Apparent transverse relaxation rates in systems with coupled carbon-13 spins

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In systems with homonuclear scalar couplings, the envelopes of spin echoes obtained with simple refocusing pulses or trains of such pulses are normally modulated so that it is difficult to extract transverse relaxation rates. It has been shown recently that echo modulations can be quenched by cumulative pulse errors that arise after applying a large number of refocusing pulses with moderate *rf* amplitudes. The resulting unmodulated decays allow one to extract apparent transverse relaxation rates. Early work on systems comprising only two nitrogen-15 nuclei or two carbon-13 spins has recently been extended to systems with coupled protons. This work focuses on systems with three coupled carbon-13 spins, which in turn are coupled to several neighbouring protons. Unmodulated echo trains can be obtained by optimizing the pulse interval, the carrier frequency and the *rf* amplitude of the refocusing pulses.

Introduction

The measurement of transverse relaxation rates $R_2 = 1/T_2$ of various nuclei can provide valuable information about internal dynamics.[1] Spin echo sequences with multiple refocusing pulses[2,3] are commonly used to extract R_2 rates of nitrogen-15 nuclei. In proteins, these nuclei are isolated from each other, in the sense that scalar couplings J_{NN} between amide nitrogen-15 nuclei can be neglected. In nucleic acids however, J_{NN} couplings in fully nitrogen-15 enriched aromatic purine and pyrimidine systems and across hydrogen bonds lead to modulations of echo trains. Likewise, homonuclear scalar J_{CC} couplings tend to mask the envelopes of echo decays in fully carbon-13 enriched molecules. Needless to say, the determination of transverse relaxation of protons is challenging in virtually all molecules because echoes are invariably modulated by scalar J_{HH} couplings.

It has recently been shown that under certain conditions, it is possible to quench echo modulations due to homonuclear scalar couplings.[4–9] In two-spin systems, so-called *apparent* transverse relaxation rates R_2^{app} can be extracted from echo decays provided that they do not suffer from significant modulations. Similar observations have been made in proton spectra of cyclosporin, which features extensive networks of coupled spins.[10]

A multiple-refocusing Carr–Purcell–Meiboom–Gill (CPMG) sequence[2,3] can be written:

$$(\pi/2)_y - (\tau - \pi_x - \tau)_{2n}. \tag{1}$$

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We shall only consider even echoes with $2n = 2, 4, 6\ldots$ The duration of each CPMG cycle is

$$\tau_c = [4\tau + 2\tau_\pi], \tag{2}$$

where τ_π is the duration of a π pulse.

In a frame rotating at the carrier frequency ω_{rf}, the Hamiltonian for a system with three spins $I = \frac{1}{2}$, $S = \frac{1}{2}$, and $R = \frac{1}{2}$ (*e.g.*, three carbon-13 nuclei) may be written:

$$H = \Omega_I I_z + \Omega_S S_z + \Omega_R R_z + 2\pi J_{IS} \boldsymbol{I} \cdot \boldsymbol{S}$$
$$+ 2\pi J_{IR} \boldsymbol{I} \cdot \boldsymbol{R} + 2\pi J_{SR} \boldsymbol{S} \cdot \boldsymbol{R} \tag{3}$$

with offsets $\Omega_I = \omega_{0I} - \omega_{rf}$, $\Omega_R = \omega_{0R} - \omega_{rf}$, and $\Omega_S = \omega_{0S} - \omega_{rf}$, where ω_{0I} is the Larmor frequency of spin I in the laboratory frame.

Note that heteronuclear couplings are not included in this Hamiltonian, *i.e.*, we assume that the protons are decoupled throughout. In actual fact, decoupling during multiple refocusing sequences may be challenging, for there can be complicated interference effects between the CPMG cycle τ_c and the cycle (or supercycle) of a (composite pulse) decoupling sequence. Even continuous-wave (CW) proton decoupling with a constant *rf* amplitude $\omega_1(^1H)$, which has a cycle $\tau_{cw} = 2\pi/\omega_1(^1H)$, can interfere with multiple refocusing of nuclei such as ^{13}C, ^{15}N, *etc.* These interference effects can accelerate the decay of echo trains, and thus increase the apparent transverse relaxation rates R_2^{app}. A detailed discussion of decoupling effects is beyond the scope of this work. A safe way to circumvent these complications is to avoid proton decoupling altogether during the multiple refocusing sequence, so that the decoupler is activated only during signal acquisition, *i.e.*, during the second half of the $2n^{th}$ echo. In the absence of decoupling, the signals of a spin S are split into multiplets by heteronuclear couplings J_{SK} with protons K. The behaviour of each separate multiplet component under the multiple refocusing sequence can be understood if we consider that it has a

different *effective offset*. Thus, in a system with a single proton K, we must consider two distinct offsets $\Omega_S + \pi J_{SK}$ and $\Omega_S - \pi J_{SK}$, instead of a single offset at the chemical shift Ω_S. In ^{13}C spectra of alanine ($NH_3^+CHCH_3COO^-$), one must also consider degenerate heteronuclear couplings that lead to quartets.

The homonuclear scalar couplings in eqn (3) have the full isotropic form,

$$\boldsymbol{I} \cdot \boldsymbol{S} = I_x S_x + I_y S_y + I_z S_z. \tag{4}$$

If the refocusing pulses are very far off-resonance, the coherences precess in each cycle τ_c under the effect of the offsets so that they accumulate phase shifts Φ,

$$\Phi_I = \Omega_I \tau_c, \ \Phi_S = \Omega_S \tau_c, \ \Phi_R = \Omega_R \tau_c. \tag{5}$$

For a system with three spins I, S, and R the carrier frequency ω_{rf} can be set on resonance for spin I ($\Omega_I = 0$). The tilt angles θ_S and θ_R of the effective fields experienced by the two other spins during refocusing pulses with an *rf* amplitude ω_1 can be described by the tilt parameters:

$$\gamma_S = \Omega_S/\omega_1, \ \gamma_R = \Omega_R/\omega_1. \tag{6}$$

The analysis of the full CPMG cycle[8] shows that the modulations of the echoes are most pronounced when

$$\Phi_S'' = \Omega_S \tau_c - \pi \gamma_S = 4k_S \pi \tag{7a}$$

$$\Phi_R'' = \Omega_R \tau_c - \pi \gamma_R = 4k_R \pi \tag{7b}$$

with integers k_S and k_R. When either of these conditions is satisfied, the scalar couplings J_{IS} or J_{IR} are said to be 'recoupled', so that the echoes are fully modulated. These conditions have superficial similarities with sidebands due to delays alternating with nutation for tailored excitation (DANTE).[11,12] We shall speak of sidebands of order k, or simply of the kth sideband. In a three-spin system with $\Omega_I = 0$, there are two distinct conditions where either J_{IS} or J_{IR} are 'recoupled' and lead to echo modulations:[9]

$$\tau_S = \frac{\pi(4k_S \omega_1 - \Omega_S)}{4\Omega_S \omega_1} \tag{8}$$

$$\tau_R = \frac{\pi(4k_R \omega_1 - \Omega_R)}{4\Omega_R \omega_1} \tag{9}$$

The intervals between these recoupling conditions are inversely proportional to the corresponding offsets:

$$\Delta \tau_S = \pi/\Omega_S \tag{10}$$

$$\Delta \tau_R = \pi/\Omega_R \tag{11}$$

These echo modulations can be simulated numerically by calculating the expectation values $\langle I_x \rangle$ at the top of the $2n^{th}$ echo (there are no phase-shifts, so that $\langle I_y \rangle = 0$ if $\Omega_I = 0$), considering the full Hamiltonian without relaxation effects. The amplitude of the echo modulations of spin I is defined over the relevant range of refocusing pulses.

$$A_{mod} = \frac{\max(\langle I_x \rangle) - \min(\langle I_x \rangle)}{2} \tag{12}$$

We shall begin by simulating the behavior of a simple two-spin system. Fig. 1 shows how the amplitude A_{mod} of the
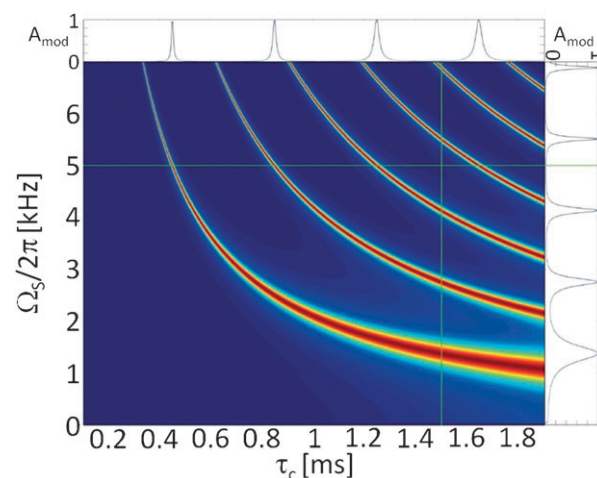
Fig. 1 Amplitudes A_{mod} of echo modulations calculated for a two-spin system as a function of the offset Ω_S of the coupling partner S when $\Omega_I = 0$, for a CPMG cycle of length $\tau_c = [4\tau + 2\tau_\pi]$ and an *rf* amplitude $\omega_1/(2\pi) = 10$ kHz. Dark blue areas correspond to regions without modulations, whereas red ridges indicate maximum recoupling conditions. The plot along the top shows A_{mod} as a function of τ_c for a fixed offset $\Omega_S/(2\pi) = 5$ kHz, while the plot along the right side shows A_{mod} as a function of the offset Ω_S of the second spin for a fixed $\tau_c = 1.5$ ms.

modulations of the expectation value $\langle I_x \rangle$ depends on the offset Ω_S of the coupling partner and on the cycle length τ_c, or, equivalently, on the duration of the defocusing and refocusing intervals τ. Sharp recoupling conditions are observed for short cycle lengths τ_c and for large offsets Ω_S, when the effective fields are strongly tilted, *i.e.*, for large values of γ_S. On the other hand, small γ_S ratios lead to broader recoupling conditions.

In systems with three or more spins, one observes a superposition of different recoupling conditions characterized by different widths. Instead of showing extensive simulations of systems with N spins in multidimensional parameter space, we shall directly compare simulations with experimental observations.

Experimental

To explore the two-dimensional parameter space shown in Fig. 1, we have used two distinct experimental approaches. In so-called hybrid experiments, we increment the delay τ (or, equivalently, the cycle time τ_c) while keeping the number $2n$ of refocusing pulses constant. This allows one to identify conditions where the echoes are not modulated. Once these favorable conditions have been found, echo decays are recorded by keeping τ constant and incrementing n, so that the apparent transverse relaxation times can be determined by fitting to a mono-exponential decay.

The measurements have been carried out with a sample of ^{13}C- and ^{15}N-enriched L-alanine (Cambridge Isotope Laboratories, U-$^{13}C_3$, 98%; ^{15}N, 98%), 0.5 M in D_2O using a Bruker Avance spectrometer with a field $B_0 = 11.74$ T (500 MHz for ^1H). Fig. 2 shows experimental echo amplitudes C^α_x (blue) and simulated expectation values $\langle C^\alpha_x \rangle$ (red) in
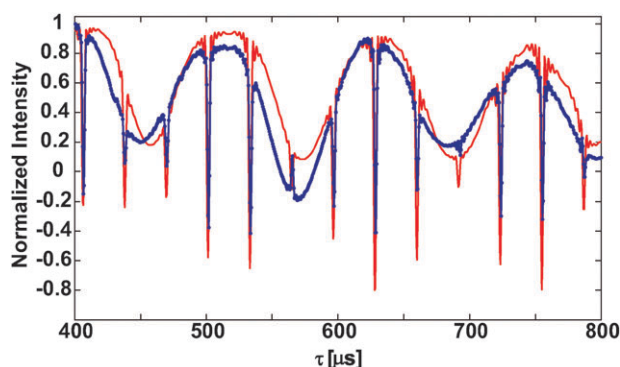
Fig. 2 Amplitudes $\langle C^\alpha_x \rangle$ of the 20th echo ($2n = 20$) in ^{13}C- and ^{15}N-enriched alanine, as a function of the delay τ. Experimental results are shown in blue, whereas simulated data are plotted in red. The ^{13}C rf amplitude was $\omega_1/(2\pi) = 20$ kHz, hence $\tau_\pi = 25$ μs. The τ delay has been varied between 400 and 800 μs in steps of 0.5 μs. The chemical shifts are $\delta(C^\alpha) = 50.37$, $\delta(C^\beta) = 16.02$ and $\delta(C') = 175.76$ ppm. At $B_0 = 11.74$ T, C^β and C' appear at offsets of -4.32 and 15.77 kHz with respect to C^α. The scalar coupling constants are $J(C^\alpha C^\beta) = 35.0$, $J(C^\alpha C') = 54.2$, $J(C^\beta C') = 1.2$, $J(C^\alpha H^\alpha) = 145.0$, $J(C^\beta H^\alpha) = 4.4$, $J(C'H^\alpha) = 4.2$, $J(C^\beta H^\beta) = 129.7$, $J(C^\alpha H^\beta) = 4.6$ and $J(C^\alpha N) = 5.7$ Hz.

alanine with $\Omega^\alpha = 0$ and $\omega_1/(2\pi) = 20$ kHz, as a function of the delay τ, using a hybrid sequence with $2n = 20$. The superposition of two different recoupling conditions is clearly visible: the narrow dips, separated by small intervals $\Delta\tau'$ are due to the $J(C^\alpha C')$ coupling to the spectrally remote C' spin ($\Omega'/(2\pi) = 15.77$ kHz, hence $\gamma' = 0.79$), while the broad dips, separated by large intervals $\Delta\tau^\beta$, are due to the $J(C^\alpha C^\beta)$ coupling to the C^β spin which has a chemical shift that is not far away from C^α, since $\Omega^\beta/(2\pi) = -4.32$ kHz, hence $\gamma^\beta = -0.22$. In order to determine the transverse relaxation rate $R_2^{app}(C^\alpha)$, it is necessary to find a flat region where the amplitude A_{mod} of the modulations is small. In the range $400 < \tau < 800$ μs, the best choice appears to be $\tau = 515$ μs.

To reduce the complexity of the problem, we can set the rf amplitude ω_1 in such a manner that the refocusing pulses (which must act like π-pulses for the on-resonance C^α spins) lead to a rotation through an angle 2π for the off-resonance C' magnetization. The effective nutation angle β_{eff} is

$$\beta_{eff} = \omega_{eff}\tau_\pi = \sqrt{\omega_1^2 + \Omega^2(C')}\tau_\pi \qquad (13)$$

which is equal to 2π when

$$\omega_1 = \frac{\Omega(C')}{\sqrt{3}} \approx 0.577\Omega(C') \qquad (14)$$

Fig. 3 shows that with this rf amplitude the narrow recoupling conditions due to $J(C^\alpha C')$ have been suppressed as expected. However, a more complex pattern appears for the $J(C^\alpha C^\beta)$ interaction. This results from the fact that the C^β carbon is attached to three H^β protons, leading to a quadruplet. The four components, which have the usual binomial amplitudes $1 : 3 : 3 : 1$, can be considered as four different effective offsets with respect to the C^α carbon, leading to four different recoupling conditions.
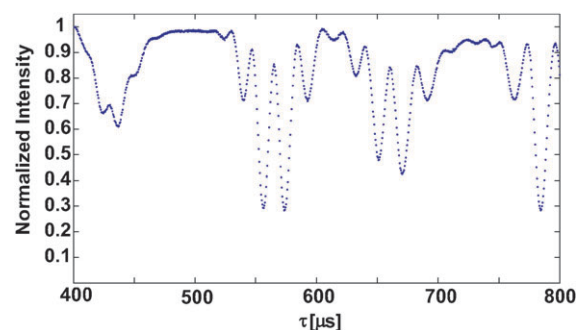

Fig. 3 Experimental echo amplitudes C^α_x in ^{13}C and ^{15}N enriched alanine, observed with a hybrid sequence $[\tau - \pi_x - \tau]_{20}$ comprising 20 π pulses. The ^{13}C rf amplitude $\omega_1/(2\pi) = 9.1$ kHz corresponds to a π pulse length $\tau_\pi = 54.92$ μs on-resonance ($\Omega^\alpha = 0$). This leads to a rotation about the tilted effective field ($\gamma' = \Omega'/\omega_1 = 1.73$) through 2π for the off-resonance C' magnetization which has an offset $\Omega'/(2\pi) = 15.77$ kHz. The τ delay has been varied between 400 and 800 μs in steps of 0.5 μs.

The regions where the echo amplitudes of C^α_x are not affected by modulations are quite narrow. Therefore it is not straightforward to measure precisely the apparent rate R_2^{app} of C^α. A simplification could be obtained by decoupling the H^β protons during the multiple refocusing sequence. However, it has been shown previously[9] that proton decoupling induces artefacts that prevent one from extracting reliable transverse relaxation rates.

Another approach to obtain unmodulated decays is to shift the carrier frequency ω_{rf} away from the C^α resonance. Fig. 4 shows the experimental (blue) and simulated (red) effects of hybrid sequences on C^α echoes when the carrier frequency is set exactly half-way between the C' and C^β resonances. The experimental decays were still not totally free from modulations.

The amplitudes A_{mod} of the C^α echoes can be calculated as a function of the delay τ and of the carrier frequency, which was stepped over a range of 6 kHz from the C^α resonance towards the C' resonance. The resulting map is shown in Fig. 5: dark blue areas correspond to regions where modulations are weak
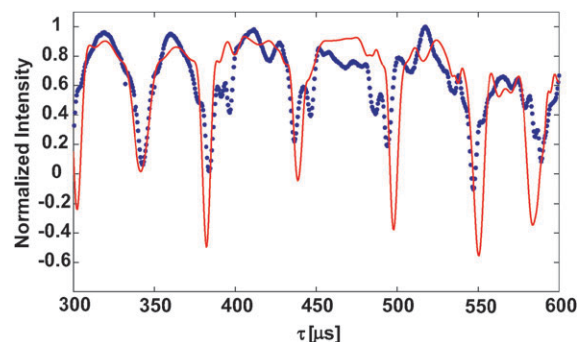

Fig. 4 Echo intensities of C^α in alanine recorded with the hybrid sequence with $2n = 20$, the carrier frequency ω_{rf} being set half-way between the chemical shifts of the two coupling partners C' and C^β. The rf amplitude was $\omega_1/(2\pi) = 19.4$ kHz, hence $\tau_\pi = 25.77$ μs, $\gamma' = 0.81$ and $\gamma^\beta = -0.22$. The delays $300 < \tau < 600$ μs were incremented in steps of 0.5 μs. Experimental results are shown in blue, whereas simulated data are plotted in red.
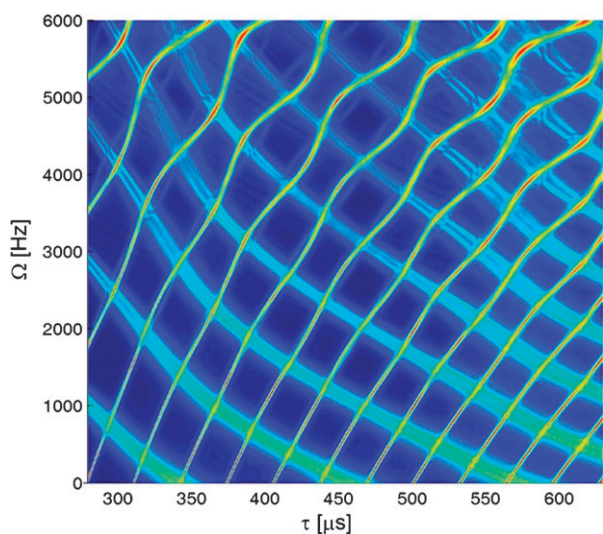
Fig. 5 Simulation of the amplitude A_{mod} of the modulations of C^{α} in alanine, with respect to the echo delay τ and the offset Ω separating the carrier frequency ω_{rf} from the chemical shift of the C^{α} carbon. In the simulations, all three carbons (C^{α}, C^{β} and C') and four protons (H^{α} and 3 H^{β}) were taken into account. The *rf* amplitude was $\omega_1/(2\pi) = 20$ kHz. Dark blue areas correspond to regions without modulations, whereas yellow ridges indicate strong modulations.

($A_{mod} \approx 0$), whereas yellow stripes indicate strong modulations ($A_{mod} \approx 1$). The thin stripes with positive slopes correspond to $C^{\alpha}C'$ recoupling, the broader stripes with negative slopes to the $C^{\alpha}C^{\beta}$ interactions. In the simulations, all three carbons (C^{α}, C^{β} and C') and four protons (one H^{α} and three H^{β}, but none of the H^N protons which were replaced by deuterium) were taken into consideration. To verify the simulations, echoes were recorded at two points on the map featuring extreme values for the amplitude A_{mod} of the modulations: at a crossing of two recoupling stripes (with an offset of $+1$ kHz from the C^{α} chemical shift and a delay of $\tau = 366.5$ μs) and in the center of a "blue field" (with an offset of $+1.275$ kHz from the C^{α} chemical shift and a delay of $\tau = 391.25$ μs). The resulting decays are shown in Fig. 6. In agreement with the simulations, the echo decay on the left,
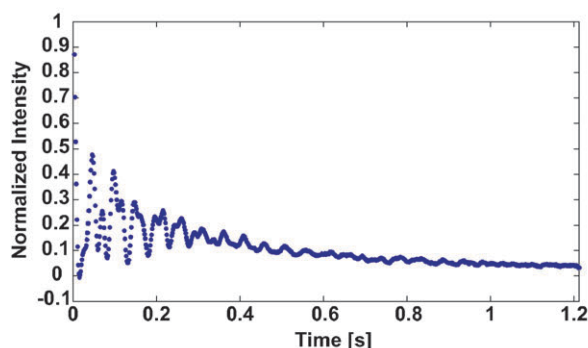
Table 1 Apparent transverse relaxation rates of C^{α} in alanine, measured in regions where the amplitudes A_{mod} of the modulations are small, according to the simulated map of Fig. 5

τ/μs	Offset/Hz of ω_{rf} w.r.t. C^{α}	R_2^{app}/s^{-1}	Simulated A_{mod}
391.25	1275	0.48	0.034
366.75	375	0.48	0.042
416.50	1125	0.47	0.040
467.50	475	0.51	0.046
492.50	1225	0.50	0.035
491.00	200	0.57	0.057
517.00	975	0.52	0.052
541.50	725	0.52	0.073
566.50	500	0.51	0.091
590.50	275	0.52	0.102
467.50	1525	0.47	0.024

recorded at a crossing of yellow stripes in Fig. 5, shows strong modulations leading to a fast decay of the echoes. On the other hand, the echo decay on the right is free of any modulations and can be perfectly fitted with a monoexponential function, leading to a transverse relaxation rate $R_2^{app} = 0.48$ s^{-1}.

Transverse relaxation rates measured in other "blue fields" in the map of Fig. 5 are listed in Table 1, together with the corresponding amplitudes A_{mod} of the modulations determined by simulations.

To verify that the resulting R_2^{app} rates are reliable, 1 M natural abundance alanine and 0.01 M uniformly enriched ^{15}N–^{13}C-alanine were mixed to guarantee the same pH and chemical exchange rates. The offset with respect to the C^{α}-resonance was 1.275 kHz and τ was 391.25 μs. The 'true' relaxation rate in the natural abundance sample was $R_2^{true} = 0.389 \pm 0.004$ s^{-1}, and the 'apparent' relaxation rate in the enriched sample was $R_2^{app} = 0.43 \pm 0.01$ s^{-1}. The difference $R_2^{app} - R_2^{true} = 0.04 \pm 0.01$ s^{-1} is slightly too large to be explained by the dipolar relaxation to the neighbouring carbon-13 nuclei C' and C^{β} that should contribute only about 0.01 s^{-1}. It is possible that, in spite of our efforts, the modulations are still not completely suppressed, so that some unresolved residual couplings still contribute to broaden the lines and accelerate the echo decays.
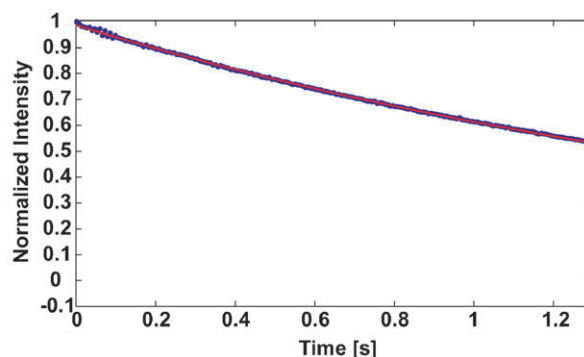
Fig. 6 Decays of C^{α} echoes under a multiple refocusing sequence $(\pi/2)_y - [\tau - \pi_x - \tau]_{2n}$, incrementing n from 1 to 800 in steps of 4. The *rf* amplitude was $\omega_1/(2\pi) = 20$ kHz, corresponding to a π pulse length $\tau_\pi = 25$ μs on resonance. The left decay was observed at a crossing of two yellow stripes in Fig. 5 with the carrier frequency at $+1$ kHz from the chemical shift of C^{α} and $\tau = 366.5$ μs. The right decay was observed in a "blue field" of Fig. 5 with the carrier frequency at $+1.275$ kHz from the chemical shift of C^{α} and $\tau = 391.25$ μs. Blue dots represent experimental data and red lines mono-exponential fits $I(t) = I_0 \exp(-R_2^{app} t)$ with $t = n(4\tau + 2\tau_\pi)$. The transverse relaxation rate was determined to be $R_2^{app} = 0.48$ s^{-1}.

Conclusions

It has been shown that, despite the interference of homo-nuclear scalar couplings J_{CC}, it is possible to measure reproducible apparent transverse relaxation rates R_2^{app} of carbon-13 nuclei. The echo decays in ^{13}C- and ^{15}N-enriched alanine are not modulated by J_{CC} couplings, provided one uses moderate *rf* amplitudes that are comparable with the offsets of the coupling partners, so that their effective fields are tilted, and provided one selects suitable τ intervals, which can be readily identified in preliminary hybrid experiments where the number of echoes n is kept constant and the delay τ is incremented. If on-resonance hybrid experiments do not reveal any useful quenching conditions, a variation of the carrier frequency may lead to conditions where the echo decays are smooth. After selecting a favourable delay τ and carrier frequency where echo modulations can be neglected, one can determine the apparent transverse relaxation rates R_2^{app} from simple mono-exponential fits.

Acknowledgements

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