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Short communication

Comparison of IRMS and NMR spectrometry for the determination of intramolecular ^{13}C isotope composition: Application to ethanol

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ABSTRACT

Isotopic ^{13}C NMR is a relatively recent technique which allows the determination of intramolecular ^{13}C isotope composition at natural abundance. It has been used in various scientific fields such as authentication, counterfeiting or plant metabolism. Although its precision has already been evaluated, the determination of its trueness remains still challenging. To deal with that issue, a comparison with another normalized technique must be achieved.

In this work, we compare the intramolecular ^{13}C isotope distribution of ethanol from different origins obtained using both Isotope Ratio Mass Spectrometry (IRMS) and Nuclear Magnetic Resonance (NMR) spectrometry techniques. The IRMS approach consists of the oxidation of ethanol to acetic acid followed by the degradation of the latter for the analysis of each fragments formed. We show here that the oxidation of ethanol to acetic acid does not bring any significant error on the determination of the site-specific $\delta^{13}C$ ($\delta^{13}C_i$) of ethanol using the IRMS approach. The difference between the data obtained for 16 samples from different origins using IRMS and NMR approaches is not statistically significant and remains below 0.3‰. These results are encouraging for the future studies using isotopic NMR, especially in combination with the IRMS approach.

1. Introduction

The $^{13}C/^{12}C$ ratio at natural abundance is recognized as a high potential tool in a wide range of scientific areas including plant physiology, geochemistry, and authentication. Due to the small range of isotopic variation at natural abundance (ca. 50‰), the precision associated with the measurement has to be high in order to obtain exploitable results. ^{13}C isotope compositions (noted as $\delta^{13}C$ and expressed in ‰ related to an international standard, V-PDB) are commonly determined using Isotope Ratio Mass Spectrometry (IRMS) that converts the analyte to CO_2 which $^{13}C/^{12}C$ ratio is further determined. Despite a high precision (i.e. 0.2‰ on the δ-scale), the major drawback of IRMS is that only the global $\delta^{13}C$ values ($\delta^{13}C_g$) of the analyte can be obtained, since a complete transformation into CO_2 of each carbon of the compound is achieved prior to its analysis. Although the determination of the ^{13}C content of the whole analyte, i.e. at the molecular level, brings important information, it is clear that the determination of the isotope composition for each carbon position of the analyte, i.e. at the intramolecular level, might increase this information to a great extent [1]. Indeed, the chemical or enzymatic reactions responsible for the isotopic variations between molecules involve specific bonds, thus leading to variations at specific positions, the other ones remaining almost unaffected. The isotope fractionation observed at the molecular level is thus "the attenuated and superficial manifestations of isotopic differences *within* molecules" [2].

Therefore, there is an increasing interest in developing methodologies able to determine the ^{13}C intramolecular isotope composition of molecules at natural ^{13}C abundance. Two main approaches have been used until now for the determination of the intramolecular isotope composition in natural molecules. The first one involves the degradation of the analyte into fragments prior to their analysis using IRMS. Ensuring that the conversion of the analytes to their fragments is quantitative or that the isotope fractionation in the fragments formation can be known precisely, the intramolecular isotope distribution in the analyte can be reconstructed from the $\delta^{13}C$ values of the fragments. The chemical and/or biochemical degradation approach is tedious and it is not appropriate for routine analyses on several samples. Recently,

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methods using on-line pyrolysis have been developed for some compounds such as acetic acid [3] or lactic acid [4]. This latter approach leads to low experimental time and thus allows the analyses of several samples.

On the other hand, approaches using isotopic ^{13}C NMR, based on the same principle as ^{2}H SNIF-NMR, has recently emerged, giving rise to important results in authentication [5,6], counterfeiting [7,8] and plant physiology [9,10]. Since NMR enables the separation and quantification of each ^{13}C isotopomer of the analyte, no degradation is necessary to get the site-specific $\delta^{13}C_i$ of the analyte using that approach. Theoretically, it is applicable on a wide range of molecules. However, while IRMS requires relatively small amount of analyte (typically much lower than 1 mg), NMR suffers from a poor sensitivity and requires, so far, typical amounts of some hundred milligrams for one analysis. Although, IRMS and isotopic NMR can be seen as complementary techniques since the drawbacks of the former are compensated by the advantages of the latter, and vice versa.

The precision (defined as "the closeness of agreement between independent test results obtained under stipulated conditions"; [11]) of both techniques can be easily established using repeatability tests. The trueness (defined as the "the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value" [11]) of IRMS can be assessed thanks to existing international calibration standards. It is not the case for the trueness of isotopic ^{13}C NMR which remains still difficult to establish.

The trueness of the NMR instrumental response itself has been monitored by using a ^{13}C-bi-labelled ethanol sample [12]. In such a chemical the $^{13}C\{^{1}H\}$ NMR spectrum consists in two doublets (due to $^{1}J^{13}C–^{13}C$ scalar coupling) centered on the $^{13}CH_2$ and $^{13}CH_3$ chemical shifts. The signal area of each doublet is strictly identical. Thus, any shift from the value of 1.000 for the ratio of these doublets should indicate a bias in the NMR measurements. Although, this protocol is very pertinent for adjusting very precisely the NMR parameters to reach the best calibration (especially the ^{1}H decoupling) for relative intensities within a given ^{13}C spectrum, there is still no direct indication on the "true" $\delta^{13}C_i$ values obtained by isotopic NMR. Since its very recent introduction as a routine tool to determine the site-specific ^{13}C content ($\delta^{13}C_i$) a few molecules have been studied by isotopic ^{13}C NMR. For all cases, the trueness has been assessed by comparing the $\delta^{13}C_i$ values between NMR experiments and IRMS after degradations from works found in literature: for glycerol [13] and [14]; for vanillin [15] and [16]; for glucose [17] and [18], respectively. A rather good trend between these two methodologies was observed. However, this comparison shows a limited interest because the samples analyzed by IRMS and NMR were not strictly identical. From the above considerations, the trueness of isotopic ^{13}C NMR spectrometry is still an issue.

The aim of the present work is to address this problem by (i) finding a molecule for which specific isotope ratios determination is possible by both IRMS and NMR, and (ii) comparing the values obtained with sufficient precision by each technique. Ethanol is a molecule which intramolecular isotope distribution can be easily determined using both isotopic ^{13}C NMR and IRMS approaches [19,20]. Its simple structure and high range of natural intramolecular variation [10] makes it a good candidate to establish the trueness of the NMR approach by comparison with results obtained using the IRMS approach. Here we present the results of such a study, from a set of samples large enough to retrieve data statistically significant: 16 samples of ethanol from different origins are presented. The conversion of ethanol to acetic acid is a prerequisite to its analysis using IRMS. This reaction has been assessed in terms of isotopic fractionation and chemical yield, as its precision.

2. Materials and methods

2.1. Samples and chemicals

Eight ethanol samples (nos. 3, 4, 5, 6, 7, 8, 9 and 11 in Table 2) were provided by the Alcohol Enterprise Head Office, New Energy and Industrial Technology (NEDO), Chiba, Japan. Their purity was above 99% (v/v).

The other ethanol samples (nos. 1, 2, 10, 12, 13, 14, 15 and 16 in Table 2) were obtained either from the fermentation of fruit juices, the fermentation of pure sugars, or from wine or liquor samples. In all cases, ethanol was purified by distillation with sufficient yield ($>95\%$) to avoid any isotope fractionation [21].

Ethanol reagent (purity 99.5%) for evaluating the oxidation method to acetic acid was obtained from Junsei Chemical Company Limited (Tokyo, Japan). Its global $\delta^{13}C$ value was measured by Solid Phase Micro Extraction–Gas Chromatography–Combustion–IRMS (SPME–GC–C–IRMS) method developed in Ref. [22], and was $-14.15\pm0.11‰$. Acetic acid reagent used as a working standard was obtained from Wako Pure Chemical Industries. Chromium oxide 95% (w/w), and sulfuric acid 98% (v/v) were purchased from Wako Pure Chemical Industries Limited (Japan).

Hexadeuterated dimethylsulfoxide (DMSO-d_6) was purchased from Eurisotop (Saint-Aubin, France) and tris(2,4) pentadionato)chromium-III [$Cr(Acac)_3$] (97%) from Acros Organics (Courtaboeuf, France).

2.2. Determination of the molecular $\delta^{13}C$ value of ethanol samples by IRMS

The global ^{13}C abundance ($\delta^{13}C_g$) of the ethanol samples nos. 3, 4, 5, 6, 7, 8, 9 and 11 were determined using sealed tube combustion followed by cryogenic separation of the resultant CO_2 using a high vacuum line system and IRMS measurement. Briefly, 6 mL of ethanol was combusted in evacuated and sealed quartz tubes with 1.5 g of CuO (Merck KGaA, Darmstadt, Germany) as the oxidant at 850 °C for 2 h. The sample tube was attached to the line system with a glass tube cracker and cooled at −78 °C for 5 min. The sample tube was broken and the released CO_2 was transferred to a 6 mm i.d. Pyrex glass tube attached to the line system at −196 °C. The Pyrex glass tube was then sealed by flame. Measurements of the $\delta^{13}C$ for the CO_2 gases in the Pyrex glass tubes were made with an isotope ratio mass spectrometer (Finnigan MAT 252 IRMS; Finnigan MAT, Bremen, Germany) with a dual-inlet system. Isotopic standardization was accomplished through comparison with reference CO_2 gas (99.9999%; Taiyo Nippon Sanso Corp., Tokyo, Japan), which was calibrated against VPDB using the international carbon isotope standard IAEA-RM8563. The off-line IRMS method yielded a $\delta^{13}C$ value of $-10.57\pm0.07‰$ (1 SD, $n=3$) for IAEA-CH-6, showing consistency with the recommended value of $-10.45\pm0.07‰$ (1 SD).

The $\delta^{13}C_g$ values of samples nos. 1, 2, 10, 12, 13, 14, 15 and 16 were determined by encapsulation and measurement by EA–IRMS with an EA Flash HT coupled with a Delta-V Advantage spectrometer (ThermoFinnigan, Courtaboeuf, France) equipped with a Porapack Q column. About 1 μL of ethanol was sealed in a tin capsule and the $\delta^{13}C$ determined by reference to a working standard of glutamic acid standardized against calibrated international reference material (NBS-22, IAEA-CH-6 and IAEA-CH-7).

In all cases, the results are expressed relative to the international V-PDB reference: $\delta^{13}C=(R-R_{st})/R_{st}$ where $^{13}C/^{12}C$ for $R_{st}=0.0112372$ is the ratio of the Pee Dee reference.

2.3. Determination of the intramolecular $\delta^{13}C$ of ethanol using the SPME-GC-py-GC-C-IRMS system

The oxidation of ethanol was accomplished by treatment with a mixture of chromium oxide and sulfuric acid, called Jones' reagent.

The oxidation protocol was conducted in the following manner: in a flask, 1 g chromium oxide was added to 10 mL of a 20% (v/v) sulfuric acid solution. Then 0.2 mL of 10% ethanol solution in water (v/v) was added with a rate of 0.2 mL/min by use of a syringe pump (Harvard Pump 11 Plus; Harvard Apparatus, MA, USA). The reaction mixture was stirred for 20 min at 25 °C.

The degree of oxidation was confirmed by (i) determining the yields of acetic acid formed with ion chromatograph (IC), (ii) qualitative analysis of by-products with SPME–GC/MS, and (iii) determining the isotope ratio of produced acetic acid by SPME–GC–IRMS [3,23].

The IC (Dionex IC 3000; Dionex Corporation, CA, USA) was equipped with an electrical conductivity detector, Dionex Ionpac AG 20 as guard column and Dionex Ionpac AS20 as separate column. GC/MS (HP6890 and 5973; Agilent technology, CA, USA) was equipped with a capillary column (PoraBondQ, 25 m × 0.32 mm i.d., 5 μm film thickness; Varian, MA, USA), and an 85 μm SPME fiber coated with carboxen/polydimethylsiloxane (Carboxen/PDMS Stable-Flex; Supelco, PA, USA) was used. The system and the conditions for SPME–GC–MS measurements were similar as those described in Ref. [23].

The acetic acid produced by oxidation was then extracted using SPME and injected in a GC-pyrolysis-GC-C-IRMS (GC-py-GC-C-IRMS) system as described in Ref. [3]. The carboxyl carbon of acetic acid was the measurement target in this system to obtain the $\delta^{13}C$ of methylene carbon ($\delta^{13}C_{CH_2OH}$) of ethanol. This aim was achieved by the measurement of CO_2 gas produced by pyrolysis at 1000 °C. The $\delta^{13}C$ value of the methyl group ($\delta^{13}C_{CH_3}$) could be calculated from the $\delta^{13}C$ value of ethanol ($\delta^{13}C_{ethanol}$) and $\delta^{13}C_{CH_2OH}$ by isotopic mass balance.

The extraction procedure in the gas phase of the SPME vial was carried out by using a 85 μm SPME fiber coated with Carboxen/PDMS. The SPME conditions were as follows: extraction temperature, 30 °C and extraction time, 60 min. Using those conditions, the isotopic fractionation induced by the SPME procedure was calculated and corrected by measurement using an acetic acid standard.

2.4. NMR spectrometry

Sample preparation: In a 4 mL vial were mixed 600 μL ethanol and 100 μL of a 0.1 M solution of $Cr(Acac)_3$ in DMSO-d_6. The relaxation agent $Cr(Acac)_3$ can partly precipitate in the presence of water, therefore the mixture was left to stand at room temperature for at least 3 h before being filtered into a 5 mm o.d. tube.

Spectral acquisition: The spectral acquisition were made on a Bruker 400 Avance I spectrometer fitted with a 5 mm i.d. dual$^+$ probe $^{13}C/^{1}H$ carefully tuned at the recording frequency of 100.64 MHz.

The temperature of the probe was set at 30 °C. The offset for both ^{13}C and ^{1}H was set at the middle of the frequency range. Inverse-gated decoupling was applied in order to avoid Nuclear Overhauser Effect (NOE). The decoupling sequence employed a cosine adiabatic pulse with appropriate phase cycles, as described previously [12]. In all the cases, the acquisition parameters were adjusted in order to obtain a signal-to-noise ratio (SNR) ≈ 2500. For each sample, 5 spectra were recorded: one measurement is considered as the mean of the results obtain for five spectra.

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: Isotope $^{13}C/^{12}C$ ratios were calculated from processed spectra essentially as described previously [8]. Briefly, the positional isotopic distribution in ethanol molecule was obtained from the ^{13}C mole fractions f_i (where i stands for the C-atom position considered) as follows: $f_i = S_i/S_{tot}$, where S_i is the ^{13}C-signal (i.e., the area under the peak associated with the C-atom position i) and S_{tot} is the sum of the two ^{13}C-signals of ethanol. Each S_i had to be corrected to compensate for the slight loss of intensity caused by satellites (^{13}C–^{13}C interactions) by multiplying by $(1+n\times 0.011)$, where n is the number of carbon atoms directly attached to the C-atom position i ($n=1$ for ethanol) and 1.1% (= 0.011) is the average natural ^{13}C-abundance (see Tenailleau et al. [15] and Silvestre et al. [8] for a detailed explanation). If F_i denotes the statistical mole fraction (homogeneous ^{13}C-distribution) at any C-atom position i, then the site-specific relative deviation in the ^{13}C-abundance is $d_i = f_i/F_i - 1$. The values of d_i were converted to $\delta^{13}C$ (‰) using the isotope composition of the whole molecule ($\delta^{13}C_g$) obtained by IRMS. That is, the site-specific deviations were expressed as $\delta^{13}C_i$ for each C-atom position of the ethanol molecule [8].

3. Results and discussion

3.1. Rate of oxidation of ethanol to acetic acid.

The on-line pyrolysis approach for the determination of the intramolecular isotope composition in natural molecules has been recently developed for acetic acid [3]. However, ethanol pyrolysis does not show ideal fragmentation for its direct analysis using this approach. Therefore, ethanol was converted to acetic acid using chromium oxide (Jones' Reagent) which was further analyzed using the method of Hattori et al. [3].

The yield of acetic acid formed and its isotopic composition ($\delta^{13}C_g$) are shown in Table 1. The yield of the oxidation was always higher than 93% (mean value = 96.2%) and the mean of the isotopic difference between starting ethanol and formed acetic acid was within 0.35‰. Therefore we assume that the isotope fractionation associated with the conversion of ethanol to acetic acid, if any, must be negligible.

3.2. Precision of the two approaches: IRMS and NMR

The long term repeatability of the whole protocol (ethanol oxidation and subsequent analysis of the acetic acid formed using SPME-GC-P-GC-C-IRMS) can be expressed by standard deviation, which has been found to be lower than 0.3‰ ($n=3$) for the IRMS approach. Moreover, each sample analyzed in this study was analyzed 3 times and the standard deviation was within 0.4‰ for each of them. The standard deviation of the internal reproducibility of the intramolecular isotopic distribution of ethanol by isotopic ^{13}C NMR was already determined in previous works: 0.3‰ [20]. Furthermore, the standard deviation over 5 measurements for each sample analyzed in this study was within 0.3‰.

3.3. Comparison of the results obtained by IRMS and NMR approaches

16 samples of ethanol from several origins were analyzed in this study (Table 2). This panel of sample covers the full range of

Table 1
Yield and isotopic composition ($\delta^{13}C$, ‰) of acetic acid formed by the oxidation of the reference ethanol sample ($\delta^{13}C = -14.15 \pm 0.11$‰; $n=3$).

Sample no.	Yield	$\delta^{13}C_{acetic\ acid}$ (‰)
1	97.1 ± 1.8%[a]	−14.04 ± 0.63‰[a]
2	93.3 ± 1.8%[a]	−14.70 ± 0.08‰[a]
3	98.2 ± 1.1%[a]	−14.43 ± 0.52‰[a]
Average	**96.2 ± 2.6%**	**−14.39 ± 0.33‰**

[a] $n=3$.

Table 2
Origin and bulk isotopic composition $\delta^{13}C$ of ethanol samples used in this study.

Sample no.	$\delta^{13}C_{ethanol}$ (‰)	Origin
1	−10.24	Maize
2	−9.79	Maize
3	−13.95	Maize
4	−10.44	Maize
5	−11.16	Maize
6	−12.33	Cane
7	−11.43	Cane
8	−12.27	Cane
9	−12.26	Cane
10	−12.41	Cane
11	−27.76	Manioc
12	−25.47	Gin
13	−26.18	Beet
14	−27.79	Beet
15	−12.29	Tequila
16	−29.51	Synthetic

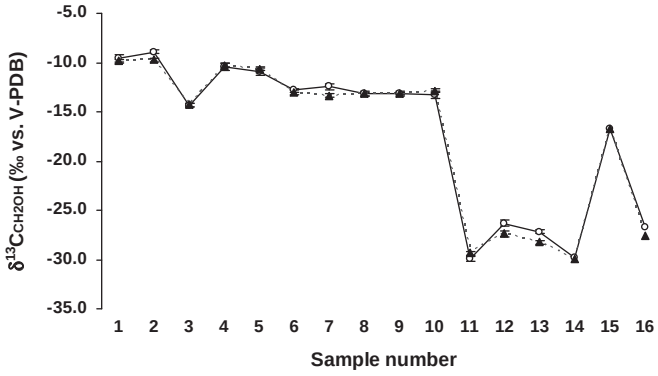

Fig. 1. Isotopic composition of the methylene site $\delta^{13}C_{CH_2OH}$ of different ethanol samples measured using IRMS (white circles; plain line) and isotopic ^{13}C NMR (black triangles; dashed line).

variation at the intramolecular level found in ethanol. The origin is the declared origin and therefore may not be ascertained: for example, sample 3 shows a rather low $\delta^{13}C_g$ value for a maize origin [6]. Such consideration has no effect on the present study and on the following discussion. The isotopic composition of the methylene site ($\delta^{13}C_{CH_2OH}$) obtained by both isotopic NMR and IRMS are depicted in Fig. 1.

As evidenced from Fig. 1, the isotope composition at the intramolecular level obtained by NMR is similar to that obtained using IRMS. The mean difference in the $\delta^{13}C_{CH_2OH}$ obtained by IRMS and NMR within 16 samples was 0.24 ± 0.54‰ (1 SD). Similar results are obtained for $\delta^{13}C_{CH_3}$ because of the symmetrical calculation. In fact, $\delta^{13}C_{CH_2OH}$ and $\delta^{13}C_{CH_3}$ are not from the same nature when using IRMS and ^{13}C NMR: for the former $\delta^{13}C_{CH_3}$ results from a subtraction between $\delta^{13}C_g$ (IRMS) and $\delta^{13}C_{CH_2OH}$ (py-IRMS) and for the latter $\delta^{13}C_{CH_3}$ is calculated as $\delta^{13}C_{CH_2OH}$ i.e. from the relative area of each NMR signal and then compared to the $\delta^{13}C$ scale by using $\delta^{13}C_g$ (IRMS). Obviously, when dealing with two peaks, this effect is minored. Interestingly, no correlation was found between the difference on one sample and its $\delta^{13}C_g$, indicating that the differences observed were not due to a systematic error but rather due to the random errors induced by each technique.

The data obtained from the correlation between the $\delta^{13}C_{CH_2OH}$ and $\delta^{13}C_{CH_3}$ values determined using NMR and IRMS were the following:

$$\delta^{13}C_{CH_2OH}(NMR) = 0.976(0.037)(IRMS) - 0.138(0.658)\,(R^2 = 0.996) \quad (1)$$

$$\delta^{13}C_{CH_3}(NMR) = 1.014(0.039)(IRMS) + 0.008(0.731)\,(R^2 = 0.996) \quad (2)$$

where numbers in bracket are uncertainties at the 95% confidence level, assuming that they include the main contribution to the total uncertainty.

The correlation coefficient is near unity supporting the consistency of the results obtained by the two techniques. Furthermore, for both carbon sites, the slope is not significantly different from unity and the y-intercept not different from zero, considering the 95% confidence level.

4. Conclusion

The intramolecular isotope distribution has been determined for 16 ethanol samples from various origins using IRMS and NMR approaches. The statistics are therefore robust and the conclusions may be ascertained. Clearly, the $\delta^{13}C_i$ values given by NMR are highly consistent with those obtained using IRMS. Besides isotopic analyses of ethanol samples, the present work should also give satisfactory results for other NMR applications where IRMS would be less efficient.

Firstly, a calibration of the $\delta^{13}C_i$ NMR values may be envisaged with the respect to the absolute international δ-scale on the basis of primary standards (such as ethanol) measured both by IRMS and NMR.

Secondly, the instrumental NMR response may be slightly different from a spectrometer to another. In this case, an intercalibration *via* the IRMS measurements would harmonize the results.

Thirdly, there are several approaches to improve the sensitivity of NMR, such as the use of cryo-probe and/or the application of polarization transfer techniques [7,24]. The resulting data may be distorted and, again, the above calibration may correct the raw data into exploitable data on the absolute δ-scale.

Work is currently in progress to assess the trueness of isotopic ^{13}C NMR measurements over a wide range of molecules and using several spectrometers. The consistency between NMR and IRMS observed in the present work is very promising for the harmony and the complementarity of both techniques.

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