Accurate Determination of Deuterium/Hydrogen Ratios in Natural Organic Compounds through a Nuclear Magnetic Resonance Time-Domain Reference Convolution Method: Application to Ethanol from Three Botanical Sources and Critical Analysis of Systematic Inaccuracies in Previous Methods

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The (D/H) ratios of three standard ethanol samples having different botanical origins (cane, grape, and beet) were measured by the official EC method (also known as the Martin method) and compared to the values obtained by a time-domain least-squares procedure, called reference convolution, which takes into account line distortions. It is shown that the official method overestimates the $(D/H)_{II}$ ratio by more than 1%, whereas no statistical difference is detected for the $(D/H)_I$ value. A qualitative proof of such a difference is presented. If line distortions are not taken into account, significantly lower $(D/H)_{II}$ values are calculated.

INTRODUCTION

A common problem in analytical NMR is the quantification of intensities of NMR signals with non-Lorentzian line shapes. Under these conditions, accurate signal integration, both in the time or in the frequency domain, becomes more difficult to achieve. In fact, the lack of an analytical function for modeling the signal does not allow setting up a reliable fitting procedure in the time domain. Likewise, in the frequency domain, the presence of line distortions makes the choice of the integration limits a very subjective procedure. The simplest way to alleviate this problem is the application of heavy weighting functions, to minimize the line width differences among signals and to measure heights instead of integrals. Even so, whereas precision usually increases (Martin et al., 1985), accuracy deteriorates, because heights can replace integrals only if all line widths in a spectrum are exactly the same.

It would be more desirable to be able to correct for non-Lorentzian line shapes and use proper signal integration. Advantage can be taken from the fact that often the obtainment of non-Lorentzian lines is a systematic phenomenon. Magnetic field inhomogeneities, for instance, produce the same type of distortion on all lines of a homogeneous sample. In many food analysis applications, a series of samples is examined in unattended mode with the aid of an automatic sample changer and autoshim routines. In this case, the same

systematic line shape distortion is observed throughout the series of spectra. Under these conditions, it is apparent that if one could model a line shape function once for all, this function could be used as a template to obtain quantitative information on signal intensities. For instance, the free induction decay (FID) of a reference signal could be used directly as a multiplier of a Lorentzian function, and the resulting function can be used to fit the signals of interest in the time domain. Methods to fit NMR signals in the time or the frequency domain have been described (Martin, 1994). Convolution/deconvolution methods have also been described to deal with non-Lorentzian functions (Wouters and Petersson, 1977; Gibbs and Morris, 1990; De Graaf et al., 1990). However, fitting procedures taking advance of convolution/deconvolution routines have never been compared with signal height-based methods.

An appropriate benchmark for this type of problem is the so-called Martin method, a procedure for determining the site-specific ratios of deuterium to hydrogen, (D/H), at the different sites of an organic molecule by ^{2}H NMR (Martin et al., 1985; Martin and Brun, 1987). It has been shown in many cases that the (D/H) ratio can be related to the biological origin of the sample (Toulemonde et al., 1983; Schmidt, 1986; Martin and Martin, 1995a; Berbeni et al., 1997; Fronza et al., 1998). In the field of food and beverage control, this analysis has long proven a valuable help in detecting commercial frauds such as sugaring of musts (Martin and Martin, 1990), addition of exogenous sugar to fruit juices (Martin and Martin, 1995b), or flavor adulteration (Martin et al., 1993). In the official wine control method of the European Union, the (D/H) ratios for methyl and methylene hydrogen nuclei in ethanol distilled from the wine are measured against a standard (tetramethylurea, hereafter TMU) and compared with data bank

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values (*Official Journal of the EC*, 1990). All measurements in the Martin method are performed on the heights of signals after application of line broadening functions. It was early recognized that the (D/H) ratios measured in this way have no physical meaning, unless they are corrected to reflect the real integral ratio of the spectral lines (Martin and Martin, 1981).

In this paper we compare and discuss the results obtained by submitting to the Martin signal height (SH) method, as well as to a time-domain least-squares analysis related to the convolution theorem, which we name reference convolution (RC) method, three different ethanol solutions currently used by EC laboratories as ²H NMR standards. We show that the RC method yields equally precise and definitely more accurate deuterium-to-hydrogen ratios than the SH method. We also show that a fitting with a simple Lorentzian model is not appropriate for quantitative purposes when the line shape is even slightly distorted.

MATERIALS AND METHODS

All the experimental work has been carried out on three standard ethanol/TMU/C_6F_6 ²H NMR samples used by EC laboratories for spectrometer adjusting purposes (Martin et al., 1994). Each sample contains a different kind of ethanol produced by fermentation of beet (B), grape (G), or cane (C) sugars and originates different (D/H) values.

The NMR spectra have been recorded at 46.13 MHz and 300 K with a Bruker AC300 spectrometer (working in SIM mode) equipped with a ²H probe, a ¹⁹F lock channel, and a sample changer. Magnetic field homogeneity has been obtained through careful manual shimming of a reference sample (RS) containing a 1 M solution of CD_3CN in a mixture of $CH_3CN/TMU/C_6F_6$. The ratio of the compounds in the RS resembles that of the ethanol samples, except for the substitution of ethanol with acetonitrile. In a single automation run, each sample was subsequently inserted into the probe, allowed to reach probe temperature while spinning, and automatically shimmed with the z^1 and z^2 coils before autogain acquisition. Ten 300-scans spectra per sample were collected over 16 k data points with a 25 ppm bandwidth and a recycle time of 7.1 s.

The (D/H) ratios where first calculated according to the official methodology. Each FID was multiplied by an exponential decay causing a 2-Hz line broadening prior to FT and manual phasing. The heights of the lines corresponding to the signals of CH_2DCH_2OH (site I) and CH_3CHDOH (site II) isotopomers, together with the one of the TMU, were measured and inserted into eqs 1A and 2A, where symbols have the usual meaning (Martin and Brun, 1987), to yield (D/H)$_I$ and (D/H)$_{II}$. We decided not to calculate and discuss (D/H)$_w$, i.e., the deuterium-to-hydrogen ratio of the CH_3CH_2OD isotopomer, since it is generally not used for wine control. The set of (D/H)'s calculated by this procedure will be hereafter referred to as the signal height (SH) set.

$$(D/H)_I^{SH} = 1.5866 \cdot \frac{m_{TMU}}{m_{EtOH} t_{EtOH}} \cdot \frac{h_I}{h_{TMU}} \cdot (D/H)_{TMU} \quad (1A)$$

$$(D/H)_I^{RC} = 1.5866 \cdot \frac{m_{TMU}}{m_{EtOH} t_{EtOH}} \cdot \frac{I_I}{I_{TMU}} \cdot (D/H)_{TMU} \quad (1B)$$

$$(D/H)_{II}^{SH} = 2.3799 \cdot \frac{m_{TMU}}{m_{EtOH} t_{EtOH}} \cdot \frac{h_{II}}{h_{TMU}} \cdot (D/H)_{TMU} \quad (2A)$$

$$(D/H)_{II}^{RC} = 2.3799 \cdot \frac{m_{TMU}}{m_{EtOH} t_{EtOH}} \cdot \frac{I_{II}}{I_{TMU}} \cdot (D/H)_{TMU} \quad (2B)$$

Reference Convolution (RC) Method. The (D/H) ratios were then calculated by the RC method as described below. In this method, line integrals are used. They are found by a

least-squares fitting of the complex FID with prior knowledge. Possible distortions from a pure monoexponential decay of each oscillating signal (corresponding to frequency domain deviations from the Lorentzian shape) are taken into account by considering that, if present, they should affect each signal to the same extent. Therefore, the FID of a sample yielding a single line (as is the case for the RS) displays the same non-Lorentzian contributions as the other samples, provided that it is recorded in the same experimental conditions. On this basis, the RS complex quadrature signal FID$_{RS}(t_N)$ is expressed as:

$$FID_{RS}(t_N) = I_{RS} \cdot X(t_N) \exp[f_{RS} t_N + i(\omega_{RS} t_N + \varphi_{RS})] \quad (3)$$

where I, ω, ϕ, and f denote its intensity, frequency, phase, and dumping factor; t_N is the time corresponding to the Nth point plus the dead time; and $X(t_N)$ is an unknown function that describes distortion of the signal from a pure Lorentzian line shape. Assuming that the distortion is the same for all signals of a spectrum, a general FID can be written as:

$$FID(t_N) = X(t_N) \sum_j^M I_j \exp[f_j t_N + i(\omega_j t_N + \varphi_j)] \quad (4)$$

where M is the number of signals in the spectrum. Recasting eq 3 for the RS FID and inserting it into eq 4 gives eq 5:

$$FID(t_N) = FID_{RS}(t_N) \sum_j^M \frac{I_j \exp[f_j t_N + i(\omega_j t_N + \varphi_j)]}{I_{RS} \exp[f_{RS} t_N + i(\omega_{RS} t_N + \varphi_{RS})]} \quad (5)$$

or

$$FID(t_N) = FID_{RS}(t_N) \sum_j^M I'_j \exp[f'_j t_N + i(\omega'_j t_N + \varphi'_j)] \quad (6)$$

The four parameters per spectral signal, indicated by the primes, can easily be found by fitting eq 6 to an experimental FID by means of one of the many least-squares optimization routines available, e.g., the Levemberg−Marquardt algorithm (Press et al., 1992), provided suitable starting values for them are supplied. The I'_j values share the same proportionality constant with the corresponding I_j and can therefore be directly used in eqs 1B and 2B to calculate $(D/H)_I^{RC}$ and $(D/H)_{II}^{RC}$. It is worth noting that this procedure is quite efficient, in that frequencies, phases, and dumping factors do not change much on passing from an ethanol/TMU/C_6F_6 sample to another: they can be roughly estimated once for all and used thereafter as starting values for the whole set.

We have chosen the name "reference convolution" because, according to the convolution theorem (Ernst et al., 1989), the frequency domain effect of the RC procedure is the convolution of the perfect Lorentzian lines of a spectrum with a non-Lorentzian shape contained in the signal of the reference sample. This name is also reminiscent of the reference deconvolution method described by Morris (Gibbs and Morris, 1990) to improve the resolution of inhomogeneously broadened signals. A simple FORTRAN program to perform the RC is available from the corresponding author upon request.

All results reported, obtained by either method, were given standard errors with 95% confidence intervals. When necessary error propagation was applied.

RESULTS AND DISCUSSION

The (D/H) ratios obtained for the SH and RC sets of data processing methods are reported in Table 1. It is immediately apparent that whereas the (D/H)$_I$ values are equal for both sets within the errors, the (D/H)$_{II}$ values are always lower for the RC than for the corresponding SH ones. Since the only difference between the values of the SH and the RC set is the use

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Table 1. $(D/H)_I$ and $(D/H)_{II}$ Values for Standard Beet (B), Grape (G), and Cane (C) Alcohol Samples Obtained by the Signal Height (SH) Official Method and the Present Reference Convolution (RC) Method[a]

sample	SH	RC	LF
		$(D/H)_I$	
B	90.21 ± 0.34	90.48 ± 0.32	90.22 ± 0.36
G	101.80 ± 0.32	102.05 ± 0.38	102.03 ± 0.46
C	109.70 ± 0.52	109.94 ± 0.57	110.06 ± 0.67
		$(D/H)_{II}$	
B	122.83 ± 0.48	120.98 ± 0.36	119.61 ± 0.44
G	131.25 ± 0.77	129.72 ± 0.70	128.37 ± 0.80
C	120.22 ± 0.45	119.02 ± 0.45	117.60 ± 0.56

[a] The values obtained by simple Lorentzian fit (LF) of the FID are also shown for comparison purposes.

of line integrals instead of line heights for the latter in eqs 1 and 2, it follows that line heights do not share the same proportionality constant to line integrals, as is the case when different line widths are present. Apparently, the 2-Hz line broadening used by the official protocol is not sufficient to level the resulting line width of ethanol and TMU to the same value.

Indeed, small differences among line widths are really sufficient to bias the results. This can be appreciated by exploiting the well-known relation among height, integral, and line width, $h = I/(\pi \cdot lw)$ (which holds for Lorentzian lines), and by putting into evidence the differences between the actual $(D/H)^{RC}$ ratio, i.e., the one that depends on the integrals, and the ratio measured by the official method, $(D/H)^{SH}$:

$$(D/H)_I^{RC} = (D/H)_I^{SH} \cdot \frac{lw_I}{lw_{TMU}} \qquad (7)$$

$$(D/H)_{II}^{RC} = (D/H)_{II}^{SH} \cdot \frac{lw_{II}}{lw_{TMU}} \qquad (8)$$

It appears that the proportionality constant between the actual and the measured (D/H) is the ratio between the width of the line of interest and the one of the TMU line. Unless the widths of the two NMR signals are exactly the same, this number, even after the application of line broadening, is close to 1 but not exactly equal to 1. This gives rise to discrepancies. For example, if the difference from unity is 1% and the $(D/H)_{II}^{SH}$ amounts to a value as typical as 130 ppm, a difference of 1.3 ppm between the two methods of calculation is computed. Moreover, since there is no reason that the three line widths should be equal, the two factors in eqs 7 and 8 are unlikely to be exactly the same. This supports the hypothesis that the results obtained through the use of line heights might not be a true measure of the (D/H) ratio.

For a qualitative demonstration that the above analysis is correct, eqs 7 and 8 can be used and the conversion factors, based on line widths, applied to the SH set to check whether (D/H) values close to the RC values appear. However, the measure of the line width cannot be performed by simple fitting of the NMR absorption line, because its shape could be intrinsically not Lorentzian, nor can an NMR pulse sequence like Carr−Purcell be used, since it is not the natural line width (depending on $1/\pi T_2$) but the apparent one (depending on $1/\pi T_2^*$) that influences the $(D/H)^{SH}$ ratio (Abragam, 1982). Therefore, each line width has been empirically estimated as the median value between the minimum and
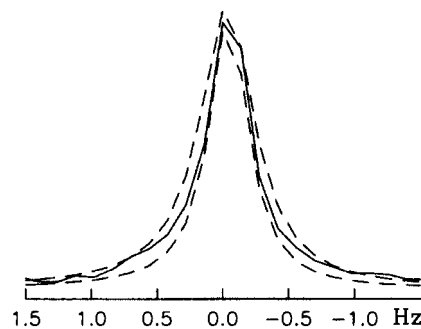

Figure 1. ^2H NMR spectrum of the TMU line (solid) together with the maximum and minimum Lorentzians which bound the signal (dashed lines). In the case shown the two Lorentzian lines, tangent to the tails and to the inner side of the TMU peak, are respectively 0.50 and 0.40 Hz wide.

the maximum width of the two Lorentzians that bound a signal (Figure 1). This procedure, applied to the total set of spectra, yields average line widths for site I, site II, and TMU of, respectively, 0.452, 0.417, and 0.448 Hz with a common standard error of 0.005 Hz. From these results, each incremented by 2 Hz to mimic the effect of the line broadening, the correction factors can be calculated as 1.002 ± 0.003 for $(D/H)_I$ and 0.987 ± 0.003 for $(D/H)_{II}$. Consequently, the $(D/H)_I^{RC}$ values are expected to be equal to the $(D/H)_I^{SH}$ values, as indeed found, because the corresponding correction factor is, within the error, equal to unity. On the contrary, the $(D/H)_{II}$ values derived by the SH set after correction are 121.23 ± 0.60, 129.54 ± 0.86, and 118.66 ± 0.57 ppm for the B, G, and C samples, respectively, different from the original $(D/H)_{II}^{SH}$ ratios and now equal within the derived error to the RC ones. This result corroborates the finding that the RC values are more accurate than the SH values, as they correspond to the values that would have been obtained if integrals, instead of heights, had been used throughout the calculations after correction for signal distortion.

It is worth pointing out that if signal distortions are not taken into account and the fitting of the same FIDs is carried out by modeling each signal as a pure damped oscillation (Lorentzian fit, LF), other $(D/H)_{II}$ results, different from both the SH and the RC ones, appear, the $(D/H)_I$ being the same within the errors (Table 1). These findings, apparently in disagreement with a recent paper (Martin, 1994), are rationalized by noting that, despite the efforts put in obtaining good shimming conditions, the spectral lines are not completely Lorentzian but slightly high-field-distorted (Figure 2). The application of a too simplified model to lines whose shape is distorted but identical (as is the case for the TMU and methyl lines) yields inaccurate integrals, which are nevertheless proportional to the actual values in such a way that their ratio equals the actual one in formula 1B. The same does not apply to the ratio of I_{II} to I_{TMU} in eq 2B because in that case the two line shapes are different, each being expressed by eq 3 with a different damping factor. The wrong model then approximates each real shape in a slightly different way, which is however sufficient to bias the result.

CONCLUSIONS

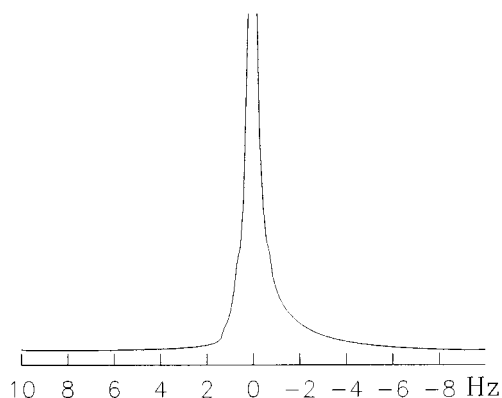In this paper a comparison between the official method for measuring (D/H) values in ethanol/TMU/

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Figure 2. ^{2}H NMR spectrum of the reference signal (RS) whose FID is used as a template in the RC procedure. The 5-fold vertical expansion shows a clear asymmetry due to a slight—but not negligible—high-field distortion.

C_6F_6 mixtures (SH method) and a numerical procedure based on the least-squares fitting of the time-domain NMR signal with modeling of signal distortion, named the RC method, has been carried out. It is shown that whereas both methods yield equal $(D/H)_I$ values, the official method systematically *overestimates* the $(D/H)_{II}$ ratio. This is due to a small—but not negligible—dependence on the signal width. It is also shown that simpler Lorentzian fit of the FID *underestimates* the $(D/H)_{II}$ ratio. The present data constitute the first unbiased measure of $(D/H)_{II}$ values for the reference beet, grape, and cane alcohols.

Of course, the $(D/H)_{II}$ ratios calculated by the official procedure are still useful, as long as all the laboratories involved in wine control use the same protocol. Care however must be taken when comparing data obtained through different procedures, because they may not be necessarily equivalent.

The use of the RC method, which is virtually insensitive to the shape or to the apparent width of each spectral line, could contribute to reduce the small discrepancies that are often detected when the same sample is analyzed by different laboratories, thus improving the overall accuracy and precision of the method.

Finally, the RC method meets the requirements of simplicity and generality to make it useful in a variety of other food analysis applications using NMR.

ABBREVIATIONS USED

FID, free induction decay; LF, Lorentzian fit; NMR, nuclear magnetic resonance; RC, reference convolution; RS, reference sample; SH, signal height; TMU, tetramethylurea.

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