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Conditions for the measurement of quantitative off-resonance ROESY intensities

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ABSTRACT: Two phenomena hamper the quantification of ROESY intensities: the off-resonance effect and the coherent magnetization transfer between scalar-coupled spins. The off-resonance application of the r.f. irradiation has been proposed to reduce these artefacts. Here the reliability of this method for the quantification of ROESY intensities measured on a sample containing a macromolecule was investigated. The threshold of irradiation offset for which the coherent magnetization transfer can be neglected was determined. It is shown that for offsets larger than this threshold, the off-resonance effect does not hinder the quantification, provided that the irradiation is alternatively applied at high and low field. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; ROE quantification; off-resonance effect; spin diffusion; off-resonance ROESY; coherent magnetization transfer

INTRODUCTION

NMR relaxation has become a widely used method for the analysis of internal dynamics of biomolecules,[1] through the observation of (^{15}N,H) and (^{13}C,H) pairs. The analysis of the (H,H) pair dynamics is, among other problems, hampered by the difficulty in measuring quantitative ROESY[2,3] intensities, because of two major phenomena: the off-resonance effect and the coherent magnetization transfer due to scalar coupling. The off-resonance effect[4] arises because the effective field experienced by each spin is tilted from the static magnetic field by an angle which depends on the Larmor frequency of that spin. The coherent magnetization transfer leads to in-phase cross-peaks associated with HOHAHA cross polarization, which induce errors in the intensity of the direct correlation between scalar-coupled spins[5,6] and also in other intensities observed in the spectrum, through the relayed magnetization transfer.[7–9]

The use of off-resonance r.f. irradiation allows the reduction of the coherent magnetization transfer and of the off-resonance effect.[10] To obtain the greatest reduction, one needs to increase the r.f. irradiation offset or to reduce its strength. However, increasing the offset leads to a decrease of the contribution of transverse relaxation which ROESY is designed to observe. In contrast, for small offset values, i.e., for a large contribution of transverse relaxation, the off-resonance effect and coherent magnetization transfer are no longer negligible. This problem has been explored theoretically;[11] this led to the conclusion that it is impossible to have pure transverse relaxation without coherent magnetization transfer.

To reduce the off-resonance effect, it was proposed to apply the irradiation carrier alternatively at high and low field.[11,12] It was shown that, in conditions of low spin diffusion,[12] this corrects the off-resonance effect to the first order. Two schemes for the variation of irradiation offset were proposed. In the first, the offset was varied during the mixing time, but small offset values were used; the experiment recorded using this scheme was defined in the literature[11] as JS-ROESY. In the second acquisition scheme, two successive transients were recorded with high- and low-field irradiation;[12] in the following, the ROESY recorded using this scheme will be referred to as the double-offset ROESY. Being interested by analyzing protein dynamics with the ROESY experiment, we were concerned with the determination of the range of experimental parameter values (offset and amplitude of the r.f. irradiation) for which the intensities measured using these schemes do not present the distortions induced by off-resonance effect and coherent magnetization transfer. Using numerical simulations and experimental evidences, we verified that the double-offset scheme is still efficient under the conditions of spin diffusion, and we determined numerically and experimentally the conditions for which the coherent magnetization transfer is lower than the other uncertainties of the measurement.

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EXPERIMENTAL

The experiments were recorded with a Bruker AMX instrument operating at 600 MHz, on a 5 mM sample of toxin γ[13] from *Naja nigricollis* venom. The toxin was dissolved in 500 μl of ^1H_2O–^2H_2O (90:10) mixture. All the experiments presented here were performed at a pH of 3.5 and a temperature of 318 K. The r.f. irradiation used adiabatic rotation.[14] It consists of a trapezoidal-shaped pulse with a time of increase of 3 ms and a total duration of 200 ms. The irradiation strength was 6.3 kHz, if not stated otherwise.

The discrepancy between two sets of intensities I^1_{ij} and I^2_{ij} (i and j being the spin indexes) was estimated numerically by calculating a relative mean difference value:

$$\varepsilon = \frac{\sum_{i,j}\left(\frac{1}{\sigma_{ij}}\right)^2 \frac{I^1_{ij} - I^2_{ij}}{I^1_{ij} + I^2_{ij}}}{\sum_{i,j}\left(\frac{1}{\sigma_{ij}}\right)^2} \tag{1}$$

where σ_{ij} is the error on the relative difference $(I^1_{ij} - I^2_{ij})/(I^1_{ij} + I^2_{ij})$, evaluated using logarithmic derivatives and uncertainty on experimental measurement and numerical simulation.

RESULTS AND DISCUSSION

Coherent transfer of magnetization

Theory. For a spin I^i, resonating at the frequency $\omega_0 + \delta_i$, where ω_0 is the frequency of the centre of the spectrum, the effective field produced by the r.f. irradiation makes an angle θ_i with the static magnetic field direction:

$$\tan\theta_i = \frac{\omega_1}{\Delta - \delta_i} \tag{2}$$

where Δ is the offset of irradiation with respect to ω_0 and ω_1 is the strength of irradiation. In the following, the spectral resonance frequencies δ_i are assumed to be located in the interval $[\omega_0 - \delta_m, \omega_0 + \delta_m]$.

During the application of the r.f. irradiation, the coherent magnetization transfer between two scalar-coupled spins is given by[5,6]

$$f(t) = \frac{\sin^2 2\phi}{2}(1 - \cos 4\pi q t) \tag{3}$$

where

$$\tan(2\phi) = \frac{[1 + \cos(\theta_i - \theta_j)]J}{2(v_i - v_j)} \tag{4}$$

$$q = \frac{1}{2}\left\{(v_i - v_j)^2 + [1 + \cos(\theta_i - \theta_j)]^2 \frac{J^2}{4}\right\}^{1/2} \tag{5}$$

v_i and v_j are the effective fields applied to spins I^i and I^j:

$$v_{i,j} = \sqrt{(\Delta - \delta_{i,j})^2 + \omega_1{}^2} \tag{6}$$

and J is the scalar coupling constant between I^i and I^j. Equation (3) describes the instantaneous coherent magnetization transfer, in a voxel of sample where ω_1 can be considered to be homogeneous. The cumulative magnetization transfer observed on the overall sample volume V is obtained by integrating this equation on the distribution $P(\omega_1)$ of ω_1 values in the sample volume V:

$$g(t) = \int_V f(t)P(\omega_1)\,\mathrm{d}\omega_1 \tag{7}$$

Because of the coherent magnetization transfer, the intensity between scalar-coupled spins i and j is scaled by a factor equal to $[1 + g(\tau_m)]$, where τ_m is the experiment mixing time. The magnitude of the distortion induced on intensity is thus evaluated by comparing $g(\tau_m)$ with 1. On the other hand, the intensity between spin i (or j) and other spins k, in presence of relayed magnetization transfer, is

$$h(\tau_m) = \int_0^{\tau_m} g(t)Q_{ik}(t)\,\mathrm{d}t \tag{8}$$

where $Q_{ik}(t)$ is the instantaneous cross-relaxation rate between spins i and k. The distortion of intensity due to relayed magnetization transfer is estimated by comparing $h(\tau_m)$ with the integral of $Q_{ik}(t)$ on the mixing time interval.

Numerical simulations. The variation $g(t)$ of coherent magnetization transfer was calculated from Eqn (7) and is plotted in Fig. 1, for a typical value of spectral width (7 kHz), and for two weakly coupled spins located at the edge of the spectrum ($J = 10$ Hz, $\delta_i = \delta_m$, $\delta_j = \delta_m - 100$ Hz). This corresponds to the worst conditions which can occur for weakly coupled spins. The θ angle was set equal to 50° and the spin-lock strength ω_1 was varied from 7 to 4.5 kHz, which is equivalent to varying Δ from 5.8 to 3.8 kHz. The distribution $P(\omega_1)$ of ω_1 values in the sample volume was obtained from an experimental measurement of ω_1 inhomogeneity (see caption of Fig. 1 for details). Figure 1 shows that for Δ values larger than $1.5\delta_m$, the magnitude of transfer $g(t)$ is always smaller than 0.05. Consequently,

$$|h(\tau_m)| \leqslant 0.05\left|\int_0^{\tau_m} Q_{ik}(t)\,\mathrm{d}t\right| \tag{9}$$

The distortions induced on ROESY intensities by coherent and relayed magnetization transfers can therefore be neglected, for Δ values larger than $1.5\ \delta_m$. In the following, the simulations will be performed and the experiments recorded under these conditions.

Experimental validation. Three ROESY experiments were recorded using the double-offset scheme, in order to provide experimental evidence for neglecting coherent magnetization in the case of Δ values larger than 1.5 δ_m. The θ value was kept constant at 45° and r.f. irradiation strengths of 5.9, 5.5 and 5.1 kHz were used. The corresponding Δ/δ_m, ratios are 1.74, 1.62 and 1.5, respectively. The variation of the r.f. offset was 400 Hz between experiments; this was shown to be sufficient to observe a large variation of intensity when the coherent

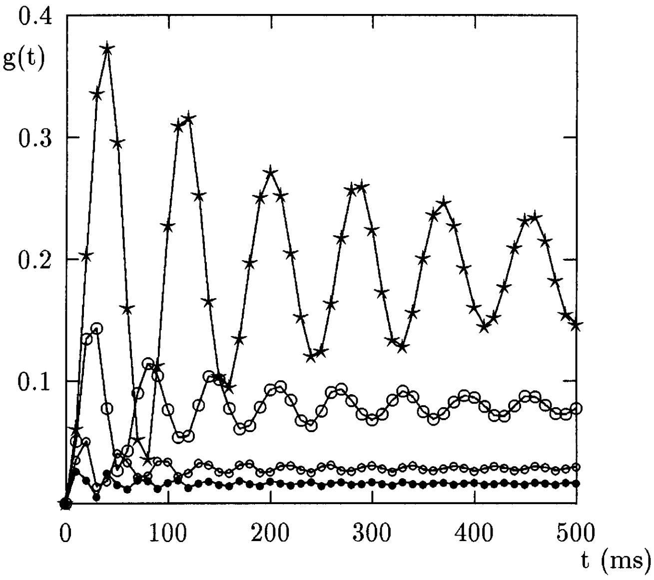

Figure 1. Variation of the coherent magnetization transfer with the time. The results are plotted for Δ/δ_m values of 1.68 (●), 1.44 (○), 1.20 (○) and 1.08 (★). The transfer was calculated for two weakly coupled spins, located at the edge of the spectrum (see text for more details). The distribution of ω_1 values used for calculating the integral of coherent magnetization on the sample volume was the sum of two non-symmetric Gaussian functions centered on the nominal ω_1 value. This function was obtained by fitting an experimental measurement of ω_1 inhomogeneity.

magnetization transfer is important.[15] For each peak, intensity values were measured in the three experiments and are compared in Fig. 2.

Relative mean difference were calculated using Eqn (1) between intensities measured for ω_1 values of 5.9 and 5.5 kHz and ω_1 values of 5.5 and 5.1 kHz. The calculation was performed on the overall set of 343 peaks, on a subset of 125 peaks correlating covalently bonded spins and on a subset of 16 peaks correlating covalently bonded spins and located on the edges of the spectrum. The relative mean difference values ε were found to be equal to 4.2 and 2.9% on the first peak set, 3.9 and 2.3% on the second set and 4.5 and 3.0% on the third set. These values are of the same order between the different strengths of irradiation and for the different sets of peaks examined, although the second and third sets of peaks are specially prone to distortions induced by coherent magnetization transfer.

The discrepancies between intensities were also estimated by two linear regression analyses (Fig. 2). Linear slopes of 0.96 and 0.97, *y*-intercepts of -0.0060 and -0.0020 and correlation factors of 0.99 were obtained. Examination of Fig. 2, the values of the relative mean differences and the results of the linear regression analyses show that the agreement between the three experiments is excellent; the intensity values were not distorted at all by coherent magnetization transfer.

Off-resonance effect

Theory. The cross-relaxation rate between spins I^i and I_j is a weighted sum of the longitudinal and transverse cross-relaxation rates R_{ij} and P_{ij}:

$$Q_{ij} = R_{ij} \cos \theta_i \cos \theta_j + P_{ij} \sin \theta_i \sin \theta_j \qquad (10)$$

where θ_i and θ_j are determined from Eqn (2). The ROESY intensity matrix **I** is calculated from the relaxation matrix $\boldsymbol{Q}$:

$$\boldsymbol{I} = \exp(-\boldsymbol{Q}\tau_m)\boldsymbol{I}_0 \qquad (11)$$

were τ_m is the mixing time and $\boldsymbol{I}_0$ the intensity diagonal matrix for a vanishingly small mixing time.

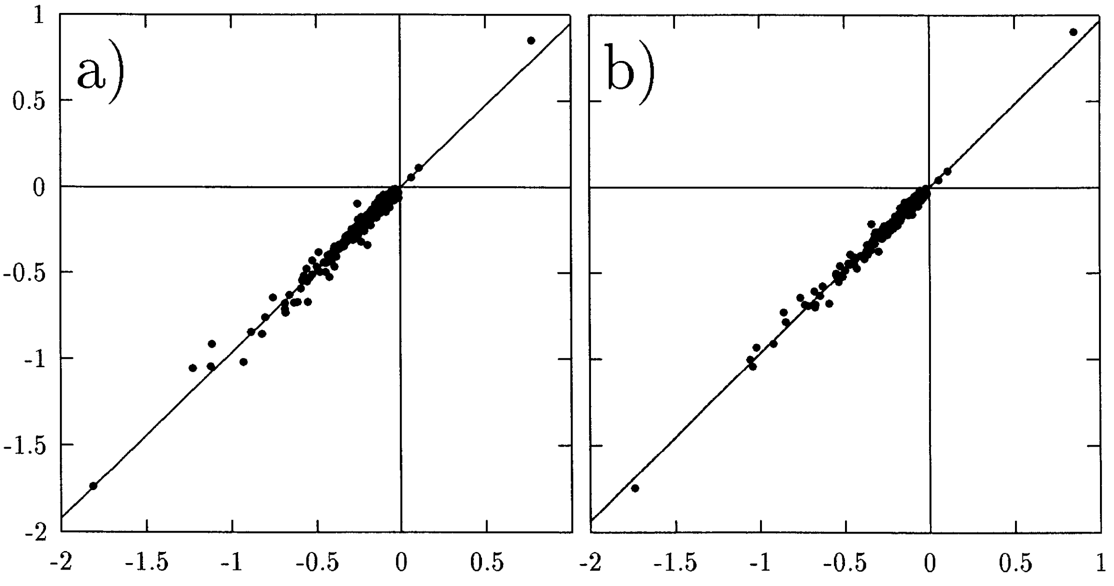

Figure 2. Comparison of experimental intensities measured at a θ value of 45° and irradiation strengths of 5.95, 5.56 and 5.12 kHz. The experiments were recorded using the double-offset method. The intensities (a.u.) are compared (a) between experiments recorded using r.f. irradiation strengths of 5.95 kHz (*x*-axis) and 5.56 kHz (*y*-axis) and (*b*) between experiments recorded using r.f. irradiation strengths of 5.56 kHz (*x*-axis) and 5.12 kHz (*y*-axis). The results of linear regression analyses are drawn with continuous straight lines.

For large Δ values ($\Delta \gg \delta_m$), the δ_i values become small relative to Δ. Thus, the spread of angles θ_i along the spectral width is negligible and the effective fields experienced by all observed spins are tilted by the same angle θ:

$$\tan \theta = \frac{\omega_1}{\Delta} \tag{12}$$

In the following, we will refer to an experiment that would be recorded without angular dispersion as the constant-θ ROESY experiment. The relaxation rate of the constant-θ ROESY is

$$Q_{ij}^{\text{cst}} = R_{ij} \cos^2 \theta + P_{ij} \sin^2 \theta \tag{13}$$

Numerical simulations. The error induced on intensities by the off-resonance effect was numerically evaluated. The program CROWD[16] was run on the proton coordinates of one of the NMR conformers[13] of the toxin γ. The average proton Larmor frequency ω_0 was taken to be equal to 600 MHz. The relaxation was assumed to result only from dipolar interaction. A model of rigid isotropic Brownian motion, characterized by a global correlation time τ_c, was used to calculate the longitudinal and transverse relaxation rates. The relaxation rates of the off-resonance ROESY and of the constant-θ ROESY were computed using Eqns (10) and (13), respectively. The θ_i values were determined from the offset Δ, the r.f. field amplitude ω_1 and the frequencies δ_i, calculated from the chemical shift values [Eqn (2)]. The chemical shifts of assigned hydrogens were taken from the literature,[13] whereas the chemical shifts of unassigned hydrogens were set equal to the random coil chemical shifts of the corresponding amino acids.[17] The intensity values were calculated from the relaxation rates using Eqn (11). In that way, the spin diffusion is completely taken into account.[18] The intensities $\boldsymbol{I}^{\text{dble}}$ of the double-offset ROESY experiment were calculated as the arithmetic mean value of the off-resonance ROESY intensities obtained for the two offsets of the r.f. irradiation. The intensities $\boldsymbol{I}^{\text{JS}}$ of the JS-ROESY at a mixing time of τ_m were calculated in the following way:

$$\boldsymbol{I}^{\text{JS}}(\tau_m) = \exp(-\boldsymbol{Q}^{\text{high}}\tau_m/2)\exp(-\boldsymbol{Q}^{\text{low}}\tau_m/2)\boldsymbol{I}_0 \tag{14}$$

where $\boldsymbol{Q}^{\text{high}}$ and $\boldsymbol{Q}^{\text{low}}$ are the relaxation matrixes for high- and low-field irradiations. Equation (14) corresponds to an irradiation offset jumped only once during the mixing time, as it was proposed[11] in the experimental scheme of JS-ROESY. Finally, the constant-θ ROESY intensity matrix $\boldsymbol{I}^{\text{cst}}$ was calculated from the constant-θ relaxation matrix $\boldsymbol{Q}^{\text{cst}}$ using Eqn (11).

The discrepancy induced on intensities by the residual off-resonance effect was calculated for different parameter values. Four series of simulations were performed by varying the experiment mixing time τ_m, the molecular global correlation time τ_c, the r.f. field amplitude ω_1 and the angle θ. In each series, the $\boldsymbol{I}^{\text{cst}}$ and $\boldsymbol{I}^{\text{JS}}$ intensities and the $\boldsymbol{I}^{\text{cst}}$ and $\boldsymbol{I}^{\text{dble}}$ intensities were compared on a subset of 425 hydrogen pairs, corresponding to NOESY intensities larger than 0.015 at a mixing time of 200 ms. The uncertainty on the intensities was consequently taken as equal to 0.0075. The discrepancy between the intensities was evaluated by calculating between $\boldsymbol{I}^{\text{cst}}$ and $\boldsymbol{I}^{\text{JS}}$ intensities, and between $\boldsymbol{I}^{\text{cst}}$ and $\boldsymbol{I}^{\text{dble}}$ intensities, the mean difference values:

$$E^{\text{dble, JS}} = \langle I_{ij}^{\text{cst}} - I_{ij}^{\text{dble, JS}} \rangle \tag{15}$$

and the relative mean difference values $\varepsilon^{\text{dble, JS}}$ [Eqn (1)].

In τ_c, τ_m and ω_1 simulation series, the $\varepsilon^{\text{dble, JS}}$ values are always smaller than 3% [Fig. 3(a)–(c)]. They increase with τ_m and τ_c values and decrease with ω_1 values. In the θ simulation series, the $\varepsilon^{\text{dble, JS}}$ variation [Fig. 3(d)] shows values larger than 3% for θ values within the 30–40° range. Moreover, $\varepsilon^{\text{dble}}$ and ε^{JS} values equal to 90% are obtained at $\theta = 35°$, and are not displayed on the plot. These large values come from the small intensities values calculated within the 30–40° θ range. Indeed, as transverse and longitudinal cross-relaxation rates have opposite signs for protein motion time-scales, Eqn (13) shows that there is a particular value of the angle θ for which the off-resonance ROESY cross-relaxation rate Q_{ij}^{cst} and thus the intensity vanish. This particular value here is close to 35°, because of the particular motion time-scale used in simulation. The observed divergence of the relative mean difference within the 30–40° range thus arises from small cross-peak intensity values rather than from an increased distortion between double-offset and constant-θ intensities. This interpretation is supported by the $E^{\text{dble, JS}}$ values, which are almost constant and smaller than 0.0015 (five times smaller than the uncertainty on the intensities) in the same θ range [Fig. 3(d)].

The intensities calculated using the scheme of the JS-ROESY present slightly larger ε and E values than the intensities calculated in the frame of the double-offset scheme. However, the difference between ε^{JS} and $\varepsilon^{\text{dble}}$ (and E^{JS} and E^{dble} respectively) values is always smaller than 1% (0.0003), and is thus negligible.

Evaluation of the overall precision of the intensity values

The relative mean difference values obtained above from the simulations and experiments are small, but the real significance of these values can be evaluated only by comparing them with the experimental reproducibility. This reproducibility was estimated by recording a series of three off-resonance ROESY spectra acquired with a θ value of 45°, and using a high-field, a low-field and a double-offset r.f. irradiation. From the double-offset ROESY spectrum, 343 peaks unambiguously assigned and displaying intensities larger than the background noise level were selected. As the double-offset ROESY experiment is obtained by summing FIDs recorded using high-and low-field r.f. irradiations,

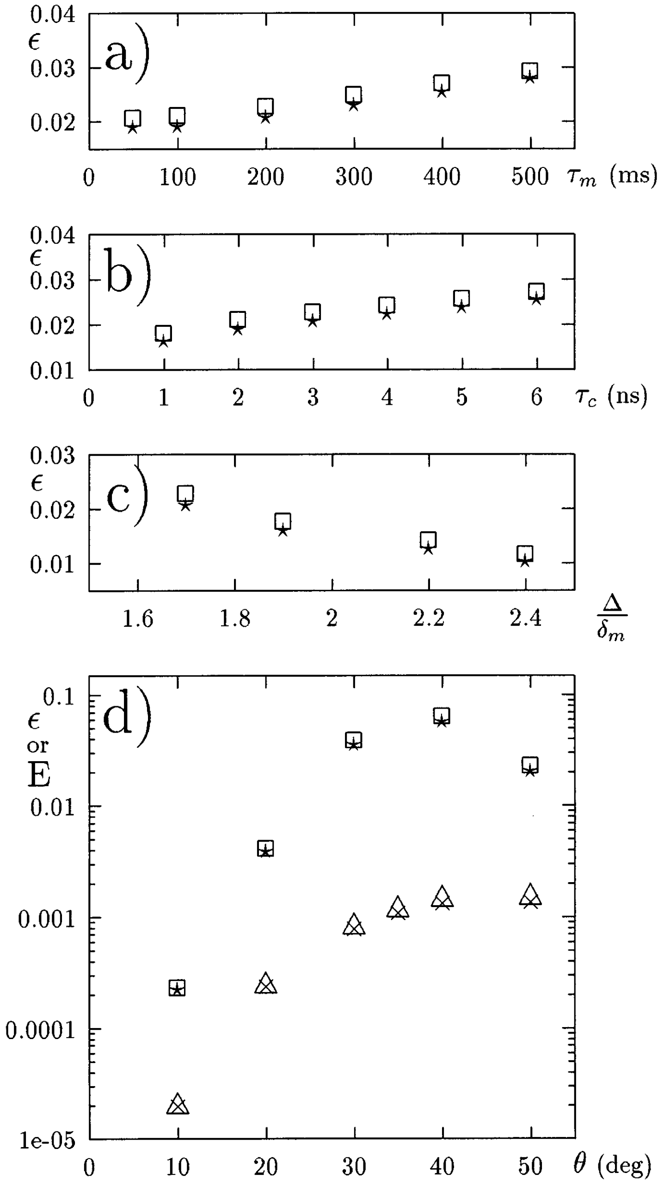

Figure 3. Relative mean differences $\varepsilon^{\mathrm{dble}}$ (★) and $\varepsilon^{\mathrm{JS}}$(□) calculated between double-offset and constant-θ ROESY intensities and between JS-ROESY and constant-θ ROESY intensities. Four simulation series were performed by varying. (a) the mixing time (50, 100, 200, 300, 400 and 500 ms); (b) the global correlation time, τ_c, from 1 to 6 ns, by steps of 1 ns; (c) the r.f. irradiation amplitude, ω_1, from 7 to 10 kHz, in steps of 1 kHz; this corresponds to Δ values varying from 5.9 to 8.4 kHz; the $\varepsilon^{\mathrm{dble,\,JS}}$ values are plotted against the value of $\Delta/\delta_{\mathrm{max}}$; in the case of the protein used here, the spectral region spreads over 6.9 kHz, and consequently δ_{max} is equal to 3.47 kHz; (d) the θ angle value from 5° to 50° in steps of 5°; for this simulation, the mean differences E^{dble} (×) and E_{JS} (Δ) are also plotted. In each simulation, the parameters held fixed have the values $\tau_m = 200$ ms, $\tau_c = 3$ ns, $\omega_1 = 7$ kHz and $\theta = 50°$.

the intensities of double-offset ROESY are expected to be equal to the mean value of intensities measured using high- and low-field irradiations. An $\varepsilon^{\mathrm{noise}}$ value was calculated [Eqn (1)] between these mean intensity values and the corresponding double-offset intensities, and was found to be 4.5%; this is of the same order as the ε values obtained above from simulations and experiments. The distortions induced on intensities by the off-resonance effect and coherent magnetization transfer are thus of the same order as those induced by ordinary lack of reproducibility of the experiment.

CONCLUSION

In this work, the distortion induced on ROESY intensities by the coherent magnetization transfer and by the off-resonance effect was examined. Numerical calculations and experimental measurements showed that the coherent magnetization transfer between weakly coupled spins can be neglected totally for offset values Δ, larger than 1.5 δ_m. The influence of the off-resonance effect on intensities was theoretically explored under the conditions $\Delta \geqslant 1.5\ \delta_m$, and was found to be of the level of the experimental noise. The irradiation offset can be varied between two scans or during the mixing time; the efficiency for the suppression of the off-resonance effect is similar.

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