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Communication

How to reduce the experimental time in isotopic ^{2}H NMR using the ERETIC method

Flore Le Grand[a], Gérard George[b], Serge Akoka[a,*]

[a] LAIEM, UMR 6006 CNRS, Université de Nantes, BP 92208, 44322, Nantes cedex 3, France
[b] DEGUSSA, Z.I. du Plan, BP 82067, 06131 Grasse cedex, France

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Abstract

Deuterium site-specific natural isotope abundance (A_i) is routinely measured by NMR spectroscopy and quantified using an internal reference peak. This study demonstrates that the substitution of the chemical compound used as internal reference by an electronically generated signal (the ERETIC method) allows a dramatic reduction of the experimental time. Measurements of A_i on eight samples of methylsalicylate have been performed using either an internal reference (TMU) or ERETIC. No significant difference in accuracy or precision had been found between the two methods. However, because the method is applicable in partially saturated conditions, the experiment time was divided by a factor of 4 and the drawbacks associated with an internal chemical reference were avoided when ERETIC was used.

Keywords: Quantitative ^{2}H NMR; Reference signal; ERETIC; Isotope abundance

1. Introduction

Quantitative deuterium NMR allows the routine determination of site-specific natural isotopic distribution [1]. This method measures significant variations in this isotopic distribution according to the origin of the molecule [2]. It has been applied to numerous domains notably the detection of chaptalization of wines [3,4], the authentication of the origin of products [5–8], and the determination of biosynthetic pathways [9,10].

The peak area (S) in an NMR spectrum is directly proportional to the number (N) of nuclei that produce this peak. However, the coefficient of proportionality depends on many parameters, which is why NMR quantitative measurements are always made relative to a calibrated reference material. The choice of an internal reference for quantitative ^{2}H NMR is often difficult. Isotope abundance and purity must be perfectly known, so the reference must be precisely calibrated or come from a commercially calibrated standard. This compound must be co-soluble with the sample, chemically stable in the preparation conditions and have no peak overlap with the sample. The number of equivalent nuclei should be high, in order to limit the quantity required. Furthermore, it is better that the longitudinal relaxation time T_1 of the reference is lower than or equal to the T_1 of the sample, in order to avoid an increase of experimental duration. The reference currently used and official for ethanol analysis is N,N,N',N'-tetramethylurea (TMU) [11].

The reference method ERETIC (Electronic REference To access In vivo Concentration) has been proposed as a mean to avoid the addition of a reference compound in in vivo NMR [12] and for concentration measurement in high-resolution ^{1}H NMR [13,14].

* Corresponding author. Fax: +33 251125712.
E-mail addresses: flore.legrand@degussa.com (F. Le Grand), gerard.george@degussa.com (G. George), serge.akoka@chimbio.univ-nantes.fr (S. Akoka).

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In the first use of ERETIC in ^{2}H NMR [15], a reduction of the total experiment time was obtained by calibrating the ERETIC peak against TMU from an acquisition with a greatly reduced number of scans. The isotope abundances were then determined on spectra obtained with a reduced repetition time. Two spectral acquisitions were therefore needed for each sample: one to calibrate the ERETIC signal and one to determine isotope abundances on the sample.

In a previous study [16], we have shown the reliability of ERETIC as an independent reference in ^{2}H NMR. Critically, it was shown that once ERETIC is calibrated against TMU, it can be used for repeated sample acquisitions leading to a substantial decrease in overall acquisition time.

In the present work, this approach has been extended to demonstrate that, with ERETIC as an independent reference, acquisition on samples can be performed in partially saturated conditions. Acquisition time can thus be dramatically reduced, without decreasing measurement accuracy. Specific isotope abundance has been measured by the two methods (chemical reference and ERETIC), on methylsalicylate samples from different origins, with different specific isotope abundance. An additional sample has been used as reference. It is shown that this generic method presents the advantages of sample and reference separation with a great saving in the experimental time.

2. Results and discussion

2.1. T_1 values

The T_1 values are presented in Table 1. The T_1 of TMU is significantly longer than those of methylsalicylate, so the longest T_1 ($T_{1\,\max}$) for ^{2}H is equal to 1.1 s. This determines the repetition time (TR) necessary between two scans to obtain a fully relaxed spectrum ($TR \geqslant 5 \times T_{1\,\max}$ allows an error $\leqslant 0.7\%$). In the case of the standard protocol, TR = 6 s was chosen.

With the ERETIC method, the T_1 of the reference does not need to be considered in the TR calculation for a quantitative acquisition. Hence, quantitative spectra on methylsalicylate can be acquired with TR = 4.25 s (5×0.85 s), leading to an experimental time of 35 min instead of 50 min per spectrum. When three spectra are acquired per sample and numerous samples are analyzed, the saving of time will be significant. For other types of samples, (e.g., lipids), the T_1 of TMU is significantly longer than those of the measured sites. For these type of compounds, the ERETIC method decreases further the TR, hence the total experimental time [16].

Table 1
Longitudinal relaxation time (T_1 in s) for the observed ^{2}H sites of methylsalicylate and TMU, determined by inversion-recovery (eight inversion delays between 5 ms and 8 s)

Site	2	3	4	5	6	TMU
T_1 (in s)	0.130	0.110	0.135	0.150	0.850	1.100

The data processing and calculations were made by the software integrated in the spectrometer.

In the second experiment (fast ^{2}H protocol), ^{2}H spectra were acquired with TR = 1.5 s. Because of their T_1, the signals of aromatic deuterons of methylsalicylate were not affected by this reduction of TR. Only the relaxation of the methoxy group (methylsalicylate) and of the TMU was not complete.

2.2. ^{1}H NMR for determination of relative concentrations

In the two methods of reference, the concentrations of the compounds in the tubes have to be perfectly known. Using weighing, the concentrations can be expressed as:

$$[\mathrm{c}] = \frac{m_\mathrm{c} \cdot n_\mathrm{i}^\mathrm{c} \cdot p_\mathrm{c}}{M_\mathrm{c} \cdot V_\mathrm{t}} \quad \text{and} \quad [\mathrm{ref}] = \frac{m_\mathrm{ref} \cdot n_\mathrm{ref} \cdot p_\mathrm{ref}}{M_\mathrm{ref} \cdot V'_\mathrm{t}},$$

where n is the number of equivalent nuclei, M and m are the molar mass and the mass in the tube, p is the purity, and V_t is the total volume of the sample.

The internal reference approach uses only one tube for the measurement ($V_\mathrm{t} = V'_\mathrm{t}$) and the evaluation of relative concentrations can be reduced to a precise measurement of masses and purities (current method for ethanol analysis [11]):

$$\frac{[\mathrm{ref}]}{[\mathrm{c}]} = \frac{m_\mathrm{ref} \cdot n_\mathrm{ref} \cdot p_\mathrm{ref} \cdot M_\mathrm{c}}{m_\mathrm{c} \cdot n_\mathrm{i}^\mathrm{c} \cdot p_\mathrm{c} \cdot M_\mathrm{ref}}. \tag{1}$$

On the other hand, the ERETIC method uses a calibration sample located in a different tube from those of the studied samples. If the gravimetric method above is employed, the total volume does not cancel out, since $V_t \neq V'_t$:

$$\frac{[\mathrm{ref}]}{[\mathrm{c}]} = \frac{m_\mathrm{ref} \cdot n_\mathrm{ref} \cdot p_\mathrm{ref} \cdot M_\mathrm{c} \cdot V_\mathrm{t}}{m_\mathrm{c} \cdot n_\mathrm{i}^\mathrm{c} \cdot p_\mathrm{c} \cdot M_\mathrm{ref} \cdot V'_\mathrm{t}}. \tag{2}$$

It is technically very difficult to measure the total volumes with the required precision, which is why, in the ERETIC method presented here, it is better to determine the relative concentrations by ^{1}H NMR spectroscopy [17]. The experimental time of a ^{1}H NMR spectrum is very short (3 min) and hardly extends the total experiment duration: a high signal-to-noise ratio (S/N) can be obtained with only 12 scans (S/N > 3000). Furthermore, it is a saving of time by avoiding purity determination (i.e., by chromatography).

2.3. A_i results

The molecule of methylsalicylate presents five interesting sites for A_i measurement in ^{2}H (Figs. 1 and 2),

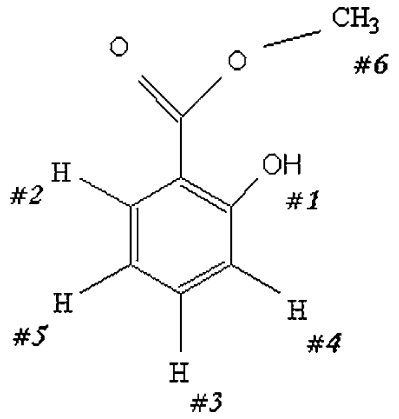

Fig. 1. Methylsalicylate molecule with ^{2}H sites numbered in decreasing chemical shift.

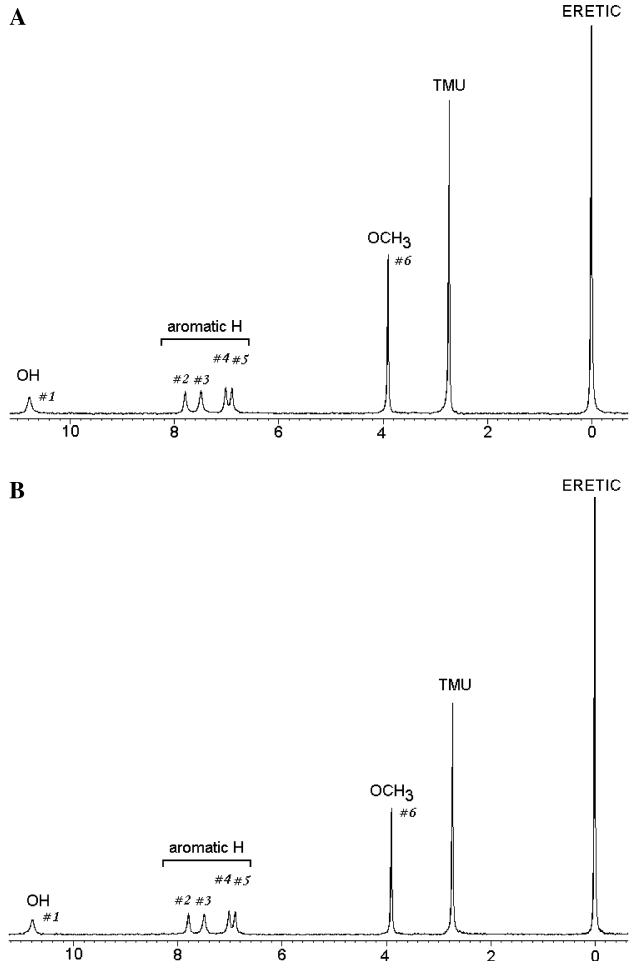

Fig. 2. Natural abundance ^{2}H NMR spectra (recorded on a Bruker DRX 500 spectrometer with a probe dedicated to ^{2}H measurement—10 mm ^{2}H–^{2}1H probe, lock ^{19}F) of methylsalicylate in the presence of the ERETIC signal. Tubes are prepared with 2.5 mL methylsalicylate, 1.5 mL TMU, and 100 μL lock-compound C_6F_6 (without additional solvent). (A) Standard ^{2}H protocol: as previously described [18,19] and in the official method [20]: flip angle $\alpha = 90°$, repetition time (TR) = 6 s, sampling period (AQ) = 4 s, number of scans (NS) = 500, spectral range (SW) = 1230 Hz, temperature (TE) = 303 K. (B) Fast ^{2}H protocol: repetition time (TR) = 1.5 s, sampling period (AQ) = 1 s. All other parameters were kept identical to (A).

since site #1 presents deuteron transfer with the medium. Specific isotope abundances measured by the three methods (TMU, standard ERETIC, and fast ERETIC), are given in Table 2. Differences between the results are always lower than the standard deviation, except for site #6, where the standard deviation is very small (because of its higher S/N ratio). However, taking into account the differences of the A_i values between different origins, this does not prevent differentiation between the three sources of the molecule (Fig. 3). The average coefficients of variation calculated for the five sites of all samples are, respectively, 0.7, 0.3, and 0.8% for TMU, standard ERETIC, and fast ERETIC. Therefore, the use of the ERETIC method does not decrease the precision of the measurement. Furthermore, the fast protocol does not significantly affect the precision of the ERETIC method.

2.4. Reduction of TR

In a previous application of ERETIC to ^{2}H NMR [15], two spectra were acquired for each sample. A fully relaxed spectrum was obtained with a reduced number of scans in order to calibrate the ERETIC peak against TMU. An equivalent isotope abundance was then assigned to the ERETIC peak and a second spectrum was performed on the sample, considering ERETIC as the new reference, with a reduced TR which did not take into account the T_1 of TMU. This strategy is time saving but only in those cases in which the T_1 values of the analyte are significantly lower than that of the reference compound.

In the method we propose here, the ERETIC signal is used as a working standard. It only has to be calibrated once on a calibration sample containing the same compound as the samples to be analyzed. This compound is the actual reference. Measurement of A_i on each sample is obtained by a single acquisition. It is not necessary to assign to ERETIC an equivalent concentration and/or an isotope abundance. The ERETIC signal properties are only used to amend the sensitivity variations that arise between the calibration spectrum and the sample spectrum. The actual reference is located in the calibration tube, with a well-known A_i. It has the same physical and chemical properties as the studied sample (nature, concentration), the relaxation times of the reference and of the sample are therefore identical (this point was experimentally verified by T_1 measurements). Thus, fully relaxed conditions are no longer necessary to perform quantitative analysis.

During a short TR, as in the fast protocol, the relaxation of the longest T_1 groups cannot be completed. That introduces a dependence on T_1 for the k coefficients in the expressions of S_i^c and S_i^{cal}. However, the magnetization of the studied sample and of the calibration sample relax in the same proportion. Therefore, the

Table 2
A_i results and standard deviations (in ppm), average on n samples, for the three origins of molecule, and for the three types of reference

Origin reference	Synthetic			Wintergreen oil			Sweet birch bark oil		
	TMU	ES	EF	TMU	ES	EF	TMU	ES	EF
A_2	150.4 ± 2.2	150.5 ± 2.3	146.8 ± 2.4	120.9 ± 2.6	122.2 ± 2.6	120.2 ± 1.2	118.8 ± 2.7	116.6 ± 2.6	115.7 ± 4.3
A_3	150.3 ± 2.2	150.3 ± 2.2	149.3 ± 2.9	151.8 ± 4.0	153.5 ± 3.8	150.2 ± 2.9	146.0 ± 3.9	143.3 ± 3.9	142.8 ± 6.8
A_4	133.3 ± 2.7	133.4 ± 2.7	132.0 ± 2.7	142.0 ± 2.0	143.7 ± 1.9	139.7 ± 3.8	125.5 ± 4.6	123.2 ± 4.6	125.3 ± 3.4
A_5	134.8 ± 3.0	134.6 ± 2.9	132.9 ± 3.3	130.5 ± 2.2	131.9 ± 2.4	130.5 ± 2.0	111.2 ± 4.2	109.1 ± 4.1	112.6 ± 2.7
A_6	141.3 ± 0.6	141.2 ± 0.6	142.3 ± 0.7	130.7 ± 0.5	132.1 ± 0.8	132.6 ± 0.5	122.8 ± 0.1	120.5 ± 0.1	119.0 ± 0.8

TMU, TMU with standard protocol; ES, ERETIC with standard protocol; EF, ERETIC with fast protocol. ($n = 4$ for synthetic and wintergreen oil, $n = 1$ for sweetbirch bark oil). Synthetic and wintergreen samples were commercial (synthetic: Rhodia, Fluka, Roth, Clariant-Lancaster; wintergreen: Aldrich, Polarome, Berjé, Citrus and Allied Essences), sweetbirch bark oil was extracted in our laboratory. The TMU employed had an isotope abundance calibrated by an official European protocol at 84.44 ppm, and was obtained from Eurofins (Nantes, France).

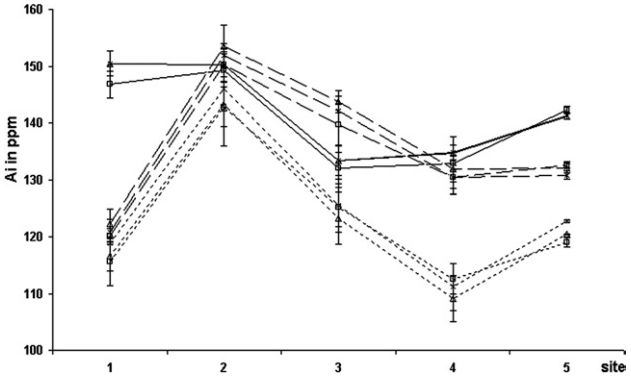

Fig. 3. A_i results, in ppm, for the observed ^{2}H sites of methylsalicylate and the three origins of the molecule (synthetic: ——; wintergreen:– – –; and sweet birch: ·····) with the three protocols employed (TMU with standard protocol: *; ERETIC with standard protocol: □; ERETIC with fast protocol: ϒ). Standard deviations are plotted as error-bars ($n = 4$ for synthetic and wintergreen, $n = 1$ for sweet birch).

T_1 dependence vanishes in Eq. (5) and the A_i determination is not affected.

That explains why the same information is obtained with TR = 1.5 or 6 s. However, the use of the fast protocol allows a dramatic reduction of the experiment duration from 50 to 12.5 min per spectrum. In the context of quantitative NMR, where several spectra per sample are required and numerous samples are analyzed, this is an effective time and cost-saving strategy.

The mean S/N on the aromatic deuterons was about 35 for the two ^{2}H NMR protocols and no significant differences were observed. On the other hand, the reduction of TR resulted in a decrease of S/N for the methoxy group (about 250 for the standard protocol and about 210 for the fast protocol), which is the site with the longest T_1 (850 ms). The S/N was much better for this site because of the number of equivalent protons and the half-height width. Therefore, its decrease was not a restrictive parameter.

Only a few molecules satisfy the criteria evoked in the introduction to define a satisfactory internal reference. ERETIC is a generic reference, which can be used with any new molecule studied. Furthermore, the sample is not polluted by the reference and it can be easily recovered for further analysis.

The dilution of the sample by introducing an internal reference has also to be considered. In the present case, adding TMU decreases the concentration of methylsalicylate by a factor of 1.6. For this study we have used an internal reference to demonstrate the accuracy of the ERETIC method. However, it could be removed without any drawbacks [16]. The signal to noise ratio would in this case be increased by 1.6 or the experiment time should be decreased by $(1.6)^2 = 2.56$.

3. Conclusion

This study shows that electronic reference ERETIC gives reliable results in quantitative ^{2}H NMR, in accordance with those obtained using the traditional internal reference, TMU.

Although it needs a prior calibration and a ^{1}H-spectral measurement, the ERETIC method offers several advantages: no problem to select the reference (solubility, peak overlap); no sample pollution or chemical interaction; cost reduction using the calibrated chemical reference only once, simplification of tube preparation (no weighing).

Furthermore, using the ERETIC method allows a dramatic saving in time: (i) removing the compound of the internal reference results in an increase in the solute concentration, and hence of the signal-to-noise ratio; (ii) because the analyte is also used as the calibration compound at a similar concentration, the reference molecule has the same T_1 in the two tubes and it is no longer necessary to choose a TR higher than $5 \times T_{1\,\max}$.

4. Experimental section

4.1. Spectra processing

After exponential multiplication (line broadening = 1 Hz for ^{2}H spectra, 2 Hz for ^{1}H spectra) and Fourier

transform on 32k data points, the peak areas were determined by fitting the spectrum to Lorentzian shapes with the PERCH software (PERCH NMR Software University of Kuopio, Finland). For each tube and each protocol three ^{2}H spectra and four ^{1}H spectra were measured.

Data from the corresponding three ^{2}H spectra were used in the calculation of A_i for each protocol. A_i and standard deviations were averaged on n samples from each origin of sample ($n = 4$ for synthetic, $n = 4$ for wintergreen oil, and $n = 1$ for sweet birch bark oil).

4.2. A_i calculation

4.2.1. Internal reference method

From the ^{2}H NMR spectra the areas:

$$S_i^c = k \cdot n_i^c \cdot [c] \cdot A_i^c \cdot V_{sd} \quad \text{and}$$
$$S^{ref} = k^{ref} \cdot n^{ref} \cdot [ref] \cdot A^{ref} \cdot V_{sd}$$

are measured. S_i^c and S^{ref} are the areas under the ^{2}H peaks for site i of the sample and for the reference respectively; n_i^c and n^{ref} are the respective statistical site population; [c] and [ref] are the concentrations of sample and reference, respectively; and V_{sd} is the sensitive volume of the ^{2}H coil. k is an unknown constant, that depends on several experimental factors, including temperature, relaxation and NOE factor. k is very difficult to control within the range of accuracy and precision required. By using relative measurements, k is eliminated and A_i^c is obtained:

$$A_i^c = A^{ref} \cdot \frac{S_i^c}{S^{ref}} \cdot \frac{n^{ref}}{n_i^c} \cdot \frac{[ref]}{[c]}. \tag{3}$$

From the ^{1}H NMR spectra, obtained on the same sample, the integral areas:

$$\sum^c = k_h \cdot n_p^c \cdot [c] \cdot V_{sh} \quad \text{and} \quad \sum^{ref} = k_h \cdot n^{ref} \cdot [ref] \cdot V_{sh}$$

are measured to determine the concentration ratios. (Note that the quantification of only one proton chemical shift of the compound is required. k_h and V_{sh} are properties of the proton coil.) giving

$$\frac{[ref]}{[c]} = \frac{\sum^{ref}}{\sum^c} \cdot \frac{n_p^c}{n^{ref}} \tag{4}$$

and

$$A_i^c = A^{ref} \cdot \frac{n_p^c}{n_i^c} \cdot \frac{S_i^c}{S^{ref}} \cdot \frac{\sum^{ref}}{\sum^c}. \tag{5}$$

4.2.2. ERETIC method

^{2}H and ^{1}H NMR spectra are measured on samples and on a "calibration tube," containing the analyte of which the isotopic abundances A_i^{cal} are known.

In this case, the reference tube contained methylsalicylate from wintergreen oil, with isotopic abundances A_i^{cal} of: 121.3 ± 2.7, 152.7 ± 1.3, 146.6 ± 5.9, 132.4 ± 1.4, and 131.6 ± 0.3 ppm for sites 2–6, respectively.

The ^{2}H spectra, obtained on the calibration tube, give the areas:

$$S_i^{cal} = k^{cal} \cdot n_i^{cal} \cdot [cal] \cdot A_i^{cal} \cdot V_{sd} \quad \text{and} \quad S'_{eretic}.$$

The ^{2}H spectra obtained on the sample tube give:

$$S_i^{cal} = k \cdot n_i^c \cdot [cal] \cdot A_i^{cal} \cdot V_{sd} \quad \text{and} \quad S_{eretic}$$

with

$$\frac{S_{eretic}}{S'_{eretic}} = \frac{k}{k^{cal}} \quad \text{and} \quad n_i^c = n_i^{cal}.$$

Hence

$$A_i^c = A_i^{cal} \cdot \frac{S_i^c}{S_i^{cal}} \cdot \frac{S'_{eretic}}{S_{eretic}} \cdot \frac{[cal]}{[c]}.$$

The ^{1}H spectra of the calibration tube give:

$$\sum^{cal} = k_h^{cal} \cdot n^{cal} \cdot [cal] \cdot V_{sh} \quad \text{and} \quad \sum'_{eretic}$$

The ^{1}H spectra of the sample tube give:

$$\sum^c = k_h \cdot n^c \cdot [c] \cdot V_{sh} \quad \text{and} \quad \sum_{eretic}$$

with

$$\frac{\sum_{eretic}}{\sum'_{eretic}} = \frac{k_h}{k_h^{cal}} \quad \text{and} \quad n^c = n^{cal}.$$

Hence

$$\frac{[cal]}{[c]} = \frac{\sum_{eretic}}{\sum^c} \cdot \frac{\sum^{cal}}{\sum'_{eretic}}. \tag{6}$$

Thus

$$A_i^c = A_{cal} \cdot \frac{S_i^c}{S_{eretic}} \cdot \frac{S'_{eretic}}{S_i^{cal}} \cdot \frac{\sum_{eretic}}{\sum^c} \cdot \frac{\sum^{cal}}{\sum'_{eretic}}. \tag{7}$$

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