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Site-specific ^{13}C content by quantitative isotopic ^{13}C Nuclear Magnetic Resonance spectrometry: A pilot inter-laboratory study

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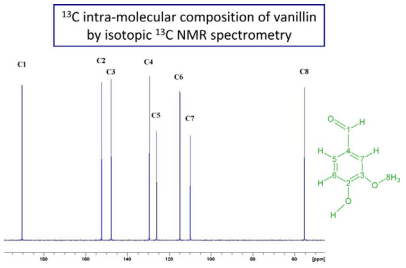
HIGHLIGHTS

- First ring test on isotopic ^{13}C NMR spectrometry.
- Evaluation of the intra- and inter-variability of the NMR spectrometers used.
- Definition of a protocol for qualification of the performance of the spectrometer.

GRAPHICAL ABSTRACT



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ABSTRACT

Isotopic ^{13}C NMR spectrometry, which is able to measure intra-molecular ^{13}C composition, is of emerging demand because of the new information provided by the ^{13}C site-specific content of a given molecule. A systematic evaluation of instrumental behaviour is of importance to envisage isotopic ^{13}C NMR as a routine tool. This paper describes the first collaborative study of intra-molecular ^{13}C composition by NMR. The main goals of the ring test were to establish intra- and inter-variability of the spectrometer response. Eight instruments with different configuration were retained for the exercise on the basis of a qualification test. Reproducibility at the natural abundance of isotopic ^{13}C NMR was then assessed on vanillin from three different origins associated with specific $\delta\ ^{13}C_i$ profiles. The standard deviation was, on average, between 0.9 and 1.2‰ for intra-variability. The highest standard deviation for inter-variability was 2.1‰. This is significantly higher than the internal precision but could be considered good in respect of a first ring test on a new analytical method. The standard deviation of $\delta\ ^{13}C_i$ in vanillin was not homogeneous over the eight carbons, with no trend either for the carbon position or for the configuration of the spectrometer. However, since the repeatability for each instrument was satisfactory, correction factors for each carbon in vanillin could be calculated to harmonize the results.

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Table 1
Symbols used in this paper.

Symbol	Meaning
$\delta\,^{13}C$	Carbon isotope composition: carbon isotopic ratio of the molecule relative to the international standard (Vienna Pee Dee Belemnite V-PDB)
$\delta\,^{13}C_g$	^{13}C mean isotopic composition of a whole molecule measured by IRMS
$\delta\,^{13}C_i$	^{13}C isotopic composition of the carbon position i measured by ^{13}C NMR
f_i	Molar fraction for a carbon site i measured by ^{13}C NMR = area of the peak corresponding to the carbon position i (S_i) divided by the sum of all the carbon sites of the molecule ($f_i = S_i/\sum_n S_i$)
F_i	Statistical molar fraction for a carbon site i: molar fraction for the carbon site i in cases of homogeneous ^{13}C distribution within the molecule (F_i = 1/8 for vanillin)

1. Introduction

Determination of isotope ratios is a tool of interest for a wide range of scientific research domains, such as archaeology, biology, climatology, ecology, environmental studies, and forensics. More particularly, the $^{13}C/^{12}C$ ratio at natural abundance is well recognized as a probe in plant physiology, geochemistry and authentication. For tens of years, mass spectrometry (isotope ratio mass spectrometry [IRMS]) has been used either (i) through combustion of a pure molecule via an elemental analyser (EA-IRMS) or (ii) on mixtures via hyphenated techniques coupled to gas chromatography (GC–IRMS) and liquid chromatography (LC–IRMS). Irrespective of the instrumental configuration, only a global ^{13}C value ($\delta\,^{13}C_g$) is obtained, i.e. the average ^{13}C contribution of all carbons of the compound under study (see Table 1 for a list of symbols used in the paper). Thus, despite the high accuracy (herein the word accuracy is defined as the contribution of trueness + precision, as recommended in ref [1–3]), IRMS does not allow access to the intra-molecular ^{13}C composition of the analyte. As a result, important information on isotope fractionation is lost. Until now there have been two main methodologies for measuring site-specific ^{13}C content. One is based on the degradation of the molecule into fragments prior to their analysis by IRMS [4]. It is a tedious approach when the monitoring of chemical and biochemical degradations must be combined, prohibiting its routine use. An appealing proposal is the use of on-line pyrolysis GC–IRMS, but this is limited to small molecules such as acetic acid [5], lactic acid [6] or ethanol [7]. On the other hand, quantitative NMR offers the possibility to determine intra-molecular site-specific $\delta\,^{13}C_i$ values at natural abundance. It directly analyses the target molecule without the need for prior chemical degradation. Site-specific natural isotopic fractionation NMR (SNIF-NMR) was developed for δ^2H determination in the 1980s and is now routinely used for metabolic and climatic analyses and as a tool in authentication [8]. However, the application of its ^{13}C analogue was made possible only recently because of the high level of precision required. Indeed, isotopic variation in natural compounds ranges from ~50‰ to ~500‰ for ^{13}C and ^{2}H, respectively, on the δ scale. Hence, isotopic ^{13}C NMR requires 10 times higher precision.

The recent development of isotopic ^{13}C NMR by means of the following steps, with an accuracy of 1‰ as a working analytical target, which is essential to detect isotope fractionation site by site: (i) mandatory efficient proton decoupling of ^{13}C–^{1}H interactions [9]; (ii) application of a protocol for operational qualification of the NMR spectrometer, including the impact of the relaxation agent (as Tris(2,4-pentadionato)chromium-III, $Cr(Acac)_3$) on the precision [10]; (iii) reduction of analysis duration by using either relaxation reagents [10] or polarization transfer pulse sequences [11,12]; (iv) verification that, in each case, the precision, expressed as long-term repeatability, is <1‰ [13]; and (v) assessment of the trueness by comparison between NMR and IRMS with ethanol as a molecular probe [7]. Each step has been successfully achieved, making it possible to apply isotopic ^{13}C NMR to a wide range of applications and providing unexpected new information with respect to ^{13}C fractionation and isotope effects (for examples, see ref [14–17]). It is thus clear that isotopic ^{13}C NMR should be an important tool for the scientific community, although to promote the technique and before its dissemination, it should be evaluated in terms of reproducibility and ease of use.

The main objective of the present work was to evaluate the method through a first collaborative inter-spectrometer variability study rather than an inter-laboratory reproducibility study. It was noted during the application of isotopic ^{13}C NMR that the configuration of the spectrometer—the combination of transmitter chain, receiver chain and probe—could be of influence on the final results. In order to limit the number of parameters of influence, it was decided to use only one manufacturer and one magnetic field strength. The data presented hereafter result from the experiments performed on eight qualified NMR spectrometers (Table 2).

Table 2
List of the spectrometers working at 9.4 T.

Spectrometer code[a]	Series level	Probe
A	Avance III	BBFO
B	Avance II	BBFO
C	Avance III	BBFO+
D	Avance I	$^{1}H/^{13}C$ dual+
E	Avance III	BBO
F	Avance III	BBFO smart probe
G	Avance III	BBFO
H	Avance III	BBFO
I	DRX	BBFO+

[a] These nine instruments and configurations were those available in the seven laboratories of the co-authors of this work.

2. Experimental

2.1. Chemicals

NMR sealed tubes containing bi-labelled ethanol were filled with a stock solution of [1,2-$^{13}C_2$] ethanol in such a way that the following composition was found in a 5 mm NMR tube: 0.6 mL of bi-labelled ^{13}C–^{13}C ethanol (99%) from Eurisotop (Saint-Aubin, France), diluted with 0.2 mL of H_2O and 0.2 mL of acetone-d_6 from Eurisotop for the lock signal. The vanillin samples were from three commercial origins: (i) ex-beans (Citrus & Allied, Lake Success, NY, USA); (ii) ex-guaiacol (Rhodia, Courbevoie, France); and (iii) ex-lignin (Booregaard, Sarpsborg, Norway). These origins are not certified and the measured $\delta\,^{13}C_i$ cannot be considered as representative of the label. Tris(2,4-pentadionato)chromium-III, $Cr(Acac)_3$, used by each participant was supplied locally.

2.2. Protocol for vanillin NMR tube preparation

$Cr(Acac)_3$ solution in acetone: A 0.1 M solution of $Cr(Acac)_3$ in 1 mL of acetone was prepared as follows: 34.9 mg of $Cr(Acac)_3$ was weighed in a pillbox of 4 mL and added to 1 mL of acetone using a glass pipette. Vanillin sample preparation: Approximately 250 mg of vanillin was weighed in a pillbox of about 4 mL. Then 400 μL of acetone-d_6 and 100 μL of $Cr(Acac)_3$ solution (0.1 M) previously prepared in acetone were added successively, these volumes having been taken using a Hamilton glass syringe. In each case, the volumes must be accurate to ensure that the T_1 values are <1.6 s (Table 3). The resulting solution was thoroughly mixed. The Pasteur pipette used during filtering (cotton wool, paper or equivalent) was placed in an oven for a few minutes before filtration to avoid crystallization

Table 3
Longitudinal relaxation times (T_1) of each carbon in vanillin using the preparation protocol described in Section 2.2.

Carbon[a]	Chemical shift (ppm)	T_1 (s)[b]
C1	190.4	1.1
C2	152.4	1.3
C3	147.8	1.5
C4	129.5	1.6
C5	126.1	0.9
C6	114.9	0.9
C7	109.9	1.0
C8	55.3	0.9

[a] See Fig. 1 for carbon numbering.
[b] Measured by the classical inversion-recovery technique.

of vanillin in the pipette. The filtration was conducted carefully to remove any remaining particles of non-dissolved $Cr(Acac)_3$. Under these conditions, the height of the sample in a 5 mm tube was about 4.2 cm.

2.3. Protocol for NMR spectrometer adjustments and qualification

Quantitative ^{13}C NMR spectra were recorded using Bruker 400 NMR spectrometers fitted with 5 mm i.d. probes (see the exact configuration for each spectrometer in Table 2), tuned at a recording frequency of 100.6 MHz. Since it had been decided that only one manufacturer would be used, the parameters used and listed in Section 2 correspond to the symbols and abbreviations of Bruker.

2.3.1. Hardware configuration and spectrometer adjustments

Experiments must be performed on a probe that includes a ^{13}C observe channel, a ^{1}H decoupler channel and a ^{2}H lock channel. The use of inverse probes must be avoided. The temperature of the sample was set at 303 K. Probe tuning and matching were performed carefully. When automatic tuning and matching is used, it must be performed using the more stringent criteria (first on the ^{13}C and then on the ^{1}H channel), followed by manual verification to check the obtained result. The pulse calibrations for both the observation channel (^{13}C) and for decoupling (^{1}H) were then carefully carried out. Focus should be on the determination of the decoupling attenuation to achieve an impulsion of 14.1 μs with the corresponding power level (parameter PL12). The pulse calibration was performed on a tube containing 10% $CHCl_3$ in acetone-d_6 and using the usual method: a $\pi/2$ ^{13}C hard pulse followed by a delay equal to 1/2 J (with J = 212 Hz) and a 14.1 μs ^{1}H hard pulse just before ^{13}C data acquisition. The ^{1}H power level was adjusted to suppress the ^{13}C doublet. Care should be taken that this parameter does not exceed the safety limit of the probe.

2.3.2. Instrumental qualification

The ability of the instrument to take measurements is assessed by using [1,2-$^{13}C_2$] ethanol (see Section 3.3) in a sealed 5 mm NMR tube. A series of acquisitions were recorded with a systematic variation of the decoupling offset (O2 parameter): O2 (in Hz) ranges from $\{½[\nu_{1H}(CH_3)+\nu_{1H}(CH_2)]-3000\}$ to $\{½[\nu_{1H}(CH_3)+\nu_{1H}(CH_2)]+3000\}$ with an increment of 500 Hz (13 values). Five independent spectra were recorded for each O2 value and then Δ_{mean} (see Section 3.3 for a definition) could be calculated.

2.3.3. Spectral acquisition conditions for vanillin analyses

The temperature of the probe was set at 303 K. The offsets for both ^{13}C and ^{1}H were set at the middle of the frequency range for each molecule. Inverse-gated decoupling was applied to avoid the nuclear Overhauser effect, and the repetition delay between each 90° pulse was set at $10 \times T_{1max}$ of the molecule under investigation to achieve quantitative relaxation of the magnetization. The typical T_1 ^{13}C values measured using the sample preparation described in Section 2.2 are displayed in Table 3. The decoupling sequence used adiabatic full-passage RF (radio-frequency) pulses with cosine square amplitude modulation (ν_2^{max} = 17.6 kHz) and offset independent adiabaticity [18] with optimized frequency sweep according to [19]. The acquisition conditions were as follows: acquisition time 1 s, repetition delay of 20 s, number of scans adjusted to reach a signal-to-noise ratio (SNR) of 700. Each measurement was made from the average of three to five independent NMR records.

2.3.4. NMR data processing

To avoid variability associated with the NMR process, the free induction decays from both experiments, [1,2-$^{13}C_2$] ethanol and vanillin samples, were sent to one place (CEISAM, Nantes, France). Data were then processed using the same parameters and the same procedure for all free induction decays: (i) an exponential multiplication inducing a line broadening of 2 Hz, (ii) manual phasing by the same operator, and (iii) automatic baseline correction, using a polynomial function (order 3). The curve fitting was carried out in accordance with a Lorentzian mathematical model using Perch Software (Perch NMR Software™, Perch Solutions Ltd., Kuopio, Finland).

2.3.5. Isotopic data

Isotope $^{13}C/^{12}C$ ratios for each carbon of vanillin were calculated from processed spectra with the method described in [20]. Briefly, the positional isotopic distribution in a molecule was obtained from ^{13}C mole fraction 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 in position i) and S_{tot} is the sum of all ^{13}C-signals of the molecule. 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 and 1.1% (=0.011) is the average natural ^{13}C-abundance (see [20] 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 d_i values were converted to $\delta^{13}C$ values using the isotope composition of the whole molecule obtained by EA-IRMS.

2.3.6. EA-IRMS

The ^{13}C abundance of the whole molecule, designated (δ $^{13}C_g$), was determined by IRMS. The sample (ca. 0.8 mg) was sealed in a tin capsule and introduced into an EA Flash HT (ThermoFinnigan, Courtaboeuf, France) equipped with a Porapack Q column to separate CO_2 and N_2. The dry CO_2 was swept on-line into a Delta-V Advantage spectrometer (ThermoFinnigan) and the δ ^{13}C determined by reference to a working standard of glutamic acid standardized against calibrated international reference material (NBS-22, IAEA-CH-6, IAEA-CH-7). Thus:

$$\delta^{13}C(‰) = \left(\frac{R}{R_{std}} - 1\right) \times 1000$$

where R is the $^{13}C/^{12}C$ isotope ratio of the sample and R_{std} is the $^{13}C/^{12}C$ isotope ratio of Vienna Pee Dee Belemnite reference standard (V-PDB) (R_{std} = 0.0112372).

3. Results and discussion

3.1. Experimental design and participants

The internal reproducibility of the isotopic ^{13}C NMR has already been established for ethanol and vanillin as molecular probes measured on spectrometer D [13]. In these previous works, it was

Table 4
Interests in using vanillin for testing the variability of the NMR spectrometer response.

Characteristic	Description	Source of variability
Sufficient signal-to-noise ratio (SNR)	High number of scans necessary	Instability of the whole spectrometer due to long analysis time
Large ^{1}H frequency range	From 3 ppm to 11 ppm	Efficiency of the ^{1}H decoupling over that range
Large ^{13}C frequency range	From 50 ppm to 200 ppm	Off-resonance effect due to an inappropriate pulse width
Quaternary carbons	Three sp^2 carbons, with long T_1, compared to the ^{1}Hs-bearing carbons	Effect of the relaxation reagent
Several origins	Three main commercial origins with specific ^{13}C profile	Capability to discriminate origins and to detect small ^{13}C content variation

clearly shown that a precision of 1‰ for $\delta\,^{13}C_i$ could not be reached unless an efficient and homogenous ^{1}H decoupling was set up: the optimum, so far, is found when using adiabatic pulse sequences [19]. Since this efficiency is directly linked to the ^{1}H frequency range to be decoupled, we decided to exclude 500 MHz NMR spectrometers because of their larger ^{1}H range that is more difficult to decouple. As a result, the experimental protocol was limited to 9.4 T spectrometers (400 MHz, ^{1}H and 100.6 MHz, ^{13}C).

Within the framework of a first ring test on isotopic ^{13}C NMR, we thought that the sources of variability could be better delineated if only one type of NMR spectrometer was used from one manufacturer, i.e. Bruker. Accordingly, participants were contacted and three questions were asked regarding (i) their interest in isotopic ^{13}C NMR, (ii) the availability of a 400 MHz Bruker spectrometer and (iii) the technical possibility to implement the adiabatic pulse sequence for ^{1}H decoupling. Nine spectrometers were aligned for the present exercise from seven laboratories (see Table 2). A protocol was sent to each participant, as well as a sealed tube containing [1,2-$^{13}C_2$] ethanol for instrumental qualification purposes and one sample of vanillin from each of the three origins (see Sections 2 and 3.2) for site-specific measurement at natural abundance.

3.2. Nature of the samples used

The application of isotopic ^{13}C NMR has successfully been applied to ethanol [21]. In this context, a comparison was achieved between spectrometers, especially from CEISAM (Nantes, France) and JRC (Ispra, Italy). It was satisfactory concerning intra- and inter-spectrometer accuracy, but used an instrumental configuration other than spectrometer I. Ethanol has characteristics that promote its isotopic ^{13}C NMR analysis: (i) a very sensitive small molecule as a liquid, and so only short durations of NMR analysis are necessary, i.e. SNR > 1500 in <25 min; (ii) restrictive ^{1}H and ^{13}C frequency ranges offering efficiency for the transmitter and receiver chains; and (iii) homogenous relaxation times, T_1, i.e. no quaternary carbon with a very long T_1. For the present ring test exercise, vanillin was chosen for its opposite capabilities: (i) a solid with a finite solubility in organic solvents; (ii) a broad ^{1}H frequency (from 3 to 11 ppm) requiring the most efficient decoupling; (iii) a broad ^{13}C frequency range; and (iv) a large variety of T_1 because of the presence of several types of carbon, from quaternary to primary C-substituted. The corresponding sources of variability which could be revealed by the ring test are summarized in Table 4. Furthermore, vanillin can be obtained from several origins with a specific isotope profile. The commercial origins used in the present work are (i) ex-bean, from an extract; (ii) ex-lignin from a semi-synthetic origin when lignin comes from wood; and (iii) ex-guaiacol from a synthetic origin using guaiacol as starting material. Subtle differences in the ^{13}C content for each carbon in vanillin may be observed for a given origin and between origins; therefore, one of the main goals of the ring test was to verify the capability of each spectrometer to show such typical ^{13}C profiles. No guarantee can be given the origins; therefore, each $\delta\,^{13}C_i$ measured in this study should not be considered as representative.

3.3. Results of NMR spectrometer qualification

The capability of the NMR spectrometer to ensure correct ^{1}H decoupling can be assessed by using bi-labelled [1,2-$^{13}C_2$] ethanol as a molecular probe and therefore can be envisaged as the performance qualification of the instrument. The principle of such an approach has been presented previously [10]. Only the main points are summarized herein. The ^{13}C NMR spectrum of the sealed tube recorded under quantitative conditions (inverse gated ^{1}H decoupling and repetition time of $10 \times T_{1\max}$ to avoid partial saturation and build-up of the nuclear Overhauser effect) shows four peaks: a doublet for $^{13}C_{CH_2}$ and for $^{13}C_{CH_3}$ resonances due to carbon-carbon scalar coupling. Therefore, for the isotopologue constituting two ^{13}C atoms, the area of both doublets should be strictly identical, independent of the level of global enrichment. In other words, the ratio of the integral of the "CH_2" doublet over the integral of the "CH_3" doublet should be equal to 1.000. Since the final $\delta\,^{13}C_i$ values are calculated from the ratio of f_i/F_i (see Section 2 and Table 1 for a definition), the ratio between the two doublets is more conveniently described by Δ set in the general expression in ‰: $\Delta = ((f_i/F_i) - 1) \times 1000$, which can be expressed for the CH_2 site as $\Delta = ((f_{CH2}/0.5) - 1) \times 1000$. A perfect accuracy observed on the [1,2-$^{13}C_2$] ethanol spectrum should give Δ = 0‰. Any shift from this value would indicate a lack of accuracy in the configuration used, and this as low as a 1‰ level. It is an efficient and rapid means to assess the performance of the decoupling over the full range of the ^{1}H frequency range, keeping in mind that other sources of variability may also contribute to the Δ value. The experimental scheme is designed to measure Δ with respect to the frequency off-set variation for decoupling around its optimum value, i.e. the

Table 5
Δ_{mean} value for each spectrometer calculated from instrumental qualification (Section 3.3).

	Spectrometer								
	A	B	C	D	E	F	G	H	I
Δ_{mean} (‰)[a]	0.7	−1.1	−0.3	0.5	0.8	0.0	0.9	0.5	−4.2
SD[b]	0.3	0.2	0.2	0.1	0.2	0.3	0.1	0.7	0.2
Confidence interval [c]	0.7	0.5	0.5	0.2	0.5	0.6	0.3	1.4	0.4

[a] $((f_{CH_2}/0.5) - 1) \times 1000$; see Section 3.3.
[b] Standard deviation.
[c] Calculated from the standard deviation and using a degree of freedom of 12 (13 values) at a 95% confidence level and a t-value = 2.1788.

Table 6
Intra-variability of each spectrometer expressed as the mean value of the standard deviation[a], calculated from the mean of the variance over the three origins of vanillin, observed during five replicates of a given sample.

	C1	C2	C3	C4	C5	C6	C7	C8	Mean of the mean[b]
A	1.2	1.3	1.0	0.6	1.3	0.8	1.5	0.5	1.1
B	0.8	1.0	0.6	0.7	1.1	0.8	1.4	0.7	0.9
C	0.9	0.8	0.8	1.0	1.2	0.9	0.9	1.1	1.0
D	0.7	0.9	0.8	0.9	1.3	1.3	0.9	0.7	1.0
E	1.0	1.1	0.7	1.1	1.3	1.0	1.2	0.8	1.0
F	0.7	0.9	1.1	1.0	1.0	1.0	1.3	0.6	1.0
G	1.0	1.1	0.6	1.2	1.3	1.4	1.0	1.2	1.1
H	1.1	1.0	1.7	1.7	1.2	1.1	0.7	1.1	1.2

[a] Mean of SD = $((\Sigma(SD)^2)/3)^{1/2}$.
[b] Mean of the mean of SD = $((\Sigma(SD)^2)/8)^{1/2}$.

Table 7
Inter-variability between the eight spectrometers as the mean value and the standard deviation of $\delta\,^{13}C_i$ for each isotopic profile of vanillin.

Vanillin origin	$\delta\,^{13}C_i$[a]	C1	C2	C3	C4	C5	C6	C7	C8
Guaiacol	Mean	−17.8	−31.3	−31.9	−26.4	−24.6	−25.3	−23.7	−53.3
	SD[b]	2.1	1.9	1.9	1.2	0.9	0.7	0.9	1.8
Bean	Mean	−18.0	−20.4	−21.1	−17.1	−24.7	−19.6	−22.5	−19.8
	SD	2.0	1.6	1.4	0.8	0.8	0.9	0.6	1.7
Lignin	Mean	−33.0	−27.8	−27.5	−24.7	−32.0	−26.4	−29.6	−24.8
	SD	1.6	1.5	1.7	1.0	0.8	1.1	1.0	2.1
	Mean of SD[c]	1.9	1.7	1.7	1.0	0.8	0.9	0.9	1.9

[a] In ‰, calculated according to Section 2.3.5.
[b] Standard deviation calculated from the eight spectrometers.
[c] Mean of the standard deviation of each vanillin sample, via variance from $((\Sigma(SD)^2)/3)^{1/2}$.

middle of the ^1H spectrum. This step has been assigned as a selection level for qualifying the instrument: if Δ is greater than ±1‰, the spectrometer is not used for the vanillin measurements at natural abundance.

Table 5 shows the mean value of Δ for 13 measurements covering the full range of proton resonances. Spectrometer C is within the acceptance criteria even if the decoupling power could not be set at the optimum value because of the safety limits imposed by the manufacturer for the given configuration. Δ for spectrometer I is −4.2‰ and, a priori, does not match the target value of 1‰. Instrument I consisted of a new probe (BBFO+) and the "oldest" NMR electronic console (DRX) tested in this work, which does not allow for easy control and setting of the actual decoupling power delivered to the probe. This may explain the higher Δ value observed for this instrument. It was therefore decided that this instrument configuration would not be included for the successive determinations on vanillin. It should be stressed, however, that spectrometer I showed a satisfactory span between the lowest and highest Δ values over the full range of decoupling offset, leading to a confidence interval lower than 1‰: this should be a good indicator to assess precision. In this respect, spectrometer H had the highest confidence interval at 1.4‰. The consequence of such findings will be further discussed in Section 3.4 in relation to the analysis of vanillin.

3.4. Intra- and inter-spectrometer variability

The repeatability of measurement for each spectrometer is expressed through (i) the precision of the spectrometer established from repeated analysis on the same tube and (ii) the dispersion of the results, including the preparation step of the NMR tube, observed during the analysis of the vanillin samples from the three origins. The analytical protocol required five independent spectra to be recorded for one analysis; the variability between the results is thus indicated by the standard deviation. Table 6 shows, across the three origins of vanillin, the mean of the mean value from the five spectra, of the standard deviation for each spectrometer, and for each carbon site of vanillin, calculated using the variances (see Fig. 1 for carbon numbering). Some heterogeneity between the carbons is observed, but there is no trend either for the chemical nature of the carbon or for the spectrometer. This variability is summarized by the average value of the mean standard deviation (calculated from the variances), which ranges from 0.9 to 1.2‰ for a given spectrometer (Table 6). Interestingly, these values are expected when considering the description of the dependence of the relative error on the NMR signal $\Delta S/S$ with the signal-to-noise ratio SNR: $\Delta S/S = 1/(2.SNR)$. Thus an instrumental precision of ∼1‰ is expected for SNR ≈ 500. The protocol used for the ring test imposed an SNR of ∼700.

The inter-spectrometer variability expresses the potential reproducibility of the isotopic ^{13}C NMR spectrometry. Such a parameter is simply described by the standard deviation from the mean value of the $\delta\,^{13}C_i$ measured by each spectrometer on vanillin samples, as shown in Table 7. The consistency of results between each vanillin origin is further confirmation of the good instrumental repeatability. As observed above, there was no homogenous behaviour for each carbon site. At the present stage of the study,

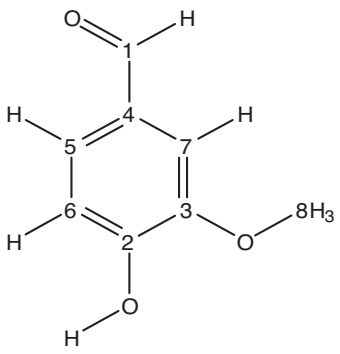

Fig. 1. Molecular structure of vanillin with carbon atoms numbered as a decreasing ^{13}C chemical shift.

there is no explanation for such discrepancies. Although the highest standard deviation is 1.9‰ (mean value calculated from the variance, Table 7), this is not a bad result, considering that it is a first test and most of the participants do not perform such analyses regularly. The discrimination power of vanillin origin using isotopic ^{13}C NMR remains for a given spectrometer. It is particularly notable that spectrometers A and B from the industrial partner (Firmenich) are well positioned in the ring test, indicating that isotopic ^{13}C NMR could be transferred from research laboratories to industrial platforms for routine applications without difficulty.

3.5. Correction factors

A proficiency test usually aims to determine a consensual value of a given mesurand. In the present round-robin test, the absolute value of $\delta^{13}C_i$ remains unknown. The trueness of the site-specific ^{13}C content could be reached by comparison to methods traceable from international standards. A previous study has shown that NMR and IRMS could be compared and inter-calibrated using ethanol as a molecular probe [7]. Spectrometer D was used for this study: if we assume that spectrometer D is traceable on the δ-international scale via IRMS, it is reasonable to use it as a reference for the other spectrometers through correction factors. Two prerequisite conditions should be fulfilled: (i) suitable precision for each spectrometer, ca. $\leq$1‰, and (ii) no global correction but a correction factor for each carbon of vanillin ((see Fig. 1 for carbon numbering). We have seen that the intra-variability of each spectrometer is equivalent to and lower than 1‰; thus, we can calculate a correction factor for each carbon in vanillin: $\delta\ ^{13}C_{i,cor} = K_{i,spec}.\ \delta\ ^{13}C_{i,D}$, where $K_{i,spec}$ is the correction factor for a given spectrometer obtained from $K_{i,spec} = <\delta\ ^{13}C_{i,spec}/\delta\ ^{13}C_{i,D}>$ (average value collected from the three origins of vanillin). It is clear that $K_{i,spec}$ obtained in this work (data not shown) is not the definitive value because the number of samples is not large enough and a full study of robustness should be performed. It is a first step for the homogenization of the results towards the absolute $\delta\ ^{13}C_i$ values.

4. Conclusions

Isotopic ^{13}C NMR spectrometry is a recent method for measuring site-specific ^{13}C content, which is at its initial stages of application. It is therefore of fundamental importance to systematically evaluate instrumental behaviour for future routine use. The aim of the present collaborative study was to evaluate the intra- and inter-variability of the spectrometers. Two aspects differed from conventional ring tests: (i) the trueness of $\delta\ ^{13}C_i$ was not the primary objective and (ii) there was no laboratory ranking. Instead, the present work focused on the internal consistency of measurement following a common protocol and its application to vanillin samples. The protocol implied a performance qualification step and adjustments of the spectrometer, so that even a participant who was inexperienced in isotopic measurement could follow the experimental scheme. It was shown previously that repeatable results depend primarily on the efficiency of ^{1}H decoupling. Accordingly, a test was designed to evaluate the homogeneity and the robustness of the optimum decoupling pulse sequence for each spectrometer. On this basis, one instrument was rejected, leaving the other eight for the round-robin test. Instrument I was the "oldest" NMR electronic console tested in this study; it did not allow for easy control of the decoupling power, as discussed earlier.

The precision of each spectrometer was evaluated as the standard deviation of repeatability, including the NMR tube preparation step. Heterogeneity was observed within the considered carbons, but on average the precision of each spectrometer was between 0.9 and 1.2‰, in full agreement with the expected value in relation to the SNR used (~700).

The mean standard deviation corresponding to the inter-spectrometer response was not the same for each carbon, but with no chemical or physical trend: it varied from 0.6 to 2.1‰. These values are higher than the repeatability (1‰) for some carbons, but remain acceptable, especially if the purpose of the analysis is to separate or discriminate between origins of vanillin samples: the three origins of vanillin are well distinguished regardless of the spectrometer used. Since the repeatability was within the target value for a given spectrometer, correction factors could be calculated using one spectrometer as a reference, ensuring a link to absolute $\delta\ ^{13}C_i$.

The results of this first round-robin test are very encouraging for a conceivably routine application of isotopic ^{13}C NMR. Some applications of the method have been already published (see references cited in the Section 1), even on the geographical origin classification [22] or on the isotope profiling [23]. Although, it is clear, from the results obtained in the present paper, that more applications will be published later. A larger ring test designed as a proficiency test could be proposed with a larger number of spectrometers (including other manufacturers) and with a larger number of samples from several origins (structure and isotopic profile).

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