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Technical Notes

Determination of Deuterium Isotope Ratios by Quantitative ^{2}H NMR Spectroscopy: the ERETIC Method As a Generic Reference Signal

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A generic method is described that minimizes the acquisition time required for the determination of the $(D/H)_i$ ratios of all the resolved chemical sites of a molecule by quantitative ^{2}H NMR. The method relies on the use as the reference of an electronically generated signal (ERETIC) that is calibrated from an acquisition with a greatly reduced number of scans. The measurement of the $(D/H)_i$ ratios can then be performed on spectra obtained with a reduced repetition time. In the case of the molecules studied in this work (derivatives of long chain fatty acids), the total acquisition time was divided by 3.4 without loss of precision.

The site-specific natural variation in deuterium distribution in natural products is readily measured by NMR spectroscopy.[1,2] This technique, SNIF-NMR, is a powerful tool to study biosynthetic pathways of natural products [2]. Essentially, the site-specific isotopic ratio $(D/H)_i$ at a given position i is obtained by measuring the area of the deuterium NMR signal for site i of the compound of interest and comparing this with the area due to a calibrated reference material.

Thus, the ratio $(D/H)_i$ can be defined by eq 1 and expressed in parts per million (ppm).

$$(D/H)_i = N_{D,i}/P_i \times N_H \quad (1)$$

where $N_{D,i}$ is the number of monodeuterated isotopomers of type i, N_H is the number of fully protonated molecules, and P_i is the number of equivalent hydrogen atoms at site i.

In the course of a detailed analysis of the nonstatistical distribution of ^{2}H at natural abundance in fatty acids, such as methyl oleate and methyl linoleate, we were confronted by two problems. First, the overlap of the ^{2}H NMR signals from hydrogens in different positions diminished the potential resolution of the analysis. Second, the acquisition time required was determined by the T_1 of the internal reference compound used (*N*,*N*′-tetremethylurea (TMU)). To overcome the first problem, methods of double bond cleavage were developed leading, in the case of methyl linoleate, to methyl-9,9′-bis(phenylthio)nonanoate **1** and 1,1′-bis(phenylthio)hexane **2**.[3,4] This chemical modification has proved to be very effective for accessing the $(D/H)_i$ ratios for a greater number of sites. However, to obtain meaningful quantitative data, the NMR measurements are particularly time-consuming (57 h/sample) because of a combination of two factors. The first of these relates to an intrinsic parameter of the analyte, namely, that the $(D/H)_i$ values for the single hydrogen at the 9 and 1 positions of compounds **1** and **2**, respectively, are extremely impoverished in deuterium [3]. Thus, to obtain an acceptable signal-to-noise ratio (>10), a high number of scans is required.

The second factor is due to the large relaxation time T_1 of the internal reference, TMU [2]. The choice of an internal reference for quantitative ^{2}H NMR is always difficult. Not only must solubility, volatility, hygroscopy, stability over time, a lack of chemical interaction, and no spectral overlap with the compound studied all be taken into account, but ideally, the same reference should be used for all compounds being compared, even though their chemical and physical properties may differ significantly. Furthermore, in the case of isotopic studies, the reference compound must have a calibrated D/H ratio. Very few molecules satisfactorily fulfill all of these criteria. To our knowledge, the only molecules for which the D/H ratio has been certified by an international validation procedure are water (V.SMOW scale)[2] and TMU.[5] In our study, water cannot be used because of its low solubility. TMU has the severe constraint that its relaxation time T_1 is generally significantly longer than those of the product studied, leading to protracted acquisition times.

Because the signal intensity from extremely depleted positions in the analyte of interest is fixed, we have focused on improving the referencing procedure. To provide a generic reference that

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can be applied independently of the nature of the solvent and analyte present, we have developed an approach in which a calibrated NMR-like, electronically produced signal is generated within the NMR magnet and collected as an integral component of the FID.[6−9] This method, electronic reference to access in vivo concentrations (ERETIC), has been shown to be effective for the determination of solute concentrations, either in vivo[6] or in high-resolution NMR,[8] and for the calibration of quantitative kinetic measurements.[9] Furthermore, it has previously been shown[10] that the evolution of the ERETIC signal in time is the same as that of the NMR signal. The short-term and long-term stability in time of this reference has already been demonstrated to be sufficiently high to introduce negligible distortion over the acquisition period required for a quantitative determination of $(D/H)_i$ ratios.[10]

In this paper, we show that the ERETIC method allows a considerable reduction in the acquisition time for quantitative ^2H NMR measurements at natural abundance without a reduction in precision. Thus, the drawbacks cited above of the internal reference method for quantitative ^2H NMR are avoided.

EXPERIMENTAL SECTION

Material. Methyl linoleate was purchased from Sigma-Aldrich.

Chemical Modification. Methyl-9,9′-bis(phenylthio)nonanoate **1** and 1,1′-bis(phenylthio)hexane **2** were obtained from methyl linoleate using the chemical modification previously described.[3]

Sample Preparation. Lipid derivatives (0.741 g for **1** and 0.596 g for **2**), solvent $CHCl_3/CCl_4$ (1:3), the external reference TMU (0.3 g), and the field frequency locking material C_6F_6 (60 μL) were mixed, filtered, and introduced into a 10-mm NMR tube fitted with an antivortex.

NMR Measurements. All experiments were performed on a DRX 500 Bruker spectrometer operating at 76.77 MHz and fitted with a ^{19}F field-frequency lock device. A dual probe ($^1H/^2H$) 10 mm in diameter was used. Three protocols were used in order to obtain NMR spectra:

(1) Standard measurement: sampling period (AQ) = 5.5 s, number of sampling points in the time domain (TD) = 2 199 890, repetition time (T_R) = 5.7 s, number of scans (NS) = 12 000.

(2) Fast measurement: AQ = 1 s, TD = 399 980, repetition time = 1.1 s, NS = 12 000.

(3) Reference measurement: AQ = 5.5 s, TD = 2 199 890, repetition time = 5.7 s, NS = 1250.

A flip angle of 90°, an observed spectral range (SW) of 1200 Hz, and a temperature of 310 K were used in the three protocols. Before Fourier transformation, all FID were zero-filled to 32 k.

An exponential multiplication was applied to the FID, inducing a line broadening of 1 Hz. An automatic baseline correction was performed before Fourier transform using the software provided with our spectrometer. Spectra were processed in the frequency domain using Interliss software(Eurofins Scientific, Nantes, France).[11]

For each compound, three spectra were acquired with the standard measurement, three with the fast measurement, and three with the reference measurement.

The T_1 values of each compound were determined by using an inversion recovery sequence [12] with 7 inversion time values ranging from 5 ms to 5 s and by using the T_1 calculation software of the spectrometer.

ERETIC Parameters. As previously described,[6,8] 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. It was derived just before the amplification stage. 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.[10] The frequency, the amplitude, and the phase of this signal were therefore freely chosen as NMR parameters by the operator. For the three protocols, the time constant and intensity of the ERETIC signal were matched to induce a line width close to that of the CH_3 group of TMU (2.5 Hz after line broadening) and an intensity close to that of TMU in the standard measurement. The ERETIC position was chosen at 10.5 ppm to avoid any overlapping with other spectral lines.

After mixing the two electronic components, the pseudo-FID was transmitted through the proton coil, which was not tuned at the operating frequency (here the deuterium frequency) and acted, therefore, as a broadband antenna for the ERETIC signal. Because this coil was also used for decoupling, the ERETIC signal was mixed with the proton irradiation by an RF coupler placed just before the probe.

Determination of the Site-Specific D/H Ratio. Standard Measurement. $(D/H)_i$ ratios were calculated from eq 2,

$$(D/H)_i = (D/H)_{TMU}\left(\frac{S}{S_{TMU}}\right)\left(\frac{p_{TMU}m_{TMU}M_{comp}}{p_i m_{comp}M_{TMU}}\right) \quad (2)$$

where P is the number of equivalent deuterium positions, M and m are the molecular weight and the mass in the tube, and S is the area under the line associated with site $_i$ of the compound or with TMU.

TMU was used as internal reference[5] and precisely calibrated on the V.SMOW scale.[2]

Fast Measurement. $(D/H)_i$ ratios were calculated from eq 3,

$$(D/H)i = (D/H)_{TMU}\left(\frac{S_i}{\lambda S_{ERET}}\right)\left(\frac{p_{TMU}m_{TMU}M_{TMU}}{p_i m_{comp}M_{TMU}}\right) \quad (3)$$

where S_{ERET} is the area under the ERETIC line, and λ is a coefficient determined from eq 4,

$$S_{TMU} = \lambda S_{ERET} \quad (4)$$

obtained from the reference measurement.

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Table 1. Relaxation Times, T_1, and Precision, σT_1, of Methyl-9,9′-bis(phenylthio)nonanoate and 1,1-Methybis(phenylthio)hexane[a]

	Methyl-9,9′-bis(phenylthio)nonanoate **1** site i						
	2	3,7	8	4,5,6	9	OMe	TMU
δ (ppm)	2.0	1.3	1.6	1.0	4.1	3.4	2.6
T_1 (s) $\pm$ σT_1 (s)	0.16 $\pm$ 0.01	0.09 $\pm$ 0.01	0.04 $\pm$ 0.01	0.09 $\pm$ 0.01	0.05 $\pm$ 0.01	0.89 $\pm$ 0.02	1.08 $\pm$ 0.02

	1,1′-Bis(phenylthio)hexane **2** site i					
	1	2	3	4,5	6	TMU
δ (ppm)	4.1	1.6	1.4	1.0	0.7	2.6
T_1 (s) $\pm$ σT_1 (s)	0.08 $\pm$ 0.01	0.09 $\pm$ 0.01	0.15 $\pm$ 0.01	0.22 $\pm$ 0.01	0.47 $\pm$ 0.02	1.15 $\pm$ 0.02

[a] For the chemical shift reference, the TMU resonance was set at 2.6 ppm, which is equivalent to tetramethylsilane (TMS) resonance in ($CHCl_3$/CCl_4) at 0.0 ppm.

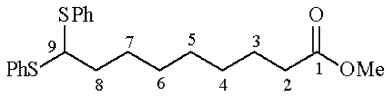
RESULTS AND DISCUSSION

The measured T_1 values for the different sites of methyl-9,9′-bis(phenylthio)nonanoate **1**, 1,1′-bis(phenylthio)hexane **2**, and TMU are given in Table 1. In the standard measurement, all of the T_1 values were taken into account to achieve quantitative conditions. The maximum T_1 value is that of TMU (1.15 s), and with a T_R of 5.7 s, the ratio T_R/T_1 was therefore equal to 4.95. Under such conditions, partial saturation due to longitudinal relaxation during the repetition time cannot induce an intensity distortion >0.71%. Under these conditions, the total acquisition time for three spectra of either **1** or **2** is 57 h.

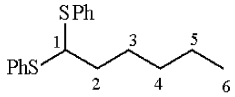
This acquisition time is excessively long and severely limits the number of samples that can be examined. As discussed earlier, it might be diminished by using another reference compound with a shorter T_1 value. However, despite examining a range of compounds that were apparently compatible with the strict constraints of quantitative isotopic ^{2}H NMR spectral acquisition—notably that could be calibrated for their D/H ratio—no alternative chemical reference could be found (data not shown). In particular, no reference compound proved to be generic.

However, the electronic strategy (ERETIC) theoretically offers considerable gains. In the case of the methyl-9,9′-bis(phenylthio)-nonanoate **1**, the highest T_1 value is that of the methoxyl group (0.89 s), and in the case of the 1,1-methyl-bis(phenylthio)hexane **2,** it is that of the methyl group (0.47 s). Thus, by calibrating the ERETIC signal against TMU and then using the ERETIC signal as the reference to determine the $(D/H)_i$ ratios, a considerable gain in time is achieved. The T_R can now be adjusted on the basis of the highest value of T_1 for the compound. Even in the case of **2**, with $T_1 = 0.47$ s for the methyl group, T_R can be reduced to only 2.35 s, leading to a 2-fold decrease in the acquisition time.

However, although this represents a significant advantage, it is possible to increase the gain in time still further by using another approach. As can be seen from Figure 2 and Table 1, the highest T_1 values for the studied compounds were obtained for the lines that have the best signal-to-noise ratio. The $(D/H)_i$ of the corresponding sites can therefore be measured with good precision from spectra acquired from a reduced number of scans. On the other hand, all the lines which exhibit low intensity in Figure 1 have T_1 values lower than 0.22 s, and the corresponding



1
Methyl-9,9'-bis(phenylthio)nonanoate



2
1,1'-bis(phenylthio)hexane

Figure 1. Structures and numbering of the products obtained from the chemical modification of methyl linoleate.

$(D/H)_i$ can be measured from spectra acquired with a much reduced T_R.

We have therefore performed two acquisitions for each compound. Three spectra were obtained with the protocol called “reference measurement”, and three other spectra were obtained with the protocol called “fast measurement” (see Experimental Section). From the former, the ERETIC signal was calibrated against TMU using eq 3, and the $(D/H)_i$ values of the methoxyl group (for the methyl-9,9′-bis(phenylthio)nonanoate **1**) and the methyl group (for the 1,1′-bis(phenylthio)hexane **2**) were determined using eq 2. From the latter protocol, the $(D/H)_i$ values of the remaining groups were determined using eq 3. The acquisition time was 5.7 h for the three spectra performed with the reference protocol and 11 h for the three spectra performed with the fast protocol. All of the spectra that were needed for the determination of all $(D/H)_i$ were therefore acquired in 16.7 h, which represents a 3.4-fold gain in time, as compared with the standard protocol.

$(D/H)_i$ values determined using the standard protocol or the fast and reference protocols are given (in ppm) in Table 2 for the methyl-9,9′-bis(phenylthio)nonanoate **1** and in Table 3 for the 1,1′-bis(phenylthio)hexane **2**. Standard deviations obtained for each site from the three spectra are also listed. Standard deviations are always on the order of 5% or lower except for site 9 of the methyl-9,9′-bis(phenylthio)nonanoate. This site has both a high level of depletion in deuterium and a small T_2, inducing the worst signal-to-noise ratio of the spectrum. The curve-fitting of the corresponding line was, therefore, the most difficult. Furthermore, it must be noted that the high standard deviation observed for

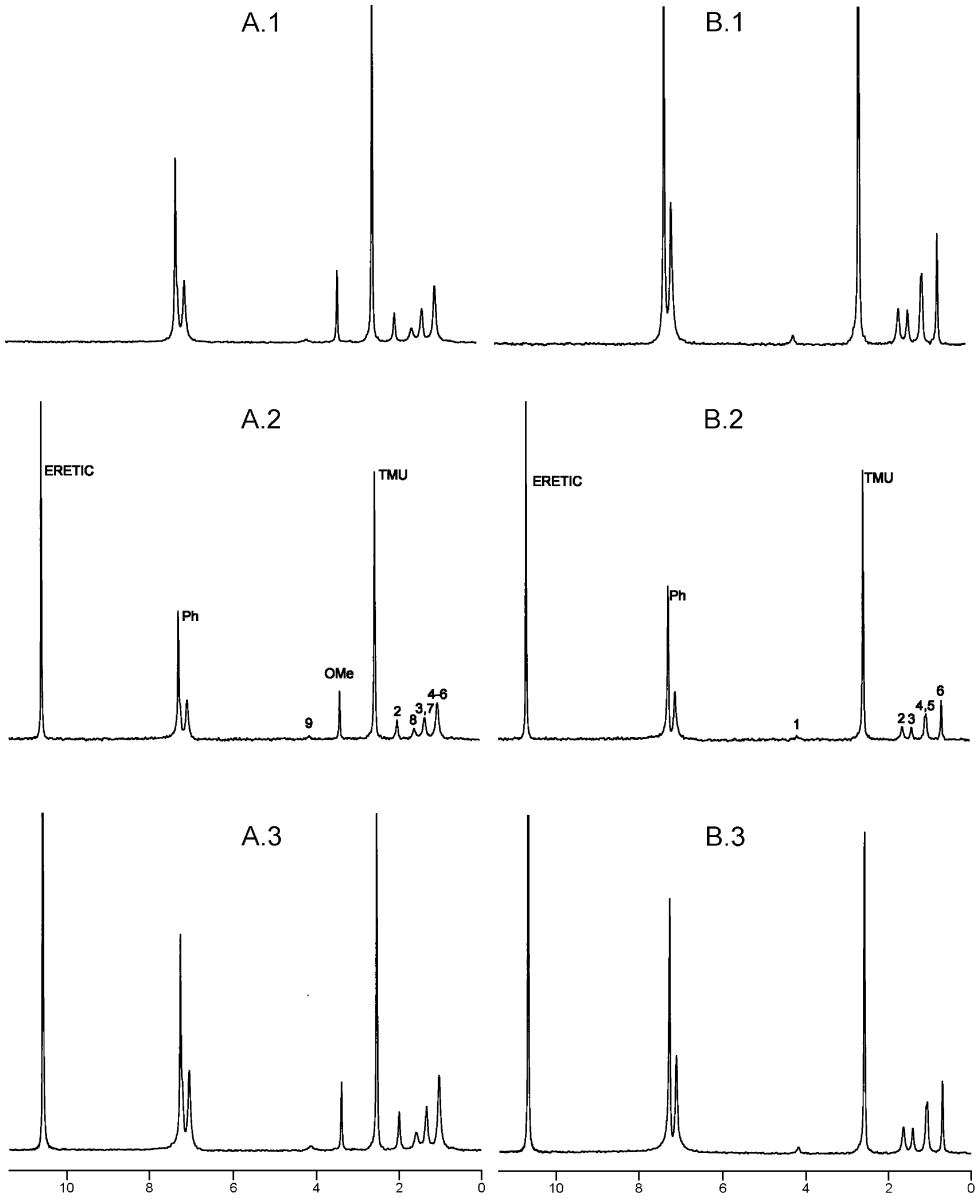


Figure 2. ^{2}H NMR spectra (76.77 MHz) of methyl-9,9′-bis(phenylthio)nonanoate **1** (A) and 1,1′-bis(phenylthio)hexane **2** (B) obtained with the standard protocol (A.1, B.1), the reference protocol (A.2, B.2) and the fast protocol (A.3, B.3).

Table 2. $(D/H)_i$ Ratios and Standard Deviations σ Measured from Three Spectra on Methyl-9,9′-bis(phenylthio)nonanoate

	site i					
	2	3,7	8	4,5,6	9	OMe
reference and fast protocol[a]	144.2 ± 1.7	109.6 ± 1.9	132.6 ± 3.6	134.7 ± 0.3	46.6 ± 12.5	126.4 ± 2.4
std protocol	147.5 ± 1.0	112.0 ± 1.9	136.8 ± 6.0	134.3 ± 1.1	37.9 ± 2.4	127.0 ± 2.4

[a] For the OMe, the $(D/H)_{OMe}$ ratio was measured on spectra obtained with the reference protocol; for other groups, $(D/H)_i$ was measured on spectra obtained with the fast protocol.

this site is due to only one spectrum measured with the fast protocol. When this measurement was removed, the variation was reduced, and the mean value (39.2 ± 2.5 ppm) was significantly closer to that obtained with the standard protocol.

The Student *t*-test for paired series was carried out on the two sets of measurements for each compound. Results obtained (t = 0.454 for **1** and t = 0.038 for **2**) show that there is no significant difference ($p < 0.01$) between the D/H values obtained by the two strategies. It can therefore be concluded that the reduction of the acquisition time does not induce any decrease in the precision of the $(D/H)_i$ determination. The acquisition of five spectra rather than three would allow a more precise statistical analysis. However, the stability of **1** and **2** in the acquisition conditions was not sufficient to prolong the analysis time further.

Table 3. $(D/H)_i$ Ratios and Standard Deviations σ Measured from Three Spectra on 1,1′-Bis(phenylthio)hexane

	Site i				
	1	2	3	4,5	6
reference and fast protocol[a]	58.9 ± 1.1	132.3 ± 2.1	97.0 ± 1.9	113.2 ± 2.1	123.3 ± 4.9
std protocol	58.3 ± 2.4	129.2 ± 3.1	96.1 ± 2.6	113.0 ± 1.2	119.5 ± 0.7

[a] For site 6, the $(D/H)_6$ ratio was measured on spectra obtained with the reference protocol; for other groups, $(D/H)_i$ was measured on spectra obtained with the fast protocol.

CONCLUSION

The new strategy presented and evaluated here to use the ERETIC method to measure $(D/H)_i$ ratios allows the acquisition time for quantitative ^2H NMR spectra to be minimized. This is particularly valuable in those cases in which the T_1 values of the analyte are significantly lower than that of the reference compound. Critically, this gain in time is obtained without any decrease in the precision of the measurement. In addition, although the validity of the method has been established by the study of long-chain fatty acid derivatives, it is generic and is equally valid for other compounds that generally exhibit low deuterium T_1 values, such as glucose derivatives.

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