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Accurate Quantitative ^{13}C NMR Spectroscopy: Repeatability over Time of Site-Specific ^{13}C Isotope Ratio Determination

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The stability over time (repeatability) for the determination of site-specific $^{13}C/^{12}C$ ratios at natural abundance by quantitative ^{13}C NMR spectroscopy has been tested on three probes: enriched bilabeled [1,2-$^{13}C_2$]ethanol; ethanol at natural abundance; and vanillin at natural abundance. It is shown in all three cases that the standard deviation for a series of measurements taken every 2−3 months over periods between 9 and 13 months is equal to or smaller than the standard deviation calculated from 5−10 replicate measurements made on a single sample. The precision which can be achieved using the present analytical ^{13}C NMR protocol is higher than the prerequisite value of 1−2‰ for the determination of site-specific $^{13}C/^{12}C$ ratios at natural abundance (^{13}C-SNIF-NMR). Hence, this technique permits the discrimination of very small variations in $^{13}C/^{12}C$ ratios between carbon positions, as found in biogenic natural products. This observed stability over time in ^{13}C NMR spectroscopy indicates that further improvements in precision will depend primarily on improved signal-to-noise ratio.

The $^{13}C/^{12}C$ isotope ratio is well established as a molecular marker and is widely exploited as a tracer in medical and biological studies and as a criterion for authenticity in traceability. For analysis at natural abundance or low enrichment, isotope ratio mass spectrometry (IRMS) provides very accurate values of ^{13}C isotopic deviation (δ‰), but only the global value of the considered compound can be obtained directly. Site-specific ^{13}C values are accessible by this technique only after controlled degradation of the molecule and IRMS measurements on the fragments.[1] This approach is tedious and prone to introducing isotopic fractionation. However, the information retrieved has proved very valuable in showing large internal ^{13}C disparity within, to cite some representative examples, glucose,[2] glycerol,[3] malic acid,[4] and vanillin.[5]

The appropriate tool for determining site-specific isotope content is quantitative NMR spectroscopy (SNIF-NMR), and its performance applied to deuterium content at natural abundance is well documented.[6] However, the application of this technique to the $^{13}C/^{12}C$ isotope ratio has been challenging due to the high level of required trueness and precision: 1−2‰ on the δ scale (a clear definition of these terms is described in ref 7). In other words, the methodology should detect a difference in the signal areas of only 0.2%. All the work so far carried out[8−12] has been confronted with the problem of attaining this level of accuracy[7] in a series of repeated measurements made over a period of time. This is especially critical when absolute values need to be determined on either the same or different samples analyzed several months apart. We have previously established satisfactory conditions with short-term repeatability that allowed a good discrimination of the origin of vanillin on the basis of relative data but did not allow access to absolute $\delta^{13}C$ values.[11] Similarly, the use of quantitative ^{13}C NMR spectroscopy for the calculation of kinetic isotope effects has been published exploiting relative data.[13−15] However, without addressing the trueness in relation to the stability of measurement over time, variation due to such factors as electronic instabilities, different rf components and their differential performance, or even changes such as in the air conditioning make it difficult to compare internal ^{13}C distribution data determined weeks or months apart.

The main source of instability of the spectrometer has been identified as uniformity in the proton decoupling.[10,12] Recently, we

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proposed a protocol for uniform and robust ^{1}H-decoupling using an adiabatic sequence.[16] By this means, very good results in terms of accuracy were demonstrated using bilabeled [1,2-$^{13}C_2$]ethanol as a model.[17] In such a molecule, the relative signal area ratio between the resonances is intrinsic to the structure and this ratio can formally be defined as exactly equal to 1.000. Any variation from this value expresses the trueness of the measurement, while the standard deviation (SD) from repetitions of measurement describes the repeatability. The experimental values observed for these two parameters on bilabeled ethanol are 0.1% and 0.02%, respectively.[17] The application of this methodology to molecules at natural abundance led to standard deviations of repeatability lower than 2‰ on the δ scale, which are satisfactory for purposes of discrimination.[18] However, the robustness of the method depends in its first instance on the stability of the measurements, that is their repeatability over a long time period. It is only realistic to embark on a collaborative interlaboratory study in order to define the reproducibility of quantitative site-specific ^{13}C NMR spectroscopy, once both trueness and repeatability have been proved satisfactory.

Therefore, with this perspective in mind, we have assessed the capacity of the technique to obtain the same results for the same compound over about a 1 year period. The key criterion tested was that the SD of the mean calculated for the long-term repeatability should not exceed the SD observed for a single measurement constituted of 5−10 repeated spectra. This internal reproducibility has been studied on bilabeled ethanol, on ethanol at natural abundance, and on vanillin at natural abundance.

MATERIALS AND METHODS

Chemicals. [1,2-$^{13}C_2$]Ethanol (99 atom %), acetone-d_6, and $CDCl_3$ were purchased from Eurisotop (www.eurisotop.fr). Vanillin was purchased from Merck (www.merck.fr). Tris(2,4-pentadionato)chromium (III) (97%) (CrAcac) was purchased from Acros Organics (www.acros.be). Four samples of biogenic ethanol were obtained by the fermentation of sugars (from grape, beet, maize, sugarcane) with *Sacharomyces cereviseae*. The commercial ethanol (99.9%) was supplied by Docks Des Alcools (France). Chloroform and acetone were purchased from VWR (fr.vwr.com).

NMR Spectroscopy Experiments. Quantitative ^{13}C NMR spectra were recorded using a Bruker DRX 500 spectrometer fitted with a 5 mm i.d. dual probe ^{13}C/^{1}H carefully tuned at the recording frequency of 125.76 MHz. The temperature of the probe was set at 303 K. The experimental parameters for ^{13}C NMR spectral acquisition were the following: pulse width 4.3 μs (90°), sampling period 1 s. The offsets for both ^{13}C and ^{1}H were set at the middle of the frequency range for each molecule. For bilabeled ethanol samples (sealed tube, 100 μL in 0.8 mL $CDCl_3$ + 200 μL of 0.1 M CrAcac solution in $CHCl_3$), 8 scans with a repetition delay of 13 s were recorded leading to a signal-to-noise ratio (SNR) $\approx$ 5000; for ethanol at natural abundance (700 μL + 200 μL distilled water + 100 μL acetone-d_6), 32 scans using a repetition delay of 101 s, leading to SNR $\approx$ 2200; for vanillin at natural abundance (250 mg + 400 μL acetone-d_6 + 100 μL of 0.1 M CrAcac solution in acetone), 92 scans using a repetition delay of 21 s, leading to SNR $\approx$ 450. Inverse-gated decoupling techniques were applied in order to avoid NOE. The decoupling sequence employed a cosine adiabatic pulse with appropriate phase cycles, as described in ref 16. Number of spectra recorded per measurement: 10 for bilabeled ethanol; 5 for ethanol at natural abundance; 5 for vanillin at natural abundance.

Data Processing. Free induction decay was submitted to an exponential multiplication inducing a line broadening of 2 Hz. The curve fitting was carried out in accordance with a Lorentzian mathematical model using Perch Software (Perch NMR Software, University of Kuopio, Finland).

Isotopic Data. The isotopic distribution in a molecule is characterized by the actual ^{13}C molar fractions f of a specific site i:

$$f_i = S_i/S_T$$

where S_i is the area of the ^{13}C NMR signal of i and S_T is the sum of the areas of all the signals for the molecule. Each S_i is corrected according to the number of carbons directly connected in order to compensate for intensity losses due to satellite lines, which are assigned to the bilabeled isotopomers. In accordance with the ^{13}C natural mean abundance of 1.1%, areas were multiplied by (1 + n0.011), where n was the number of carbons directly connected.[9] The shift from the random distribution of ^{13}C, which is the main discriminatory factor of the origin of the molecule, may be expressed as the ratio f_i/F_i named the reduced molar fraction, where F_i is the statistical molar fraction (for further information on these parameters, see ref 10). To express the trueness of the ^{13}C NMR measurement on bilabeled ethanol, we have used the ratio of the integral of the methylene doublet over the integral of the methyl doublet that should be equal to 1.000. If the phenomenon is described in terms of experimental molar fractions f and statistical molar fractions F, the parameter Δ (units of %), the extent of the variation of the measured ^{13}C/^{12}C ratio from the true value, can be described as

$$\Delta = (f_i/F_i - 1) \times 100$$

where i is either the methylene or the methyl group. In the current work, the methylene group has been used.

Periods of Measurement. For bilabeled ethanol: 5 determinations from 02/2006 to 03/2007. For ethanol ex-grape: 7 determinations from 09/2005 to 07/2006. For ethanol ex-sugarcane: 9 determinations from 09/2005 to 01/2007. For ethanol ex-maize: 8 determinations from 09/2005 to 01/2007. For ethanol ex-beet: 9 determinations from 09/2005 to 01/2007. For commercial ethanol: 12 determinations from 07/2005 to 01/2007. For vanillin: 17 determinations from 05/2006 to 02/2007.

RESULTS AND DISCUSSION

Stability of Measurement of ^{13}C/^{12}C Ratio Determination in Bilabeled Ethanol. As previously shown, the shift of the Δ value from 0.000 is mainly associated with the efficiency of ^{1}H-decoupling.[12,16] Further variability will be observed depending on the stability of the transmitting device. From the five series of measurements on the bilabeled ethanol collected between 02/

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Table 1. Mean Isotopic Value ($\delta^{13}C$) over *N* Determinations, Measured by ^{13}C NMR Spectroscopy for Ethanol Samples[a]

sample origin	*N*	$\delta^{13}C$ (‰) CH_2	SD (‰)	$\delta^{13}C$ (‰) CH_3	SD (‰)
beet	9	−26.5	0.5	−28.2	0.5
grape	7	−23.0	0.5	−26.5	0.5
sugarcane	9	−13.0	0.3	−11.4	0.3
maize	8	−11.8	0.6	−8.4	0.6
commercial	12	−32.7	0.3	−26.0	0.3

[a] The standard deviation (SD) from the *N* determinations is expressed on the δ-scale (‰).

2006 and 03/2007, a mean value for $\Delta = 0.07\%$ is obtained. The standard deviation is calculated to be 0.02%, which is of the same order as that generated from one measurement consisting of 10 spectra. It should be noted that this value for Δ (0.07%) is significantly different from that which we have previously published (−0.08%),[17] although the two Δ both are <0.1%. Between these two sets of measurements, the amplifier was changed, illustrating the potential problem of interinstrument variation if standard conditions are not used. This emphasizes the need for a standardized protocol and standardized reference materials to ensure both intra- and interlaboratory and instrumental consistency.

Stability of Measurement of $^{13}C/^{12}C$ Ratio Determination in Ethanol at Natural Abundance. Ethanol is a challenging molecular probe to assess internal reproducibility for a number of reasons. First, the differences obtained by fragmentation and IRMS between the $\delta^{13}C$ values for the isotopic deviation of the methylene and the methyl groups are only a few permil, with ambiguities in both the sign and magnitude.[2,8,12,19,20] Second, it can test effectively whether ^{13}C-SNIF-NMR spectroscopy is able to detect consistently small differences of $\delta^{13}C$ over a long time period. Third, it can be used to examine to what extent other sources of variability contribute to the standard deviation apart from the SNR. For this, ethanol is compared with vanillin (see below), for which both the range of ^{13}C chemical shifts is larger and the SNR is smaller. Both sets of results are expressed as the mean of the means and the SD of the means.

For the natural abundance ethanol samples (Table 1), the differences between $\delta^{13}C$ of methylene and $\delta^{13}C$ of methyl are positive for ethanol derived from the C3-type plants grape and beet (+3.5‰, +1.7‰) and negative for ethanol derived from the C4-type plants sugarcane and maize (−1.6‰, −3.4‰). This difference is consistent and very stable over time since the SD is ≤0.6‰, which is of the same order as, or even below, the SD calculated from one measurement resulting from 5 spectra.

This data is qualitatively in agreement with that of Caer et al.,[8] who in their pioneering work on quantitative ^{13}C NMR spectroscopy also found that it is the methylene monolabeled isotopomer which is ^{13}C-enriched in C3-type plants and the methyl monolabeled isotopomer which is ^{13}C-enriched in C4-type plants. However, solely based on the $\delta^{13}C$ values for the C1, C2, C5, and C6 positions of glucose derived by chemical degradation,[2] it should be the methylene monolabeled isotopomer which is ^{13}C-enriched in both types of plants. Evidently, further work is required to elucidate the biochemical origin of the measured differences between C4- and C3-type plants and the present method is well adapted to tackle these problems.

Table 2. Mean Isotopic Value ($\delta^{13}C$) and Standard Deviation (SD) for Each Carbon of Vanillin, Over 17 Determinations

	carbon number[a]							
	C1	C2	C3	C4	C5	C6	C7	C8
$\delta^{13}C$ (‰)	−29.2	−31.4	−31.9	−24.6	−19.8	−26.0	−18.8	−41.7
SD (‰)	0.8	1.1	1.1	0.9	1.2	0.9	0.7	0.8

[a] See Figure 1 for numbering in relation to chemical shift.

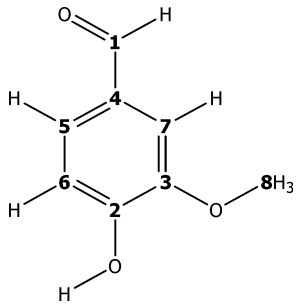

Figure 1. Molecular structure of vanillin with carbon atoms numbered in terms of decreasing ^{13}C chemical shift in the NMR spectrum.

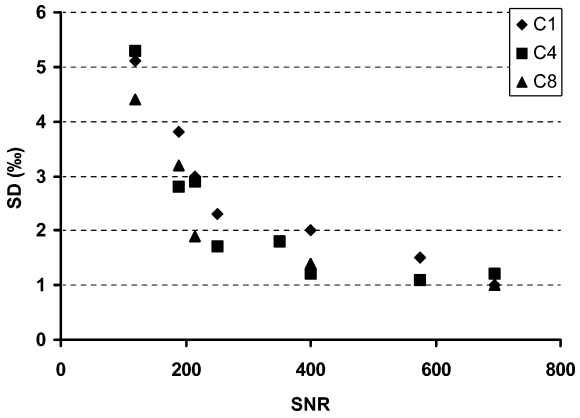

Figure 2. Evolution of the standard deviation (SD) in permil as a function of the signal-to-noise ratio (SNR) for carbons numbered 1, 4, and 8 in vanillin, according to numbering of Figure 1.

Stability of Measurement of $^{13}C/^{12}C$ Ratio Determination in Vanillin at Natural Abundance. Vanillin presents a different set of problems to ethanol. First, the ^{1}H chemical-shift range is much larger, stipulating a much more rigorous regulation of the ^{1}H-decoupling. Second, since vanillin is a solid, the number of molecules that can be observed in the NMR probe is limited by the solubility of vanillin, leading to smaller SNRs than for ethanol during an acceptable experimental time period. As can be seen (Table 2), the SD of the mean of each carbon, calculated from 17 measurements, is between 0.7 and 1.2‰. On average, this range is smaller than that calculated from 5 spectra (1−2‰). This is very satisfactory, confirming that ^{13}C-SNIF-NMR spectroscopy should be a good tool for differentiating between origins of vanillin.

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CONCLUSIONS

The present work shows for the first time that good within-laboratory repeatability can be achieved for quantitative ^{13}C NMR spectroscopy to determine absolute values of $^{13}C/^{12}C$ isotope ratios. Since the measurements are stable in time, further improvements in precision will depend solely on improved SNR. The maximum error (ΔS) on the signal area is inversely proportional to the SNR:[21]

$$\Delta S/S \leq 1/(2 \times \mathrm{SNR}) \quad (1)$$

A comparison of the SNR in bilabeled ethanol spectra (≈5000), in ethanol spectra at natural abundance (≈2200), and in vanillin spectra at natural abundance (≈450), associated with standard deviations, from one measurement, of 0.2‰, 0.6‰, and 1.5‰, respectively, shows that the present data fit this rule. Furthermore, when three types of carbon in vanillin, C1, C4, and C8 (see Figure 1 for numbering) are taken as examples (Figure 2), it can be observed that the SD from the average of five spectra rapidly decreases from SNR = 100 to SNR = 400, whereafter significant improvement of precision is much harder to achieve. As predicted from eq 1, a good linear relationship between SD and 1/SNR is obtained: $y = 584.9x + 0.312$ ($r = 0.983$) for C1, $y = 605.9x + 0.081$ ($r = 0.967$) for C4, and $y = 492.5x + 0.167$ ($r = 0.958$) for C8. Thus, it can easily be calculated that increasing SNR (and therefore experimental time) is rapidly exhausted as a means to gain in SD and to improve precision. We are currently working on other approaches to reduce the experimental duration without degrading the performance quality, such as increased scan frequency through enhanced relaxation rate.[17]

To gauge fully the discriminatory value of the data herein obtained for ethanol and vanillin will require a more extensive assessment of the robustness of the methodology through interlaboratory testing on larger sample sets. What is evident is that quantitative ^{13}C NMR spectroscopy provides an accurate and reliable technique to determine consistently and reproducibly the small differences in $^{13}C/^{12}C$ ratios in ethanol. Because it resolves both the methyl and methylene groups, which show different ratios depending on the source of ethanol, it is a more challenging probe than global $\delta^{13}C‰$ determined by IRMS, in which this discriminatory parameter is lost. As it is presented in this paper, ^{13}C-SNIF-NMR has the capability for studies of metabolism at natural abundance or authentication of natural products.

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