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The application of the ERETIC method to 2D-NMR

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Abstract

As a means of obtaining precise quantitative data in 2D NMR spectroscopy, the use of an electronic reference signal (ERETIC) has been examined. The results presented demonstrate that the ERETIC method can be used in different situations encountered in 2D-NMR spectroscopy: homonuclear and heteronuclear spectra, phased or magnitude mode, symmetrization. The main restriction to introducing ERETIC in 2D spectra could be the need for several spectrometer channels. However, most modern NMR spectrometers are equipped with at least three channels, that allows the implementation of the ERETIC method as reference for 2D-NMR spectroscopy without needing any hardware modification.

Keywords: ERETIC; Quantitative; Reference; 2D-NMR

1. Introduction

Quantitative 1D or 2D magnetic resonance spectroscopy is a powerful tool to determine concentrations because of the direct proportionality of signal intensity to the quantity of analyte present. It is widely used in many fields of chemistry, biology, and medicine [1–4]. However, one of the critical point is the method used to calibrate the signal.

In vitro the reference peak is most frequently one of the peaks of a compound added to the sample, the so-called internal reference. The choice of a reference compound is not always an easy task because it has to satisfy many criteria: co-solubility with the sample, chemical shifts different from those of the sample, no chemical interaction with the sample components. The use of this method can be difficult in many cases, such as the study of biological fluids.

On the other hand, except for one application for the study of rat kidney [5], it is very difficult to find suitable exogenous markers in vivo. The use of an external standard for reliable absolute in vivo quantification is therefore necessary. All the methods proposed estimate metabolite concentrations by comparison with the NMR signal coming from a calibration solution. The main problem with these strategies involving several acquisitions is that in vivo and in vitro calibration acquisitions have to be performed with the same parameters and coil loading.

In order to avoid these drawbacks, the ERETIC method has been developed [6–9]. A pseudo FID, electronically generated, is transmitted during acquisition of the NMR signal. After Fourier transform, it provides an additional line, which is calibrated against a standard solution and used as reference. Until now, it has been used in 1D-NMR [7–9] and in MRI [10]. In this paper, the adaptation of the ERETIC method for 2D-NMR spectroscopy has been evaluated.

2. Method

The ERETIC signal is created by RF channels different from those used for observation. These RF channels must be coherent in phase because the ERETIC and NMR signals are simultaneously detected. For this purpose, we have used a phase reset which brings channel phases to zero at the same time.

This reset cannot be applied during the pulse sequence because it would change the relative pulse phases. Therefore, reset occurs before the first RF-pulse.

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Moreover, the Δ delay between the phase reset and the ERETIC onset must remain constant from one acquisition to another so that ERETIC phase $\varphi_{\mathrm{ERET}} = 2\pi\nu_{\mathrm{ERET}}\Delta$ is kept constant. That is obtained by decrementing the delay between the phase reset and the first pulse of the sequence when the delay t_1 is incremented.

The COSY sequence adapted to the ERETIC method is shown in Fig. 1.

Thanks to the method described above, the ERETIC peak appears at the centre of the spectrum along the ω_1 dimension. If a phase increment $\Delta\varphi_{\mathrm{ERET}} = (2\pi\omega_1^{\mathrm{ERET}})/(\Omega_1)$ is applied, the ERETIC line is shifted to ω_1^{ERET}, where ω_1^{ERET} is the shift in frequency of the ERETIC line in comparison of the central frequency and Ω_1 the spectral width along the ω_1 dimension. $\Delta\varphi_{\mathrm{ERET}}$ must therefore be added to the ERETIC phase with each t_1 increment.

Symmetrization procedures are generally applied to some 2D spectra about one of the diagonals (COSY) or the zero frequency in the ω_1 dimension (J-resolved). A single ERETIC peak would therefore vanish after such a processing. In order to avoid this, another ERETIC signal must be used. These two ERETIC signals are characterized by $(\omega_2^{\mathrm{ERET1}}, \Delta\varphi_{\mathrm{ERET1}})$ and $(\omega_1^{\mathrm{ERET2}}, \Delta\varphi_{\mathrm{ERET2}})$.

For a COSY sequence, it becomes:

$$\Delta\varphi_{\mathrm{ERET}_1} = \frac{2\pi\omega_2^{\mathrm{ERET}_2}}{\Omega_1} \quad \text{and} \quad \Delta\varphi_{\mathrm{ERET}_2} = \frac{2\pi\omega_2^{\mathrm{ERET}_1}}{\Omega_1}$$

in order to obtain two peaks symmetrical about the diagonal.

For a J-resolved homonuclear, we must have after tilt:

$$\omega_1^{\mathrm{ERET}_1} = -\omega_1^{\mathrm{ERET}_2} \quad \text{and} \quad \omega_2^{\mathrm{ERET}_1} = \omega_2^{\mathrm{ERET}_2}$$

Before the tilt, the two signals must be therefore characterized by:

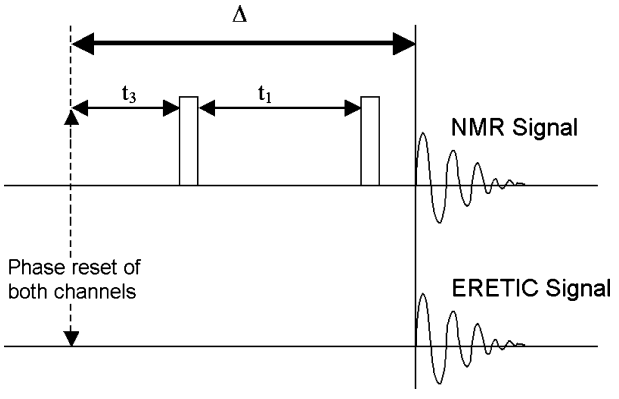

Fig. 1. NMR signal and ERETIC are both started at a delay Δ before acquisition. The ERETIC signal is created by an RF channel different from that used for observation. These two RF channels must be coherent in phase. This command, called "reset," brings to zero both channel phases at the same time. Moreover, the delay Δ between phase reset and the ERETIC start must remain constant from one acquisition to another so that $\varphi_{\mathrm{ERET}} = 2\pi\nu_{\mathrm{ERET}}\Delta$ is kept constant.

$$\left(\omega_2^{\mathrm{ERET}} - \omega_1^{\mathrm{ERET}}, \Delta\varphi_{\mathrm{ERET}_1} = -\frac{2\pi\omega_1^{\mathrm{ERET}}}{\Omega_1}\right) \quad \text{and}$$

$$\left(\omega_2^{\mathrm{ERET}} + \omega_1^{\mathrm{ERET}}, \Delta\varphi_{\mathrm{ERET}_2} = \frac{2\pi\omega_1^{\mathrm{ERET}}}{\Omega_1}\right).$$

Almost all experiments, that involve transfer of magnetization or coherence between different spins, do not yield information concerning the signs of the ω_1 frequencies. Without this sign information, two-dimensional Fourier transformation can therefore only lead to a spectrum folded in ω_1 about the transmitter reference frequency. Discriminating the signs of the frequencies indirectly detected during t_1 in a two-dimensional NMR experiment can be done by different ways. The approach most commonly used is to convert the modulation of the signals during t_1 to one of phase rather than amplitude. Unfortunately, this method has an intrinsic unavoidable penalty in the form of phase-twist lineshape, which necessarily results whenever signals phase-modulated in t_1 are subjected to two-dimensional Fourier transformation [11]. It is, therefore, clearly desirable to obtain ω_1 sign discrimination in some alternative manner which avoids the need for phase modulation, and this is achieved by time-proportional phase incrementation TPPI [12].

In this procedure, the time t_1 is advanced in intervals of $1/(2\Omega_1)$, where Ω_1 is the required spectral width in the ω_1 dimension. Each time t_1 is incremented, the phase of the desired coherence evolving during t_1 is shifted by $\pi/2$ radians [13].

In order to adapt the ERETIC method to this mode of detection, it is first necessary to obtain an amplitude-modulated signal. Therefore, two ERETIC signals are added with:

$$\omega_2^{\mathrm{ERET}_1} = \omega_2^{\mathrm{ERET}_2} \quad \text{and} \quad \Delta\varphi_{\mathrm{ERET1}} = -\Delta\varphi_{\mathrm{ERET2}}.$$

As the real and imaginary parts of the signal are sampled alternately, the shift in phase seen by each part is twice the value of $\Delta\varphi_{\mathrm{ERET}}$ applied.

Thus, $\Delta\varphi_{\mathrm{ERET1}} = -\Delta\varphi_{\mathrm{ERET2}} = \pi/2$ radians must be applied after each data point is taken to set the ERETIC peak at the centre of the spectral width in the ω_1 dimension.

3. Results and discussion

The ERETIC signal, whose parameters (ω_2^{ERET} and $\Delta\varphi^{\mathrm{ERET}}$) are freely controlled from the spectrometer console, can easily be positioned at will anywhere on the spectral plain. Fig. 2 illustrates how the ERETIC signal may be shifted in the first dimension of the spectrum. The position of the ERETIC peak in the ω_2 dimension is given by ω_2^{ERET} and its position in the ω_1 dimension is only determined by $\Delta\varphi^{\mathrm{ERET}}$ with the strategy we have developed.

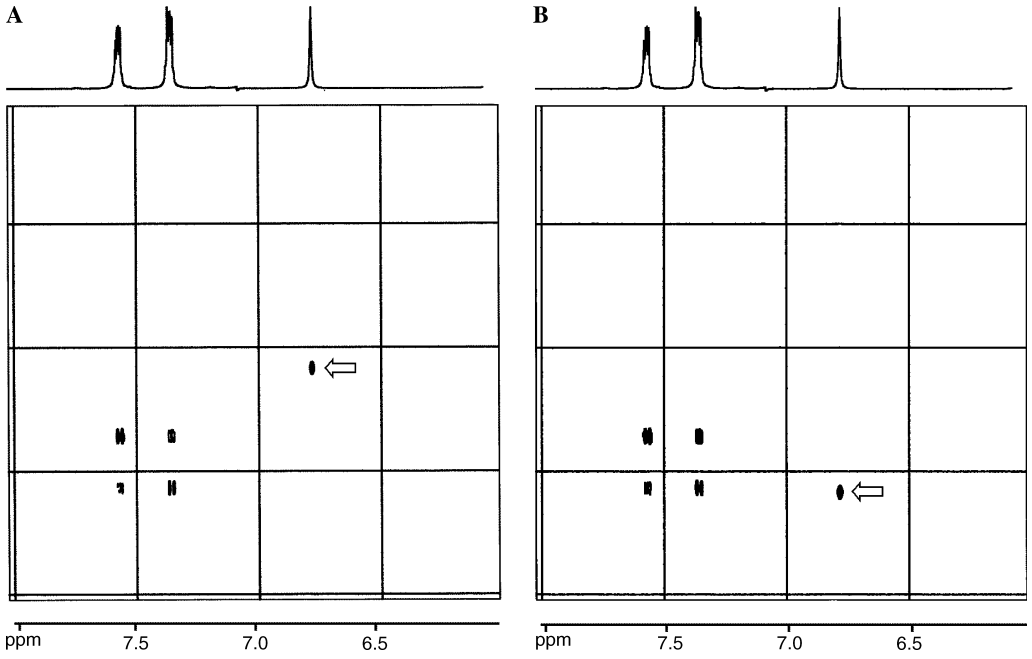

Fig. 2. (A) COSY spectrum of orthodichlorobenzene with ERETIC peak (arrow) at the centre of spectral width along dimension 1, $\omega_1^{\mathrm{ERET}} = 0$. (B) $\omega_1^{\mathrm{ERET}} \neq 0$, a phase increment was applied, signal was shifted to ω_1^{ERET}, this phase increment must be $\Delta\varphi_{\mathrm{ERET}} = (2\pi\omega_1^{\mathrm{ERET}})/(\Omega_1)$. For this spectrum, $\Delta\varphi^{\mathrm{ERET}} = \pi/2$.

An apparently more simple approach would be to start the ERETIC signal with the first RF-pulse of the sequence. Detected signal would thus be modulated in phase during t_1 with the frequency ω_2. Because its frequency would have the same sign, the ERETIC signal would therefore be along the second diagonal. Indeed, as *p*-peaks are selected, NMR signal frequencies are of opposite sign in the two dimensions [14]. However, the main drawback of this method is that it is no longer possible to shift the ERETIC peak independently in the two dimensions. The method we have used is therefore to be preferred, as it provides greater freedom of choice.

Fig. 3 displays symmetrized COSY and *J*-resolved spectra with two ERETIC peaks. For COSY or *J*-resolved spectra, symmetrization improves spectral quality by suppression of t_1 noise. However, if only one ERETIC signal is generated, it will be eliminated. Symmetrization is an interesting method but incompatible with the use of only one ERETIC signal. *J*-resolved and COSY spectra need therefore two ERETIC signals thus at least three channels are needed on the spectrometer; one for NMR and two for ERETIC. This requirement limits the method to those spectrometers which are suitably equipped.

However, the t_1-noise essentially comes from spectrometer fluctuation. In fact, as spectrometers become more and more stable, the t_1-noise is less and less important and symmetrization becomes less indispensable.

Fig. 4 shows a COSY DQF spectrum in phased mode. This technique improves the spectral resolution [15], as can be seen by comparing Fig. 2 with Fig. 4. As in the case of symmetrization, an additional channel is needed for ERETIC.

In order to obtain several ERETIC signals, another solution could be to generate series of shaped pulses containing both ERETIC signals with a shift in phase, and to program them sequentially. This method has not been further developed because this kind of "shape cycle" is not already available on NMR-spectrometers.

Fig. 5 illustrates a heteronuclear correlation spectrum. The same method as for COSY spectra was employed to introduce the ERETIC signal. Thus, a phase reset has been used which brought to zero the channel phases at the same time. Furthermore, the Δ delay between the phase reset and the ERETIC onset from one acquisition to another remained constant. It must be noted that three channels are needed in a heteronuclear experiment,.

In this work, we have used a modified probe [16], although such a device is not strictly necessary. In the case of homonuclear correlations, the pseudo-FID could be transmitted through an unused coil (i.e., the carbon coil), which is not tuned at the operating frequency and acts, therefore, as a broadband antenna for the ERETIC signal [7]. In the case of heteronuclear correlations, the pseudo-FID could be transmitted through the decoupling coil; the ERETIC signal must therefore be mixed with the decoupling irradiation by an RF coupler placed just before the probe [17].

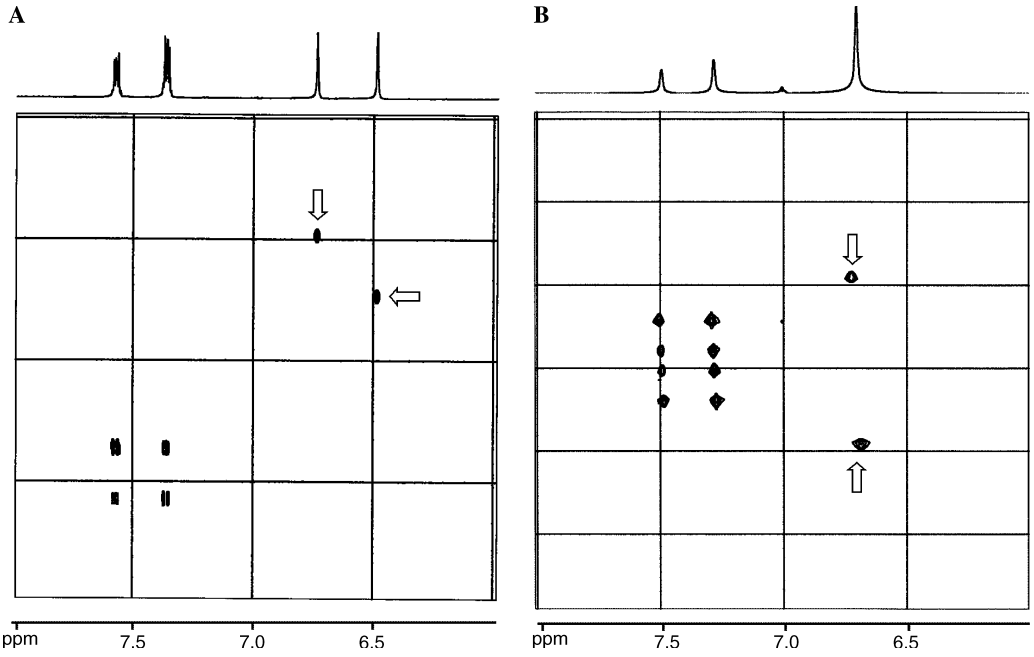

Fig. 3. (A) Symmetrized COSY spectrum of orthodichlorobenzene with two ERETIC peaks (arrows). These two ERETIC signals are characterized by ($\omega_2^{\mathrm{ERET_1}} = -125$ Hz, $\Delta\varphi_{\mathrm{ERET1}} = -\pi/4$) and ($\omega_2^{\mathrm{ERET_2}} = -250$ Hz, $\Delta\varphi_{\mathrm{ERET2}} = -\pi/2$). Indeed, $\Delta\varphi_{\mathrm{ERET_1}} = (2\pi\omega_2^{\mathrm{ERET_2}})/(\Omega_1)$ and $\Delta\varphi_{\mathrm{ERET_2}} = (2\pi\omega_2^{\mathrm{ERET_1}})/(\Omega_1)$ with $\omega_2^{\mathrm{ERET}} = 3500$ Hz (the frequency shift of the ERETIC peak in comparison to the centre frequency). (B) For a *J*-resolved homonuclear spectrum, after tilt $\omega_1^{\mathrm{ERET_1}} = -\omega_1^{\mathrm{ERET_2}} = 10$ Hz and $\omega_2^{\mathrm{ERET_1}} = \omega_2^{\mathrm{ERET_2}} = -100$ Hz, so before tilt the two signals are characterized by ($\omega_2^{\mathrm{ERET}} - \omega = -90$ Hz, $\Delta\varphi_{\mathrm{ERET_1}} = -((2\pi\omega_1^{\mathrm{ERET_1}})/(\Omega_1)) = -\pi/3$) and ($\omega_2^{\mathrm{ERET}} + \omega = -110$ Hz, $\Delta\varphi_{\mathrm{ERET_2}} = ((2\pi\omega_1^{\mathrm{ERET_2}})/(\Omega_1)) = \pi/3$).

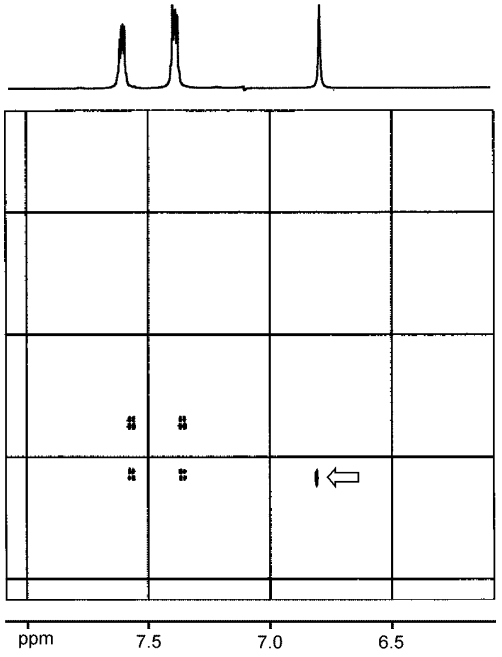

Fig. 4. COSY DQF spectrum of orthodichlorobenzene with an ERETIC peak (arrow). It is necessary to obtain an amplitude-modulated signal to adapt the ERETIC method to this mode of detection. So the ERETIC signals with $\omega_2^{\mathrm{ERET_1}} = \omega_2^{\mathrm{ERET_2}}$ and $\Delta\varphi_{\mathrm{ERET1}} = -\Delta\varphi_{\mathrm{ERET2}}$ are added. As the real and imaginary parts of the signal are sampled alternately, the shift in phase seen by each part is twice the value of $\Delta\varphi_{\mathrm{ERET}}$ applied. Thus, $\Delta\varphi_{\mathrm{ERET1}} = -\Delta\varphi_{\mathrm{ERET2}} = \pi/2$ radians must be applied after each data point to set the ERETIC peak at the centre of the spectral width. In this case, the ERETIC peak is shifted of $\Omega_1/2$ in the ω_1 dimension. Here, characteristics of ERETIC signals are $\omega_2^{\mathrm{ERET_1}} = \omega_2^{\mathrm{ERET_2}} = -150$ Hz and $\Delta\varphi_{\mathrm{ERET1}} = -\Delta\varphi_{\mathrm{ERET2}} = \pi/2 + \pi/4 = 3\pi/4$.

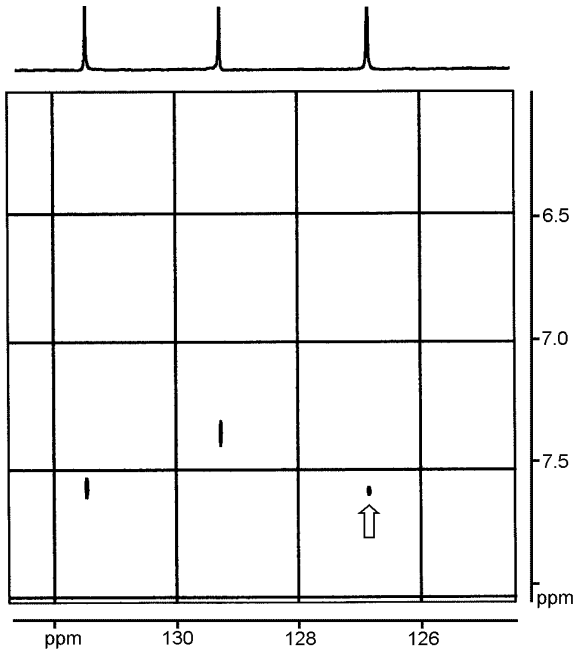

Fig. 5. Heteronuclear correlation spectrum of orthodichlorobenzene. A similar method as for the COSY spectrum was employed to introduce the ERETIC signal (arrow). Thus, a phase reset has been used which brought to zero the channel phases at the same time and the Δ delay between the phase reset and the ERETIC onset from one acquisition to another remained constant.

4. Conclusion

Several experiments have been performed that demonstrate that the ERETIC method can be successfully applied to the different situations encountered in 2D-NMR spectroscopy: homonuclear and heteronuclear spectra, phased or magnitude mode, symmetrization.

The use of the ERETIC reference can be carried out on a commercial spectrometer with minimal modification. For ERETIC implementation, there is no need for any additional hardware. Probe modification can be avoided using NMR coils already present.

The main limit of this method is that several RF channels are necessary. Nevertheless, recent spectrometers are often equipped with three RF channels that allow homonuclear 2D spectra (even in phase mode) and heteronuclear correlation in absolute mode for two nuclei: these techniques correspond to the main approach used.

Future prospects for this technique will be for absolute quantification with 2D-NMR in biological media (in vivo, biological fluid, cell medium, biopsy HR-MAS). Indeed, in cases where it is not possible to add a compound to the sample, the ERETIC method makes quantitative analysis feasible.

5. Experimental

All experiments were performed on a DRX 500 Bruker spectrometer operating at 500.13 MHz for proton NMR. A 5 mm dual probe (proton/carbon) was used. The probe was modified by inserting inside an additional broadband antenna placed just below the NMR coil, consisting of a copper wire connected to the end of a coaxial cable [16]. During NMR experiments, the probe and sample temperatures were controlled and kept at 298 K.

For COSY spectra, fully relaxed spectra were acquired with the following parameters: flip angle 90°, sampling time 1 s, repetition time 5 s, number of scans 16, number of dummy scans 16, $SW_1 = 2$ ppm, $SW_2 = 2$ ppm, $td_1 = 128$, and $td_2 = 1200$, data matrix size after double Fourier transformation: 1024*1024. A sinus multiplication was applied in the ω_1 dimension and a square sinus multiplication in the ω_2 dimension.

For COSY DQF spectra, the same conditions were used except for $td_1 = 150$ and $td_2 = 2002$. Two sinus multiplications were applied in ω_1 and ω_2 dimensions.For 2D-heteronuclear shift correlation, all parameters were identical except $SW_1 = 8.3$ ppm and $SW_2 = 2$ ppm.

For homonuclear J-resolved 2D correlation, $SW_1 = 0.12$ ppm, $SW_2 = 2$ ppm, $td_1 = 128$, and $td_2 = 1400$. A sinus multiplication was performed in ω_1 dimension and a gaussian multiplication in ω_2 dimension. Data were processed in absolute mode for every acquisition except for the COSY DQF acquisition, which was processed in phase mode.

The ERETIC signal was obtained by multiplication of a sinusoidal signal (high frequency component) and an exponentially decreasing signal (low frequency component). The high frequency component was provided by one of the channels of the NMR spectrometer. Our spectrometer, as with most modern NMR spectrometers, is equipped with hardware able to synthesize RF shaped pulses. An exponential shape was therefore numerically defined and used as the low frequency component. The second channel of our spectrometer has been used to produce the ERETIC signal and the third channel when two ERETIC signals were necessary.

A sealed sample tube has been used which contained 5% of orthodichlorobenzene and 1% of TMS in acetone-d6, used for the lock signal.

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