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Measurement of T_1/T_2 relaxation times in overlapped regions from homodecoupled ^{1}H singlet signals

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ABSTRACT

The implementation of the HOmodecoupled Band-Selective (HOBS) technique in the conventional Inversion-Recovery and CPMG-based PROJECT experiments is described. The achievement of fully homodecoupled signals allows the distinction of overlapped ^{1}H resonances with small chemical shift differences. It is shown that the corresponding T_1 and T_2 relaxation times can be individually measured from the resulting singlet lines using conventional exponential curve-fitting methods.

1. Introduction

The measurement of relaxation rates by Nuclear Magnetic Resonance (NMR) spectroscopy can provide important insights into the dynamics of molecules in solution [1]. Longitudinal spin–lattice T_1 relaxation times are usually determined from the Inversion-Recovery (IR) experiments [2,3] whereas transverse spin–spin T_2 relaxation times are measured from Carr–Purcell–Meiboom–Gill (CPMG) sequences [4,5]. Recently, an improved compensated CPMG sequence that achieves Periodic Refocusing Of J Evolution by Coherence Transfer (PROJECT) has been proposed to minimize the effects of J evolution during the echo periods, allowing a more accurate extraction of T_2 values by fitting the experimental data to a clean exponential decay of pure-phase, non-J-modulated signals [6,7]. A common feature of all these experiments is that measurements are based on exponential signal decays that can be described by first-order differential equations. In spectral regions with well resolved peaks the corresponding time constants are easily determined from nonlinear least-squares fits of each decaying signal to a separate mono-exponential function. However, simple data analysis are hampered in spectral regions with significant peak overlap, where the observed signal decays may be the result of superposition of several individual decays which are difficult to distinguish and require the use of sophisticated fitting methods [8–10]. Several NMR approaches have been proposed to avoid signal overlapping in relaxation experiments, such as the initial use of selective coherence by TOCSY transfer from an isolated signal [11], although the improved signal dispersion achieved in 2D/3D NMR experiments has become the common technique to study the conformational and dynamics aspects of biomolecules in solution [12].

On the other hand, a number of broadband homodecoupled NMR methods have been reported to obtain simplified ^{1}H singlet signals without the typical fine J(HH) multiplet structure [13–24], and recently an excellent overview of the homodecoupling techniques and applications has been reviewed [18]. The most recent applications, that have been encompassed under the term "pure-shift NMR", are based on the original Zangger–Sterk (ZS) experiment [14]. Basically exists two different acquisition protocols: (i) a time-consuming pseudo-2D acquisition mode based on adding the first part of different interferograms [14,15], and (ii) a real-time one-shot mode that reduce the experimental time and do not need for sophisticated processing tools [17]. Most of them use spatial encoded techniques, and therefore pronounced sensitivity losses due to slice selection are unavoidable that requires long acquisition times. Other homodecoupling methods using the BIRD module [19] do not suffer of sensitivity penalties but their applications are limited to heteronuclear correlation experiments [24]. Alternatively, a novel HOmodecoupled Band-Selective (HOBS)

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approach [25,26], closely related to the instant ZS experiment [17] has been proposed. The HOBS technique is not a broadband homodecoupling method that covers all the spectral width, rather it is a frequency-selective inverse homodecoupled method. However, it has been shown to be a sensitive and valuable practical tool when focusing specifically on a narrow part of the whole spectrum and applications have been provided for enantiodifferentiation studies [27], discrimination of diastereoisomers [28] or the measurement of heteronuclear coupling constants [29]. The main drawback is that it is a frequency-selective experiment and only a particular part of the ^{1}H spectrum can be monitored in a single experiment. As a major advantage, the HOBS method omits the spatial encoding gradient applied simultaneously with the selective pulses in the original instant scheme, avoiding any sensitivity loss and allowing its performance with reasonable experimental times. This communication reports the straightforward implementation of the HOBS technique in standard IR and PROJECT experiments (Fig. 1) with the aim to resolve overlapped ^{1}H resonances with small chemical shift differences. Thus, T_1 and T_2 relaxation times can be accurately measured from the resulting singlet lines using conventional exponential curve-fitting methods, without need for additional data analysis based on deconvolution or line fitting techniques [30,31].

2. Results and discussion

The major novelty with respect to the original experiments is the incorporation of the homodecoupled element during the detection period that consists of a pair of hard/selective 180° ^{1}H pulses (represented as solid and shaded shapes) at the middle of $2\Delta = AQ/n$ periods, where AQ is the acquisition time and n the number of concatenated loops [25,26]. In addition, a ^{1}H-selective gradient echo has been inserted prior to acquisition to select the area of interest, where the involved selective 180° ^{1}H pulse is the same as used for homodecoupling. For a perfect broadband homodecoupling, these experiments should be applied to particular areas of the ^{1}H spectrum where appear overlapped protons that are not mutually J coupled.

HOBS experiments can use the same automated data acquisition, processing and fitting analysis subroutines as the original experiments. A series of 1D ^{1}H spectra are sequentially recorded as a function of the recovery delay (τ) or the total echo time ($\tau_e = 4m\tau'$) in IR (Fig. 1A) and PROJECT (Fig. 1B) experiments, respectively. Fig. 2 compares the experimental results obtained for the IR and HOBS-IR experiments applied to the H_α proton region in the peptide cyclosporine. Good agreement is observed between the T_1 measured for all isolated signals with both methods demonstrating that the incorporation of homodecoupling does not distort the measurement (Table 1). The excellence of the method is illustrated by distinguishing the individual decays of the overlapped H_7 and H_8 resonances at 4.78–4.80 ppm. Clearly, the successful analysis of the two resolved singlets (separated by 13 Hz) allows an accurate determination of each distinct T_1 value without resorting to more complex data analysis. The same strategy can be applied for T_2 measurements. The simplicity and the accuracy of the measurements is demonstrated when comparing the equivalent CPMG, PROJECT and HOBS-PROJECT spectra, all of which acquired with a total echo time of 156 ms (Fig. 3B–D). Whereas the standard CPMG spectrum shows strong multiplet distortions due to the unavoidable J_{HH} evolution, perfect in-phase multiplets are obtained from both PROJECT spectra.

Clearly, the in-phase properties are fully retained in the HOBS-PROJECT spectra (Fig. 3D), where improved sensitivity and resolution are obtained due to the efficient multiplet collapsing. The method works equally well for mutually J-coupled protons that experience the effect of the selective 180° pulse, and therefore they are not fully homodecoupled. T_2 measurements on the partially decoupled olefinic $H1_\varepsilon$ and $H1_\zeta$ protons (asterisks in Fig. 3D) can be also monitored efficiently from the simplified doublet patterns.

The HOBS methods can be very useful to simplify highly congested areas, such as those found in the aliphatic region of the steroid progesterone (Fig. 4). Three resonances with complex multiplet patterns appear completely overlapped at 2.0 ppm. The simplified HOBS spectrum shows clean singlets for each of these signals, with small chemical differences of 14–18 Hz. Note the equivalence between IR and HOBS-IR data by observing the same exact null point for the strong methyl signal (see experimental details and experimental T_1/T_2 values in the supporting information).

Experimentally, the HOBS technique requires a very simple and fast implementation. Only two parameters need to be defined in a

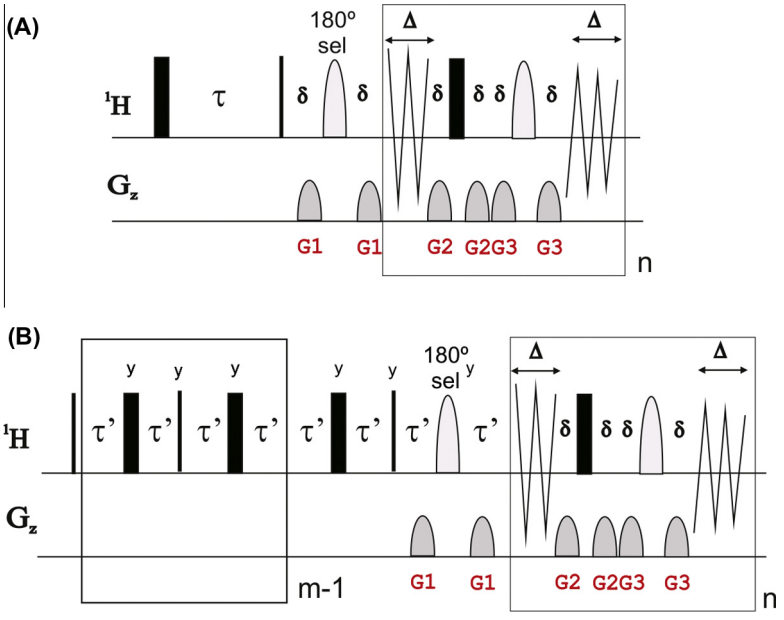

Fig. 1. NMR pulse schemes of the HOBS-IR and HOBS-PROJECT experiments used to measure T_1 and T_2 relaxation times, respectively, in overlapped proton signals.

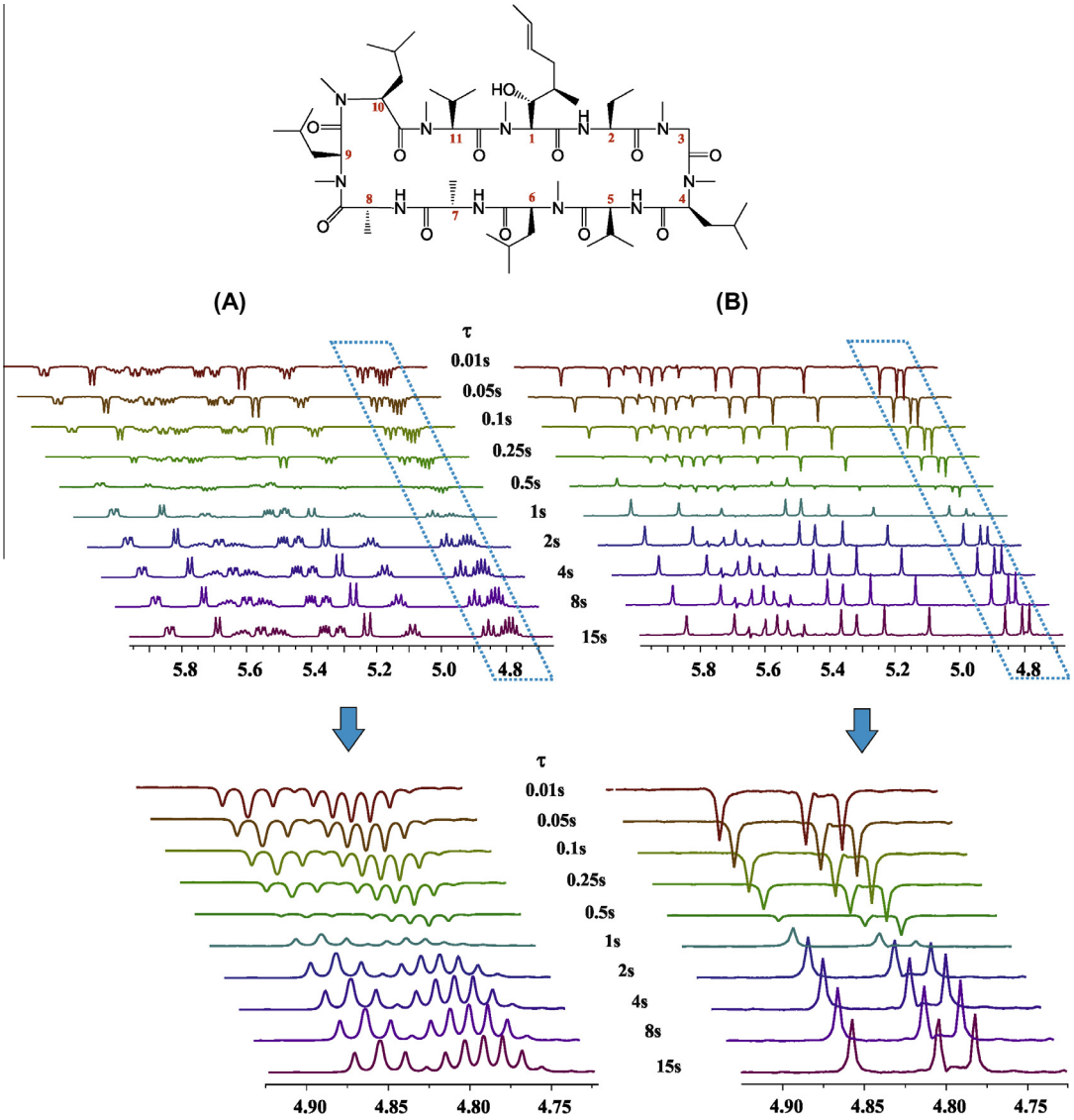

Fig. 2. 600 MHz ^{1}H NMR spectra obtained from the (A) conventional IR and (B) HOBS-IR experiments to determine T_1 values for all H_α protons on 25 mM cyclosporine in benzene-d_6. At the bottom, the clean differentiation between the overlapped H7 and H8 protons is shown. All spectra were collected under the same experimental conditions and plotted at the same absolute vertical scale. Homodecoupling was achieved using the detection scheme described in Fig. 1A with a 5 ms REBURP 180° pulse, Δ = 8.9 ms, AQ = 569 ms and n = 32.

Table 1
Experimental T_1 and T_2 values obtained from IR, HOBS-IR, PROJECT and HOBS-PROJECT experiments for H_α and olefinic protons in cyclosporine, calculated using a simple mono-exponential decay.

Proton	δ (ppm)	T_1 measurement (s)		T_2 measurement (s)	
		IR	HOBS-IR	PROJECT	HOBS-PROJECT
H9	5.83	0.41 ± 0.01	0.43 ± 0.01	0.37 ± 0.01	0.36 ± 0.01
H1	5.69	0.60 ± 0.01	0.60 ± 0.01	0.29 ± 0.02	0.33 ± 0.01
H4	5.55	0.89 ± 0.01	0.88 ± 0.01	0.32 ± 0.01	0.34 ± 0.01
H6	5.36	0.55 ± 0.01	0.57 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
H10	5.31	0.41 ± 0.01	0.42 ± 0.01	0.27 ± 0.01	0.30 ± 0.02
H11	5.23	0.81 ± 0.02	0.85 ± 0.01	0.41 ± 0.03	0.35 ± 0.04
H2	5.09	0.94 ± 0.01	0.92 ± 0.01	0.35 ± 0.02	0.32 ± 0.01
H5	4.85	0.88 ± 0.02	0.88 ± 0.01	0.41 ± 0.01	0.38 ± 0.02
H8	4.80	1.14 ± 0.01	0.98 ± 0.02	0.48 ± 0.01	0.47 ± 0.01
H7	4.78		1.22 ± 0.01		0.50 ± 0.01
H1ε	5.61	1.41 ± 0.01	1.39 ± 0.02	0.57 ± 0.01	0.56 ± 0.01
H1ζ	5.50	1.27 ± 0.01	1.29 ± 0.01	0.56 ± 0.01	0.55 ± 0.02

single-scan 1D acquisition mode: the offset and the selectivity of the 180° ^{1}H pulse as a function of the crowded area to be analyzed. It is also worth to mention that maximum sensitivity is retained, although multiple experiments would be required to monitor different overlapped areas. This fact no means a severe impediment to the method as proton relaxation times do not consume large amounts of spectrometer time. Alternatively, broadband homodecoupled for all signals present in the ^{1}H spectrum should be feasible using the instant ZS experiment [17], simply applying a gradient during the selective 180° pulses in schemes of Fig. 1, but high levels of sensitivity would be lost due to spatial encoding selection. Moreover, we can anticipate that the HOBS technique could be successfully implemented to improve the analysis in other related relaxation methods [32–34], including the measurement of selective T_1 relaxation times (T_{1sel}) [35,36] or spin–lattice relaxation times in the rotating frame (T_{1rho}) [11]. Other potential applications should be the study of reaction kinetics in complex areas or the determination of individual diffusion coefficients in

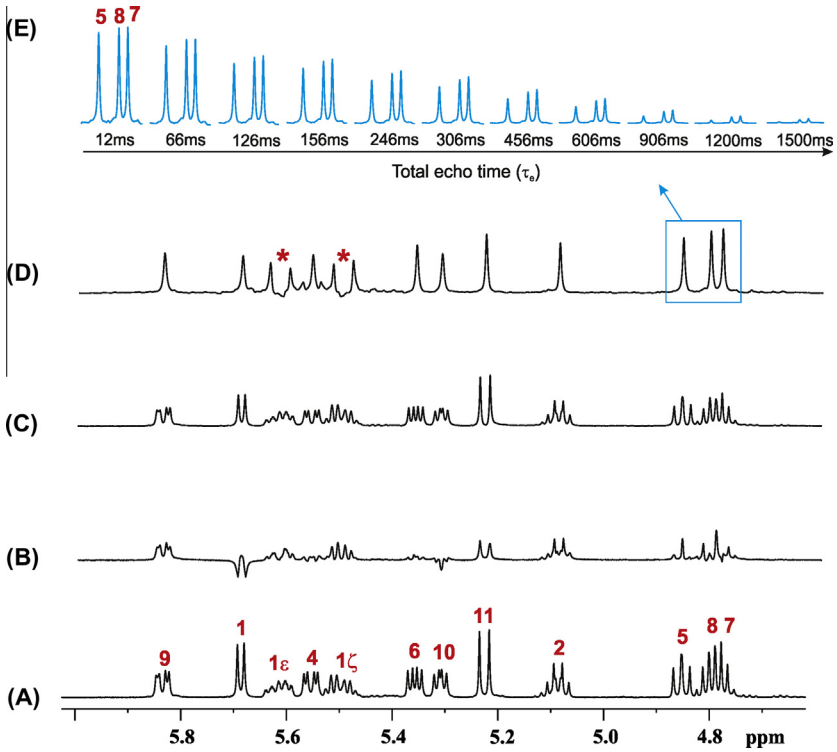

Fig. 3. Comparison of 1D (A) conventional ^{1}H, (B) CPMG, (C) PROJECT, and (D) HOBS-PROJECT spectra of cyclosporine acquired with a total echo time of τ_e = 156 ms (m = 26 and τ' = 1.5 ms). All spectra were collected under the same experimental conditions as described in Fig. 2 and are plotted at the same absolute vertical scale. (E) Signal T_2 decays for the H5, H8 and H7 protons in the HOBS-PROJECT experiment.

multi-component systems as similarly reported for analogous pure-shift DOSY experiments [15,16].

3. Conclusions

In summary, homodecoupling can improve the appearance of crowded areas of the ^{1}H spectrum by collapsing multiplet structure to singlet lines. The implementation of the HOBS technique in standard IR and PROJECT experiments can enhance the simplicity and accuracy by which T_1 and T_2 relaxation times are measured from overlapped resonances, while the sensitivity of the original experiments are retained. It has been shown that in absence of signal overlapping, individual mono-exponential decays from simplified singlet signals can be easily monitored using standard fitting procedures.

4. Methods and materials

All NMR experiments were collected at 298 K on a Bruker AVANCE spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 600.13 MHz proton frequency, equipped with a 5 mm triple resonance inverse probe and a z-axis pulsed field gradient accessory (maximum strength of 53.5 G/cm) acquired and processed using the software TOPSPIN 3.1 (Bruker BioSpin, Rheinstetten, Germany).

The two samples used in this work were 25 mM cyclosporine (in benzene-d6) and 100 mM progesterone (in DMSO-d6). Hard 90° ^{1}H pulses of duration 7.8 μs (for cyclosporine) and 8.3 μs (for progesterone) were used in each sample. A 180° band-selective REBURP shaped pulse of 5.0 ms (for cyclosporine) and 20 ms (for progesterone) was used for both excitation and homodecoupling in HOBS experiments. The strengths of the G1, G2 and G3 gradients were set to 9.1, 21.9 and 33.7 G/cm, respectively, with durations of 500 μs followed by a recovery delay of 20 μs (δ = 520 μs). The ^{1}H spectral width was set to 7200 Hz and 8 K complex points were recorded during an acquisition time of 569 ms. 32 (for cyclosporine) and 23 (for progesterone) loops (n) were concatenated with Δ periods of 8.9 and 12.37 ms, respectively ($\Delta = AQ/2n$). The first and the last chunks are half size ($AQ/2n$) relative to the rest of chunks (AQ/n).

10 experiments with different values of recovery delay τ(0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, 4, 8 and 15 s) were acquired for each IR and HOBS-IR experiment, using 8 scans and 15 s of recycle delay. 12 experiments with different number of echoes ($m - 1$ = 1, 5, 10, 20, 25, 40, 50, 75, 100, 150, 200 and 250) and a relaxation delay τ' of 1.5 ms were acquired for each PROJECT and HOBS-PROJECT experiments, using a single scan and 10 s of recycle delay. 1D time-domain data were transformed without any sensitivity or resolution enhancement, and the same phase and baseline corrections were applied for all resulting 1D spectra.

The standard IR and CPMG experiments were recorded using the *t1ir* and *cpmg1d* pulse programs that are available in the Bruker library. Pulse programs codes for Bruker spectrometers are available in our blog (http://sermn.uab.cat).

The calculation of longitudinal T_1 relaxation times was carried out with the subroutine t1guide included into the TOPSPIN3.1 software package. A set of 1D spectra recorded with different recovery delays τ were stored in a 2D data set and T_1 values were extracted by fitting the data to the equation:

$$\frac{A}{A_0} = 1 - 2e^{\left(-\frac{\tau}{T_1}\right)} \quad (1)$$

where A is the integrated area of the peak in the spectrum and A_0 is the area when $\tau \to \infty$.

The transversal T_2 relaxation times values were extracted from fitting the integrated area of a given signal as a function of total echo time τ_e assuming single exponential decay process. This natural exponential function can be rewritten in natural logarithmic

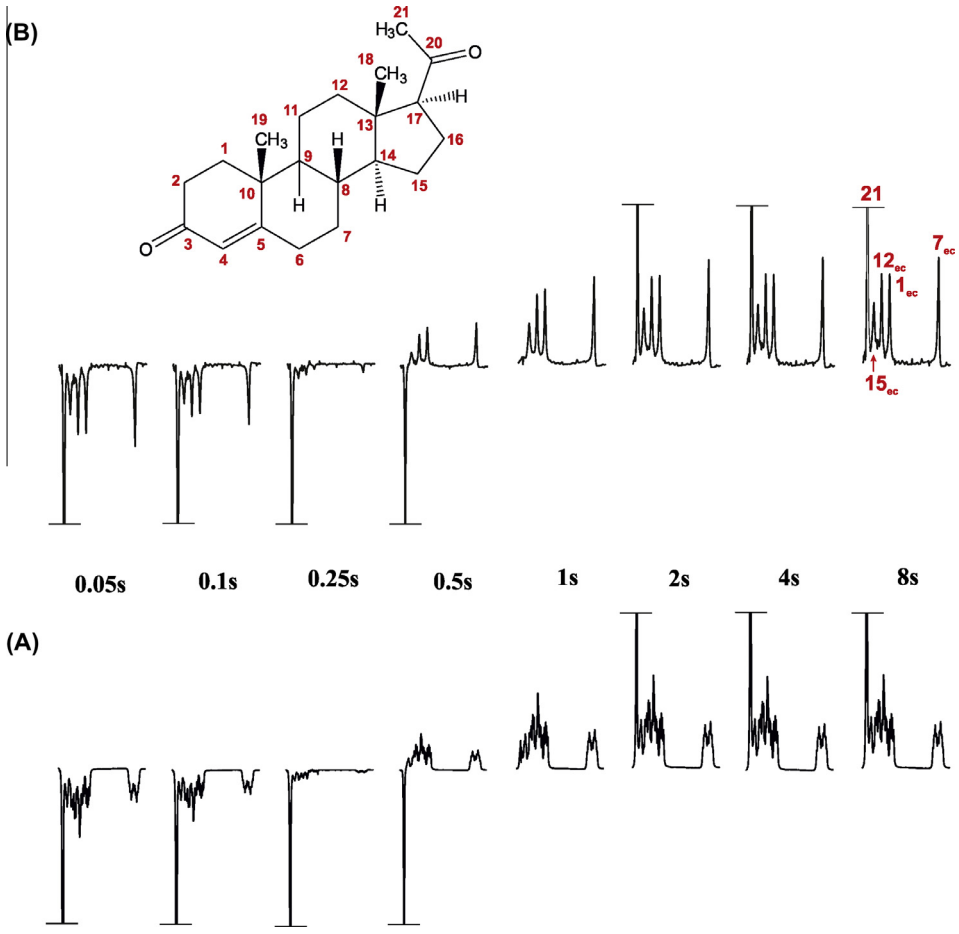

Fig. 4. Experimental decays in (A) IR and (B) HOBS-IR experiments of all protons resonating in the region 1.8–2.1 ppm in progesterone. All spectra are plotted in the same absolute vertical scale. Homodeocupling was achieved using the detection scheme described in Fig. 1A with a 20 ms REBURP 180° pulse, Δ = 12.37 ms, AQ = 569 ms and n = 23.

form where A and τ_e present a linear dependence and T_2 can be extracted from the slope:

$$A = A_0 \cdot e^{\left(-\frac{\tau_e}{T_2}\right)} \ln A = \ln A_0 - \frac{1}{T_2}\tau_e \quad (2)$$

where τ_e is calculated as $\tau_e = 4m\tau'$.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jmr.2014.04.003.

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