

Practical Aspects of Two-Dimensional Transverse NOE Spectroscopy

AD BAX AND DONALD G. DAVIS

Laboratory of Chemical Physics, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

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Recently, Bothner-By and co-workers proposed a new method for measuring homonuclear NOE effects under spin-locked conditions, which they named the CAMELSPIN experiment (1). We prefer to refer to this experiment as rotating-frame Overhauser enhancement spectroscopy (ROESY) since this name suggests a similarity with the closely related 2D NOESY experiment (2, 3). The ROESY method is particularly suitable for molecules that have a motional correlation time, τ_c , near the condition $\omega\tau_c = 1$, where ω is the angular Larmor frequency. In this case the laboratory-frame NOE effect is near zero, whereas the rotating-frame NOE (under spin-locked conditions) is always positive and monotonically increases for increasing value of τ_c , and can be significantly large under those conditions.

The pulse scheme of the ROESY experiment is sketched in Fig. 1. At the end of the evolution period, a strong rf field is switched on for a duration τ_m . During this mixing time, τ_m , projections of the magnetization vectors present at the end of the evolution period, t_1 , onto the vector of the effective rf field remain spin locked along this effective rf field vector. Spin exchange among spin-locked magnetization components of different nuclei can then occur, analogously to spin exchange in the regular NOE experiments, where magnetizations are aligned along the $\pm z$ axis. The spectral density functions describing spin exchange in the ROESY experiment differ from those in the NOESY experiment and cause the effect to be positive for all values of τ_c . We will analyze some experimental considerations that are relevant to this new technique. In particular, we will consider the effect of limited rf field strength of the spin-locking field as well as artifacts that can occur in this type of experiment.

First, as is the case for the NOESY experiment, it is very advantageous to record the ROESY spectrum in the 2D absorption mode. As will be shown below, there is an additional advantage in this type of experiment to the use of absorption-mode spectra: distinction between positive and negative peaks facilitates the identification of certain types of artifacts. A 2D hypercomplex Fourier transformation, as outlined by Müller and Ernst (4) and States *et al.* (5) is conveniently used in this type of experiment. A simple description of this procedure is presented elsewhere (6). A minimum of four experiments with phase cycling according to Table 1 is required

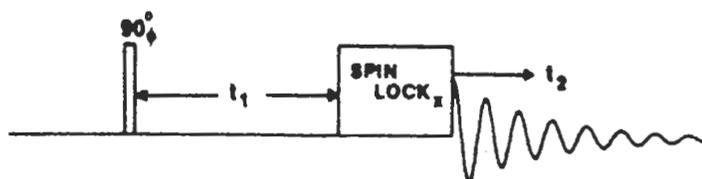


FIG. 1. Pulse scheme of the 2D ROESY experiment. The rf phase of the spin-lock field is cycled according to Table 1, and data acquired for odd- and even-numbered scans are stored separately.

for this experiment. In practice, we use a 16-step experiment, which incorporates CYCLOPS phase cycling (7, 6) for suppression of quadrature artifacts.

Before addressing spurious resonances that may occur in the 2D ROESY spectrum, we qualitatively treat the effect of rf offset on the magnitude of the measured ROESY cross peak. During the mixing period, τ_m , the effective rf fields for two spins A and B make angles α_A and α_B with the positive z axis. If the spin-locking field is applied along the x axis, only the spin exchange of the A and B spin-magnetization components parallel to the x axis is governed by the expressions given by Bothner-By *et al.* (1). Since the fraction of the transverse magnetization that can be spin-locked depends on $\sin \alpha$, the x component of this spin-locked magnetization is proportional to $\sin^2 \alpha$. Hence, the magnitude of the ROESY cross peak between spins A and B will be proportional to $\sin^2 \alpha_A \sin^2 \alpha_B$, while the intensity of the diagonal peaks will be proportional to $\sin^2 \alpha_A$ and $\sin^2 \alpha_B$, for spin A and spin B, respectively. The effect of rf offset on $T_{1\rho}$ is not accounted for here.

Upon application of the ROESY experiment we found two different types of spurious cross peaks, both due to coherent transfer between scalar coupled spins. The first type is due to the fact that the long spin-lock pulse also acts as a mixing pulse for antiphase magnetization, analogously to the 90° mixing pulse in the COSY experiment. Cross peaks induced by this effect will be referred to as COSY-type cross peaks. The second source of artifacts is due to Hartmann-Hahn-type (8) spurious transfer. In the following, the COSY and the Hartmann-Hahn-type transfer

TABLE 1

Phase, ϕ , of the Initial 90° Pulse, and the Way the Data Are Added to and Subtracted from Memory in Successive Scans of the Experiment Sketched in Fig. 1

Scan	ϕ	Acq. ^a
1	x	+
2	y	+
3	-x	-
4	-y	-

^a Data acquired for odd- and even-numbered scans are stored in separate locations.

will be discussed separately. However, a full treatment shows that under the condition where the difference in effective field strength for two coupled spins is on the order of the scalar coupling or less, the two effects are closely related.

Consider two weakly coupled protons, A and B, with coupling constant, J . At the end of the evolution period there will be A spin magnetization that is in antiphase with respect to B. In the operator formalism nomenclature (9) the $\pm x$ components of this magnetization are denoted by $I_{Ax}I_{Bz}$. The effect of a θ_y mixing pulse on this magnetization is described by

$$I_{Ax}I_{Bz} \xrightarrow{\theta_y} I_{Ax}I_{Bz}\cos^2\theta - I_{Az}I_{Bz}\sin\theta\cos\theta + I_{Ax}I_{Bx}\cos\theta\sin\theta - I_{Az}I_{Bx}\sin^2\theta. \quad [1]$$

It is the fourth term on the right-hand side of the arrow that denotes the antiphase magnetization that has been transferred from A to B. The magnitude of this transfer for a long pulse ($\theta \gg 2\pi$) and an inhomogeneous rf field will be proportional to $\langle \sin^2\theta \rangle = 0.5$. It is assumed here that flip angles θ_A and θ_B are identical for both spins A and B. A more detailed analysis shows that θ_A and θ_B differ if the rf offsets of the two nuclei are different, because in this case the effective rf field strengths will not be equal for the two spins. For offsets Δ_A and Δ_B from the transmitter frequency, and an average rf field strength, ν , with $\Delta_A, \Delta_B \ll \nu$, the effective rf field strengths are to a good approximation given by

$$\nu_A = \nu + \Delta_A^2/(2\nu) \quad [2a]$$

$$\nu_B = \nu + \Delta_B^2/(2\nu). \quad [2b]$$

For the case where $|\Delta_A| > |\Delta_B|$, we can consider the mixing pulse of duration, τ , as the sum of two pulses: a pulse with flip angle θ_B applied to both spins followed by a pulse with flip angle $\theta_A - \theta_B$ applied to spin A exclusively. The difference in flip angle is given by

$$\theta_A - \theta_B = \pi\tau(\Delta_A^2 - \Delta_B^2)/\nu. \quad [3]$$

No transfer between A and B will occur for $\theta_A - \theta_B = (n + \frac{1}{2})\pi$, or

$$\tau(\Delta_A^2 - \Delta_B^2)/\nu = n + 1/2 \quad [4]$$

where n is an integer number. The dependence of the difference in flip angle, $\theta_A - \theta_B$, on the inhomogeneous rf field strength of nominal value, ν , also causes the COSY-type cross peak to vanish under the condition

$$\tau(\Delta_A^2 - \Delta_B^2)/\nu \gg 1. \quad [5]$$

This effect has been studied experimentally and follows theoretical predictions. Note also that the integral over a COSY-type cross multiplet is zero.

The second source of spurious cross peaks is due to Hartmann-Hahn transfer through scalar J coupling between mutually coupled protons. This effect has been described in detail by Müller and Ernst (4) and by Chingas *et al.* (10) for the case of a heteronuclear spin system and has also a strong similarity with the coherence transfer in a TOCSY experiment (11). If spins A and B have opposite but equal offsets from the transmitter, the two effective rf fields for spins A and B, ν_A and ν_B , are identical in magnitude and provide a perfect match for homonuclear Hartmann-

Hahn transfer. More generally, the efficiency of the Hartmann-Hahn transfer depends on the difference in the offsets, $|\Delta_A|$ and $|\Delta_B|$, the magnitude of the coupling, J , and the angles α_A and α_B between the effective rf fields and the z axis. The transfer between A and B is an oscillatory function of the mixing time, τ_m . Provided that $\nu \gg J$, it can be shown by using simple density matrix arithmetic that the maximum amount of net magnetization transfer, R_{AB} , from spin A to spin B is given by

$$R_{AB \text{ max}} = [J^2\{1 + \cos(\alpha_A - \alpha_B)\}^2] / [4(\nu_A - \nu_B)^2 + J^2\{1 + \cos(\alpha_A - \alpha_B)\}^2]. \quad [6]$$

From Eq. [6] it is seen that it is advantageous to use a relatively small value for the rf field strength, ν , since this maximizes $|\nu_A - \nu_B|$ (see Eq. [2]), and therefore minimizes this type of spurious cross peak. Note also that the Hartmann-Hahn cross peaks will have the same phase as the diagonal peaks, whereas ROESY cross peaks have opposite phase. The brief analysis presented above is oversimplified; there is also an oscillatory interchange between the COSY and the Hartmann-Hahn-type signals (4, 10, 11). A complete treatment will be presented elsewhere.

The ROESY experiment has been applied to a 0.1 M solution of cyclo(D-Ala-L-Pro-L-Ala)₂ in 90% CDCl₃/10% C₆D₆. Experiments were performed on a Nicolet 270 MHz spectrometer. Regular NOE effects in this sample at 270 MHz are small and positive. The results of two ROESY experiments are shown in Figs. 2 and 3, both recorded with a mixing time of 200 ms. Figure 2 shows the result obtained by using a low rf field strength (2 kHz) and positioning of the carrier frequency at 5.12 ppm. The contour plot only displays resonances that have opposite phase to the diagonal, i.e., resonances that are due to transverse NOE effects. A large number of NOE peaks can be seen in this spectrum. In the lower inset, for example, the cross section parallel to the F_1 axis taken at the chemical shift of the L-Ala amide proton is displayed, showing relatively weak NOE effects between this proton and the L-Ala methyl group, the α , γ , and δ protons of the proline residue, the L-Ala α proton, and the D-Ala amide proton. Conclusions regarding the conformation of this peptide will be presented elsewhere.

Figure 3 shows the result of an experiment where a strong spin-lock field (5 kHz) is used and the carrier frequency is positioned at 3.08 ppm. In this contour plot only resonances that have the same phase as diagonal resonances are displayed; i.e., all cross peaks visible in the contour plot are artifacts, due to Hartmann-Hahn transfer. Two cross sections through this spectrum, shown as insets in Fig. 3, demonstrate that the size of the artifacts can be many times larger than the NOE effect. For example, in Fig. 2 an intense negative NOE cross peak was observed between the C α protons of the alanine residues and their methyl protons, whereas in Fig. 3 this cross peak is positive, due to Hartmann-Hahn transfer.

We have found that using a limited rf field strength for the mixing period in the ROESY experiment is beneficial not only for the lifetime of the probe, but more importantly for the suppression of spurious resonances that are due to COSY and Hartmann-Hahn-type transfers. For the same reason, the carrier frequency has to be chosen in such a way that a Hartmann-Hahn match is avoided. The fact that only little rf power is needed, 0.5 W or less, allows the use of the ROESY experiment on commercial spectrometers, without probe or amplifier modification. Since cross peaks due to Hartmann-Hahn effects are opposite in sign to transverse NOE cross

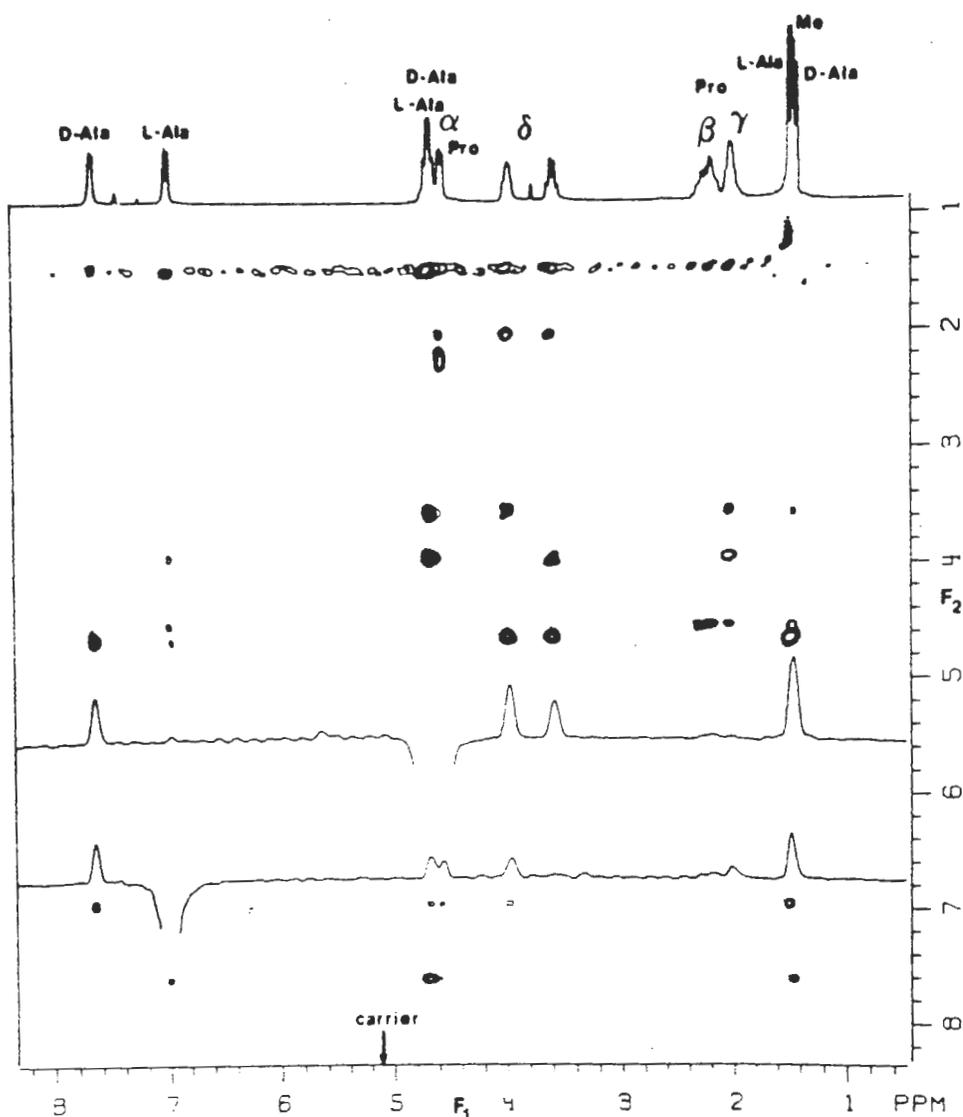


FIG. 2. 2D ROESY spectrum of a 0.1 *M* solution of cyclo(D-Ala-L-Pro-L-Ala)₂ in 90% CDCl₃/10% C₆D₆, recorded at 270 MHz. The carrier frequency is positioned at 5.12 ppm and a 2 kHz spin-lock field has been used during the 200 ms mixing period. 32 scans are performed for every *t_i* value and two 128 × 512 data sets were acquired. Zero filling was used to lead to a 512 × 512 size of the displayed absorptive part of the spectrum. The contour plot shows the resonances that have a sign opposite to the diagonal peaks and are due to NOE. Two insets (with different vertical scale) show cross sections taken parallel to the *F₁* axis at the *F₂* frequencies of the L-Ala amide proton and of the overlapping L-Ala and D-Ala α protons. In these sections, the negative resonance is the diagonal and all positive resonances represent transverse NOE.

peaks, even a small Hartmann-Hahn effect can decrease the size of the observed NOE cross peak significantly. Examination of Eq. [6] is necessary to estimate the maximum possible error that may be introduced by Hartmann-Hahn effects. For

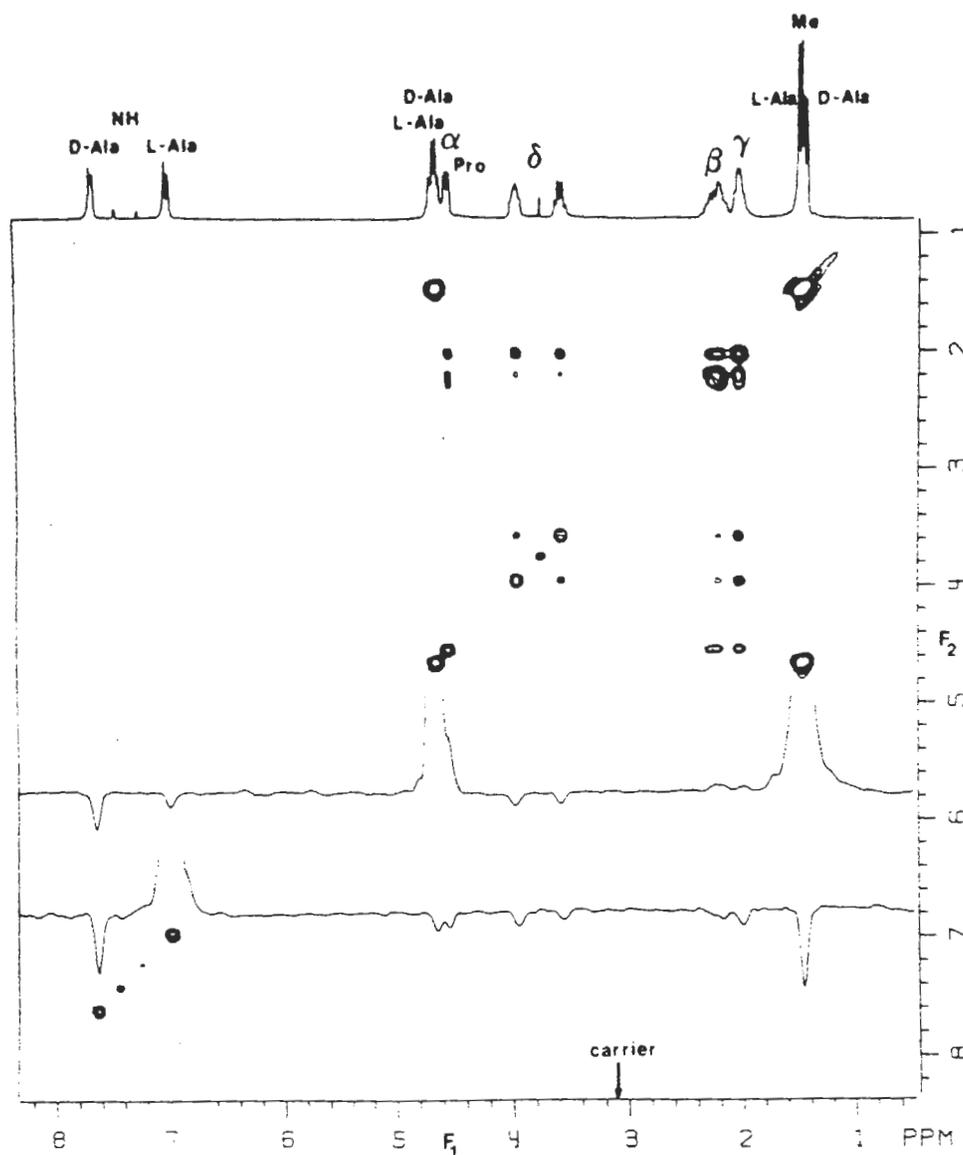


FIG. 3. 2D ROESY spectrum of the same compound as in Fig. 2, recorded under identical conditions with the exception of a 5 kHz rf field strength and a carrier frequency at 3.08 ppm. This contour plot displays only resonances that have identical sign as the diagonal resonances, i.e., Hartmann-Hahn peaks. The two insets are cross sections taken through this spectrum at the F_2 frequencies of the L-Ala amide and of the partially overlapping D-Ala and L-Ala α protons. Negative resonances in these sections are due to transverse NOE.

obtaining quantitatively reliable results at least two spectra with different transmitter frequencies should be recorded. A second problem encountered in quantitative experiments is calibration of NOE intensities. The commonly used procedure, to measure the NOE between nonequivalent geminal protons and use of the known

interproton distance for calibration purposes, can be troublesome since it is often difficult to avoid Hartmann-Hahn effects for nonequivalent geminal protons (large J value and often small difference in chemical shift). However, it is our experience, also based on the study of a number of larger molecules than shown in this paper, that the ROESY experiment can be very useful for conformational studies if used in a careful fashion.

We also wish to point out that Hartmann-Hahn effects can lead to erroneous results in $T_{1\rho}$ experiments on homonuclear coupled spin systems. In a future publication we will demonstrate that Hartmann-Hahn-type transfer can be extremely powerful for the determination of connectivity in homonuclear coupled spin systems.

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REFERENCES

1. A. A. BOTHNER-BY, R. L. STEPHENS, J. LEE, C. D. WARREN, AND R. W. JEANLOZ, *J. Am. Chem. Soc.* **106**, 811 (1984).
2. J. JEENER, B. H. MEIER, P. BACHMANN, AND R. R. ERNST, *J. Chem. Phys.* **71**, 4546 (1979).
3. S. MACURA AND R. R. ERNST, *Mol. Phys.* **41**, 95 (1980).
4. L. MÜLLER AND R. R. ERNST, *Mol. Phys.* **38**, 963 (1979).
5. D. J. STATES, R. A. HABERKORN, AND D. J. RUBEN, *J. Magn. Reson.* **48**, 286 (1982).
6. A. BAX, *Bull. Magn. Reson.*, in press.
7. D. HOULT AND R. E. RICHARDS, *Proc. R. Soc. London Ser. A* **344**, 311 (1975).
8. S. R. HARTMANN AND E. L. HAHN, *Phys. Rev.* **128**, 2042 (1962).
9. O. W. SØRENSEN, G. W. EICH, M. H. LEVITT, G. BODENHAUSEN, AND R. R. ERNST, *Progr. Nucl. Magn. Reson. Spectrosc.* **16**, 163 (1983).
10. (a) G. C. CHINGAS, A. N. GARROWAY, R. D. BERTRAND, AND W. B. MONIZ, *J. Chem. Phys.* **74**, 127 (1981); (b) R. D. BERTRAND, W. B. MONIZ, A. N. GARROWAY, AND G. C. CHINGAS, *J. Am. Chem. Soc.* **100**, 5227 (1978), (c) G. C. CHINGAS, A. N. GARROWAY, R. D. BERTRAND, AND W. B. MONIZ, *J. Magn. Reson.* **35**, 283 (1979).
11. L. BRAUNSCHWEILER AND R. R. ERNST, *J. Magn. Reson.* **53**, 521 (1983).