.), John Wiley, Chichester, U.K., pp. 172–175.

Blechta, V. and Freeman, R. (1993) *Chem. Phys. Lett.*, **215**, 341.

Blechta, V., Del Rio-Portilla, F. and Freeman, R. (1994) *Magn. Reson. Chem.*, **32**, 134–137.

Geen, H., Wu, X-L., Xu, P., Friedrich, J. and Freeman, R. (1989) *J. Magn. Reson.*, **81**, 646–652.

Hadamard, J. (1893) *Bull. Sci. Math.*, **17**, 240–248.

Kaiser, R. (1974) *J. Magn. Reson.*, **15**, 44–63.

Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.

Kay, L.E., Xu, G.Y. and Yamazaki, T.J. (1994) *J. Magn. Reson.*, **A109**, 129–133.

Krezel, A., Khasibhatla, C., Hidalgo, P., McKinnon, R. and Wagner, G. (1995) *Protein Sci.*, **4**, 1478–1489.

Krishnamurthy, K. (2001) *J. Magn. Reson.*, **153**, 144–150.

Kupče, E. and Freeman, R. (1993a) *J. Magn. Reson.*, **A102**, 122–126.

Kupče, E. and Freeman, R. (1993b) *J. Magn. Reson.*, **A105**, 234–238.

Kupče, E. and Freeman, R. (1993c) *J. Magn. Reson.*, **A105**, 310–315.

Kupče, E. and Freeman, R. (1994) *J. Magn. Reson.*, **A108**, 268–273.

Kupče, E. and Freeman, R. (1996) *Chem. Phys. Lett.*, **250**, 523–527.

Kupče, E. and Freeman, R. (2003a) *J. Magn. Reson.*, in press.

Kupče, E. and Freeman, R. (2003b) *J. Magn. Reson.*, in press.

Kupče, E. and Freeman, R. (2003c) *J. Magn. Reson.*, in press.

Levitt, M.H., Freeman, R. and Frenkiel, T. (1982) *J. Magn. Reson.* **47**, 328–330.

Marshall, A.G. and Comisarow, M.B. (1978) Multichannel methods in spectroscopy, In *Transform Techniques in Chemistry*, Griffiths, P.R. (Ed.), Plenum Press, New York.

Nishida, T., Widmalm, G. and Sandor, P. (1995) *Magn. Reson. Chem.*, **33**, 596–599.

Nishida, T., Widmalm, G. and Sandor, P. (1996) *Magn. Reson. Chem.*, **34**, 377–382.

Schraml, J., van Halbeck, H., De Bruyn, A., Contreras, R., Maras, M. and Herdewijn, P. (1997) *Magn. Reson. Chem.*, **35**, 883–888.

Sloane, N.J.A. (1982) In *Fourier, Hadamard and Hilbert Transforms in Chemistry*, Chapter 2, Marshall, A.G. (Ed.), Plenum Press, New York.

Van de Ven, F.J.M. (1995) *Multidimensional NMR in Liquids. Basic Principles and Experimental Methods*, VCH Publishers, New York, NY.

Yamazaki, T., Lee, W., Revington, M., Mattiello, D.L., Dahlquist, F.W., Arrowsmith, C.H. and Kay, L.E. (1994) *J. Am. Chem. Soc.*, **116**, 6464–6465.