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Authentication of the Origin of Vanillin Using Quantitative Natural Abundance ^{13}C NMR

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The use of ^{13}C isotopic distribution as an efficient means to determine the origin of vanillin has been substantiated. Using quantitative ^{13}C NMR, the $^{13}C/^{12}C$ ratios at all eight carbon positions can be exploited. On a set of 21 samples of vanillin from five different origins, complete discrimination can be achieved. It is shown that, for many purposes, a rapid analysis in which only five sites are used is sufficient. However, improved discrimination using all eight sites is preferable to differentiate between different methods of production from natural ferulic acid or between natural and lignin-derived vanillin on the basis of the $^{13}C/^{12}C$ ratios characteristic of different origins. The C1 and C8 positions are demonstrated to be the most significant sites for discrimination using principle component analysis. However, aromatic carbon positions make an essential contribution, notably in differentiating between natural and lignin-derived vanillin.

KEYWORDS: Nuclear magnetic resonance spectroscopy; carbon-13; quantitative; vanillin; ferulic acid; authentication

INTRODUCTION

Vanillin (**Figure 1**) is one of the most widely used flavoring compounds currently used in the food industry. However, the demand for vanillin—estimated at 12000 Mton in 1990 (*1*)—far outweighs its natural supply, ∼20 Mton in 1990. Its natural origin is the partially dried and fermented pods of *Vanilla planifolia*, now widely cultivated across the tropical regions. The extract of *V. planifolia* contains not only vanillin but also a range of related phenylpropanoid (C_6-C_3) compounds (notably *p*-hydroxybenzaldehyde), which combine to give the unique subtlety to natural vanilla flavor (*1*). In many applications, however, synthetic vanillin is satisfactory for providing the required organoleptic properties. Much of this is obtained by chemical transformation or by fermentation of other natural phenylpropanoids, notably ferulic acid or lignin (**Figure 2**). Entirely synthetic vanillin from guaiacol is also available. More recently, biotechnological production by microbial fermentation of ferulic acid has been developed (*2*, *3*).

Natural vanillin commands a high price, whereas the various synthetic or semisynthetic substitutes are much cheaper. Thus, to avoid the fraudulent substitution of synthetic for natural vanillin, the means are required to authenticate the origin of different vanillin samples. The problem is compounded by the additional need to distinguish "nature-identical" from synthetic sources (*3*). Three basic approaches to resolving this problem have been taken: ash analysis (*4*), compositional profiling (*5*, *6*), and isotopic profiling (*4*, *6*−*12*). Of these, the use of isotopic analysis has proved to be the most effective.

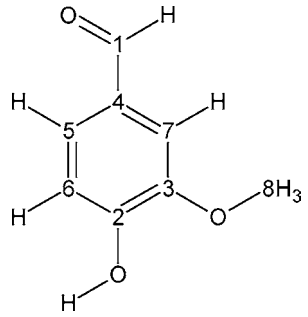

Figure 1. Vanillin molecule with carbon sites numbered in decreasing chemical shift.

The potential of isotopic analyses as a tool for origin discrimination is based on the fact that the distribution of isotopes on the different sites of the molecule is not statistical but rather that it depends on the origin of the precursor and on the type of process to which the precursor has been subjected (*13*). Vanillin contains three elements (H, C, O) that can all be used for isotopic discrimination. Thus, $\delta^{18}O$ values obtained by IRMS have been used to distinguish natural from guaiacol- and lignin-derived vanillin (*14*), but this parameter is susceptible to chemical exchange for the aldehyde position during laboratory

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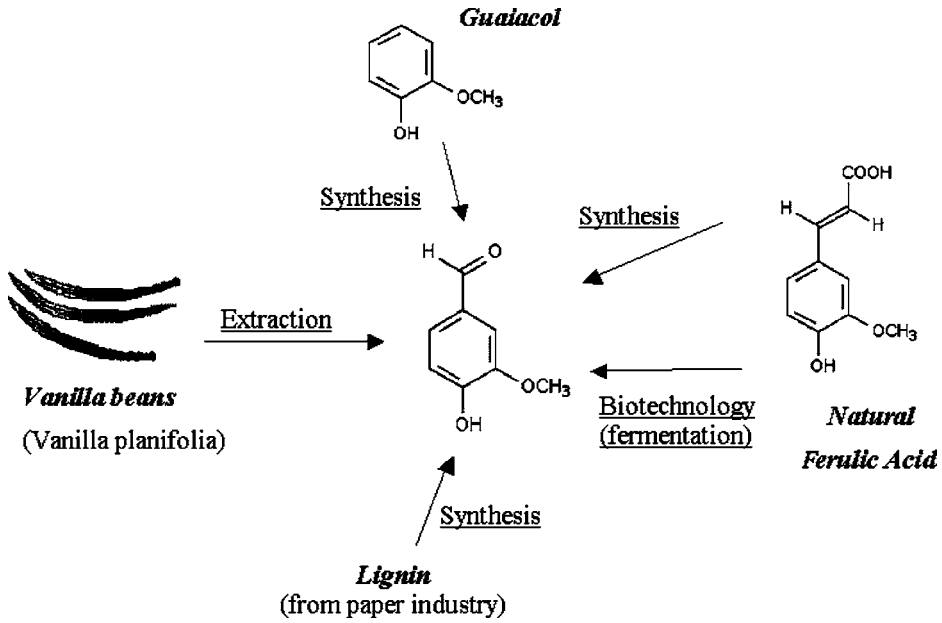

Figure 2. Principal sources for the production of vanillin.

or industrial procedures (*9*). The site-specific ^{18}O isotopic content in vanillin also gives additional data for distinguishing between origins, but the main drawback of this approach is the chemistry required to access this information. Quantitative deuterium nuclear magnetic resonance (^2H NMR) provides a very powerful tool for distinguishing between natural and synthetic origins of the aromatic ring (*7*) but is insufficient to differentiate between the different ways by which chain shortening (C_6-C_3 to C_6-C_1) has been conducted (*9*). Also, aromatic hydrogen exchange under certain conditions cannot be ruled out.

The carbon skeleton, however, is not susceptible to the same restrictions, and all eight atoms of carbon present should contain $^{13}C/^{12}C$ ratios that reflect their origin. Thus, Krueger and Krueger (*15*) were able to distinguish natural and falsified samples on the basis of their $\delta^{13}C$ values, and this capacity was extended to on-line GC-C-IRMS by Bréas et al. (*16*). The attractiveness of using $^{13}C/^{12}C$ ratios as an isotopic fingerprint for vanillin is enhanced by the fact that *V. planifolia* exploits CAM metabolism, thus producing a product with $\delta^{13}C$ values intermediate to those for C3 or C4 plants. Hence, whereas C3 plants typically produce phenylpropanoids of $\delta^{13}C \approx -26$ to -30‰ (*17*) and C4 plants phenylpropanoids of $\delta^{13}C \approx -12$ to -15‰, Remaud et al. (*12*) found the $\delta^{13}C$ for natural vanillin to vary very little, with $\delta^{13}C = -18.2 \pm 0.6$‰ ($N = 30$).

However, IRMS suffers from providing only global mean $\delta^{13}C$ values and is insufficient to counter more sophisticated fraudulent practices, in which the mean isotopic value is adjusted by the judicious addition of labeled material. This practice was already detected in the mid 1980s (*18*) with C1-enrichment and, more recently, by ^2H NMR, when Remaud et al. (*12*) showed adulteration of the ^2H content of the methoxyl group. Although chemical degradation (*9*) can go some way toward resolving this complication, this approach is itself subject to technical problems and reproducibility limitations (*9*). These problems can largely be overcome by the on-line pyrolysis described by Dennis et al. (*10*). This well-established technique has, however, the drawback that it provides only an average $^{13}C/^{12}C$ ratio of positions C_1, C_2, and C_8 and therefore cannot be as reliable as the ^{13}C NMR technique.

The site-specific isotopic $^{13}C/^{12}C$ ratios of carbon at natural abundance are accessible by ^{13}C NMR spectroscopy. However, although this technique is a priori very attractive, accessing natural $^{13}C/^{12}C$ ratios with a suitable degree of accuracy has proved to be challenging. Nevertheless, in a pioneering study, Caer et al. (*11*) were able to obtain limited natural abundance site-specific isotopic $^{13}C/^{12}C$ ratios and to show distinct differences between different origins. Their approach required the separate observation of three domains of the spectrum and proved to be very sensitive to acquisition conditions. Crucially, the feasibility of this approach was demonstrated. We have recently demonstrated (*19*) that with improved technology—notably in stability and in the broad-band decoupling ability of modern NMR spectrometers—it is now possible to obtain precise and accurate measurements of specific isotopic molar fractions by ^{13}C NMR.

The purpose of the present work is to evaluate a methodology adapted to the determination of site-specific ^{13}C abundance of vanillin and to estimate its discriminating potential to identify vanillin from different origins. Two approaches are presented: one providing a rapid screen for differences, the other a more refined discrimination based on absolute measurements.

MATERIALS AND METHODS

Material. Twenty-one samples of vanillin from various origins were investigated: 2 samples were extracted from beans; 3 were synthetically produced from lignin; 10 were synthetically produced from several different batches of guaiacol; 4 samples were produced by biotechnology from natural ferulic acid (bacterial process); and 2 samples were produced from natural ferulic acid according to two different synthetic procedures.

NMR Experiments. Samples (500 mg) of vanillin were prepared, quantitative ^{13}C NMR spectra were recorded at 125.76 MHz, and data were processed as described previously (*19*).

The interpulse delay D was equal to 131 or 21 s. Indeed, to obtain quantitative measurements for all eight sites with an accuracy of 1‰, a repetition time (D) equal to 7 times the longest T_1 was chosen: $7 \times 18.7 = 131$ s. By omitting sites 2−4, the maximum T_1 value becomes that of site 8 (2.95 s) and D becomes 21 s instead of 131 s. Under these conditions, measurements are no longer quantitative for sites 2−4, but experiment time becomes 32 min instead of 3.5 h as described previously (*19*).

Isotope Ratio Mass Spectrometry (IRMS) Experiments. The overall carbon isotope ratios of the samples were measured using a

Table 1. Partial Reduced Molar Fractions [± Standard Deviation ($n \geq 3$) or Range ($n = 2$)] for the 21 Samples of Vanillin from ^{13}C NMR Spectra Acquired with $D = 21$ or 131 s[a]

f_i/F_i partial	ex-guaiacol ($n = 10$)		ex-lignin ($n = 3$)		biotech ex-ferulic acid ($n = 4$)		ex-beans ($n = 2$)		synth ex-ferulic acid ($n = 2$)	
	$D = 21$ s	$D = 131$ s	$D = 21$ s	$D = 131$ s	$D = 21$ s	$D = 131$ s	$D = 21$ s	$D = 131$ s	$D = 21$ s	$D = 131$ s
1	1.016 ± 0.002	1.015 ± 0.003	1.007 ± 0.002	1.006 ± 0.004	1.009 ± 0.003	1.008 ± 0.002	1.011 ± 0.002	1.011 ± 0.002	1.002 ± 0.003	1.003 ± 0.003
5	0.996 ± 0.003	0.997 ± 0.003	0.987 ± 0.002	0.989 ± 0.002	0.996 ± 0.002	0.997 ± 0.002	0.987 ± 0.001	0.989 ± 0.003	0.996 ± 0.002	0.996 ± 0.002
6	0.996 ± 0.001	0.996 ± 0.002	1.000 ± 0.003	0.998 ± 0.003	1.001 ± 0.002	1.001 ± 0.002	1.001 ± 0.001	1.001 ± 0.001	1.004 ± 0.002	1.002 ± 0.002
7	0.999 ± 0.002	0.999 ± 0.003	0.991 ± 0.004	0.992 ± 0.002	1.000 ± 0.003	1.000 ± 0.002	0.991 ± 0.002	0.993 ± 0.002	1.002 ± 0.003	1.002 ± 0.002
8	0.993 ± 0.003	0.993 ± 0.003	1.016 ± 0.005	1.016 ± 0.001	0.994 ± 0.002	0.994 ± 0.002	1.070 ± 0.002	1.007 ± 0.002	0.996 ± 0.001	0.997 ± 0.002

[a] Results have been averaged over each origin. The f_i/F_i standard deviations over the four measurements made on the same sample were typically 0.002 (0.2%); n is the number of samples analyzed for each source, four NMR spectra being averaged for each sample.

Finnigan Delta E mass spectrometer equipped with a Carlo Erba microanalyzer. All values obtained had a standard deviation of ≤0.3‰ over five measurements.

Principal Component Analysis (PCA). Data treatment by PCA was used to process table-crossing vanillin samples (as individuals) and specific isotopic deviations (as variables). The principal components were computed via the covariance matrix (centered data) because all measurements of the variables were in the same units (*20*, *21*).

Isotope Analysis. The overall carbon isotope ratios (δ^{13}C ‰) of the samples were measured by isotope ratio mass spectrometry as described in ref *19*, and specific carbon abundances (A_i) were calculated using

$$A_i = \frac{f_i}{F_i} \times A_c \quad (1)$$

where f_i is the molar fraction of the isotopomer monolabeled in position i, relative to the total ^{13}C content

$$f_i = \frac{S_i}{\sum_i S_i} \quad (2)$$

with S_i the area of the peak corresponding to the molecular site i; F_i is the population of site i in the case of a random distribution of the stable isotopes

$$F_i = \frac{P_i}{\sum_i P_i} \quad (3)$$

with P_i the number of equivalent carbons for the molecular site i; and A_c is the overall ^{13}C abundance measured by isotope ratio mass spectrometry.

In the following, we define the term f_i/F_i (5) as the *partial reduced molar fraction* with only the sites 1 and 5−8 used and $D = 21$ s and the term f_i/F_i (8) as the *total reduced molar fraction* with all sites 1−8 used and with $D = 131$ s.

The specific carbon isotopic deviations, the total reduced molar fraction, and the partial reduced molar fractions were all calculated as described previously (*19*).

RESULTS AND DISCUSSION

In a previous publication (*19*), the optimal conditions for determining the ^{13}C distribution in the molecule of vanillin were described. It was shown that the $\delta^{13}C_i$ values can be determined accurately for all eight carbon atoms of vanillin by the combined use of NMR and IRMS. However, within the context of authentication, a comparison of the molar fractional distribution between samples can prove to be sufficient, obviating the need for the additional determination of $\delta^{13}C_i$. Furthermore, not all eight sites were found to contribute significantly to the differentiation of origin, allowing data acquisition times to be substantially reduced.

Partial Reduced Molar Fractions f_i/F_i (5). To test the robustness and discriminating power of the partial reduced fractions method, these were calculated for 21 vanillin samples from ^{13}C NMR spectra acquired with an interpulse delay (D) of 21 s. Under these conditions, comparative data only are acquired and acquisition time is rapid (30 min per sample). Because measurements for sites 2−4 were not quantitative, partial reduced molar fractions were determined from peak areas for only sites 1 and 5−8 (*19*).

Results were averaged over each origin, and values are given in **Table 1** and plotted in **Figure 3a**. No significant differences in the mean and in the standard deviations ($p < 0.001$) were seen when these values were compared with the total reduced molar fractions (**Table 2**). Hence, it can be concluded that no loss of intra- or extramolecular reproducibility has resulted from the use of partial reduced molar fractions.

PCA was performed in the space of the five partial reduced molar fractions calculated from the 84 ^{13}C NMR spectra (4 spectra of each of the 21 samples of vanillin) acquired with $D = 21$ s. In **Figure 3b**, the 21 samples are represented in the plane of the two main axes (CP1 and CP2) and the relative component weightings are indicated in parentheses. Because four spectra were recorded for each sample, one sample is represented by four points on the PCA. Each point represents one spectrum from one sample. A variance analysis reveals that major contributions came only from site 8 (73%) for CP1 and only from site 1 (67.5%) for CP2. Other sites contributed <14% each.

Differentiation is effectively achieved between the various origins of vanillin. Thus, vanillin produced chemically from guaiacol is distinguished from that derived from natural precursors, ferulic acid or lignin. Naturally produced vanillin from vanilla pods groups well away from ferulic acid-derived vanillin but is rather close to lignin-derived vanillin, although the groupings are distinct. Similarly, the distinction between vanillin derived from ferulic acid by either hemisynthesis or biotechnology is insufficient.

Hence, it is apparent that measurement under these conditions is adequate for certain samples but may prove to be inadequate to categorize with confidence in the beans/lignin and in the ferulic acid synthesis/biotechnology regions of the PCA plane. The relatively short acquisition time and the avoidance of determining the δ_i values by IRMS make this the method of choice for an initial screening of samples. However, more discriminatory power is necessary for those cases that fall near these interfaces.

Total Reduced Molar Fractions f_i/F_i (8) and Specific Isotopic Deviations δ_i. Total reduced molar fractions and specific isotopic deviations δ_i (‰) were calculated for the 21

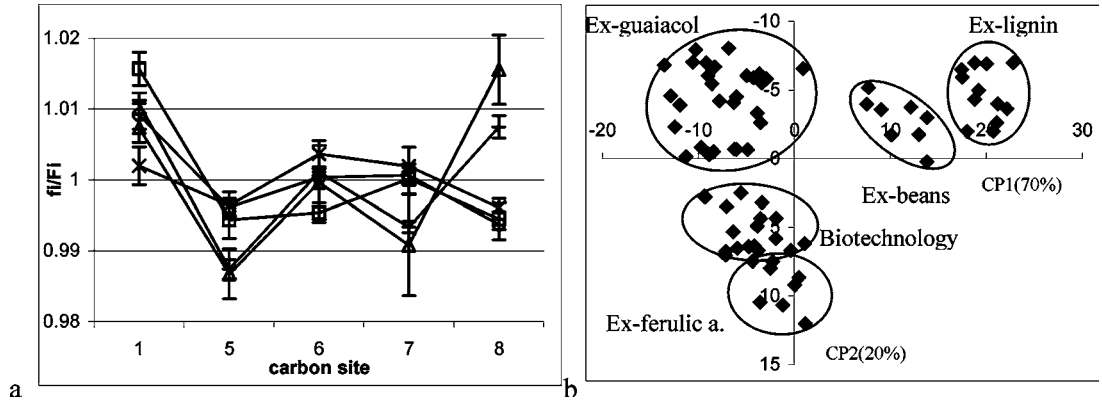

Figure 3. Partial reduced molar fractions of sites 1 and 5–8 of vanillin calculated from ^{13}C NMR spectra acquired with $D = 21$ s. (**a**) Results have been averaged over each origin, and standard deviations (ex-guaiacol, $n = 3$; ex-lignin, $n = 3$; biotechnology, $n = 4$) or range (ex-pod, $n = 2$) are plotted as error bars: □, ex-guaiacol; △, ex-lignin; ×, synthetic ex-ferulic acid; ○, biotechnology ex-ferulic acid; +, ex-beans. (**b**) Bidimensional representations of the PCA performed on partial reduced molar fractions f_i/F_i of sites 1 and 5–8 of vanillin calculated from ^{13}C NMR spectra acquired with $D = 21$ s. The 21 samples are represented in the plane of the two main axes (CP1 and CP2), and the relative weights are indicated in parentheses.

Table 2. Specific Isotopic Deviations and Total Reduced Molar Fractions [± Standard Deviation ($n \geq 3$) or Ranges ($n = 2$)] of the Eight Sites of Vanillin Calculated from ^{13}C NMR Spectra Acquired with $D = 131$ s[a]

	ex-guaiacol ($n = 10$)		ex-lignin ($n = 3$)		biotech ex-ferulic acid ($n = 4$)		ex-beans ($n = 2$)		synth ex-ferulic acid ($n = 2$)	
site i	f_i/F_i	δ_i (‰)	f_i/F_i	δ_i (‰)	f_i/F_i	δ_i (‰)	f_i/F_i	δ_i (‰)	f_i/F_i	δ_i (‰)
1	1.017 ± 0.003	−12.1 ± 3	1.007 ± 0.003	−19.6 ± 1	1.003 ± 0.001	−33.1 ± 1	1.007 ± 0.001	−12.7 ± 0.5	0.998 ± 0.001	−36.4 ± 2
2	0.998 ± 0.002	−30.2 ± 2	0.993 ± 0.001	−29.0 ± 1	1.006 ± 0.002	−30.0 ± 1	1.002 ± 0.001	−18.1 ± 0.5	1.011 ± 0.006	−23.8 ± 8
3	0.996 ± 0.002	−32.7 ± 2	0.997 ± 0.001	−30.2 ± 1	1.002 ± 0.001	−34.2 ± 2	1.000 ± 0.003	−20.2 ± 3	1.008 ± 0.001	−26.9 ± 3
4	1.002 ± 0.001	−26.5 ± 2	1.003 ± 0.000	−23.6 ± 1	1.011 ± 0.001	−25.2 ± 1	1.011 ± 0.004	−9.3 ± 4	1.009 ± 0.002	−26.5 ± 0
5	0.997 ± 0.001	−31.9 ± 1	0.988 ± 0.000	−38.4 ± 0	0.993 ± 0.001	−42.9 ± 1	0.986 ± 0.002	−34.4 ± 3	0.991 ± 0.001	−44.1 ± 2
6	0.996 ± 0.001	−32.2 ± 2	0.995 ± 0.000	−31.8 ± 2	0.997 ± 0.001	−39.4 ± 2	0.998 ± 0.002	−22.3 ± 1	0.995 ± 0.003	−39.8 ± 1
7	0.999 ± 0.001	−30.1 ± 2	0.991 ± 0.000	−35.3 ± 1	0.996 ± 0.001	−40.0 ± 1	0.989 ± 0.002	−30.7 ± 2	0.996 ± 0.001	−39.0 ± 3
8	0.992 ± 0.003	−36.8 ± 3	1.016 ± 0.001	−10.8 ± 2	0.989 ± 0.001	−46.4 ± 1	1.004 ± 0.004	−16.6 ± 3	0.988 ± 0.002	−46.1 ± 1
Ar	0.998 ± 0.002	−30.6 ± 2	0.995 ± 0.002	−31.4 ± 5	1.001 ± 0.001	−35.3 ± 6	0.998 ± 0.009	−22.5 ± 9	1.002 ± 0.002	−33.3 ± 8
δ global		−29.2 ± 0.4		−27.3 ± 0.7		−36.4 ± 0.4		−20.5 ± 0.4		−35.3 ± 2

[a] Results have been averaged over each origin. The f_i/F_i standard deviations over the four measurements made on the same sample were typically 0.002 (0.2%); n is the number of samples analyzed for each source, four NMR spectra being averaged for each sample. Ar gives the average value over the aromatic carbons. δ global is the mean $\delta^{13}C$ determined by IRMS.

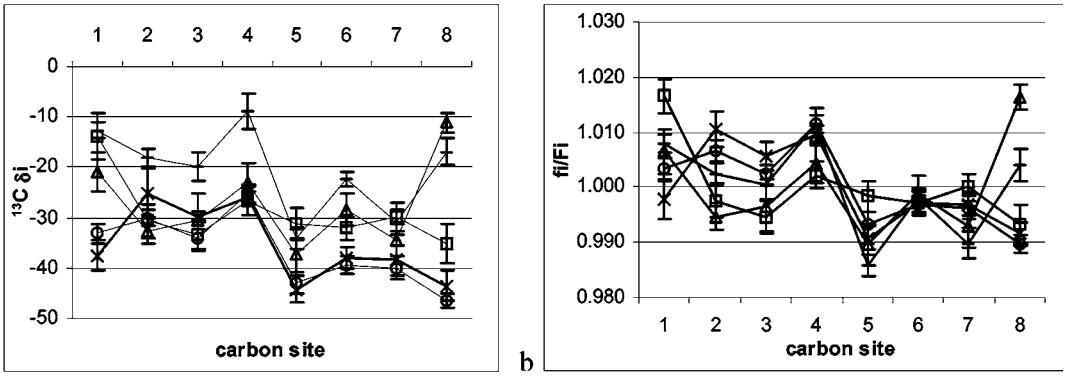

Figure 4. Specific isotopic deviations (**a**) and total reduced molar fractions (**b**) of the eight sites of vanillin calculated from ^{13}C NMR spectra acquired with $D = 131$ s. Results have been averaged over each origin, and standard deviations ($n \geq 3$; ex-guaiacol, ex-lignin, and biotechnology) or ranges ($n = 2$; synthetic vanillins ex-ferulic acid and ex-beans vanillins) are plotted as error bars: □, ex-guaiacol; △, ex-lignin; ×, synthetic ex-ferulic acid; ○, biotechnology ex-ferulic acid; +, ex-beans.

vanillin samples from ^{13}C NMR spectra acquired with $D = 131$ s (**Table 2**). Because isotopic deviations are calculated from A_i, they depend on molar fractions and on overall isotopic deviations measured by IRMS (eq 1). Like deviations, total reduced molar fractions on the eight sites are origin-specific. Hence, the evolution of δ_i can be expected to follow closely that of f_i/F_i (8). That is the case as can be seen by comparing panels **a** and **b** of **Figure** 4.

PCA was performed on the data set containing all 84 analyses (21 vanillin samples and 4 spectra per vanillin sample) in the space of the eight total reduced molar fractions f_i/F_i ($i = 1-8$) calculated from the ^{13}C NMR spectrum acquired with $D = 131$ s (**Figure 5a**). The 21 samples are represented in the plane of the two main axes (CP1 and CP2), and the relative weights are indicated in parentheses. A variance analysis reveals that major contributions came only from sites 1 and 8 (30 and 23%,

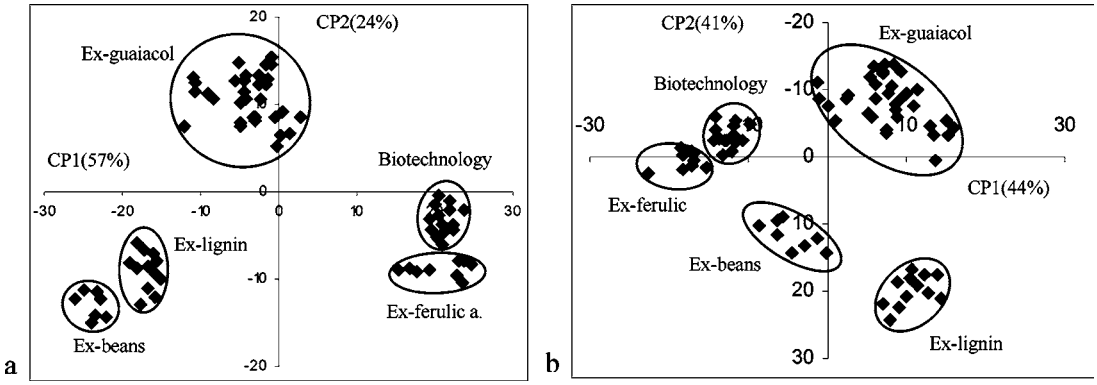

Figure 5. Representation of the PC plane of PCA performed on isotopic deviations $\delta_i^{13}C$ (**a**) and total reduced molar fractions f_i/F_i (**b**). The four measurements from each of the 21 samples are represented in the plane of the two main axes (CP1 and CP2), and the relative weightings are indicated in parentheses.

respectively) for CP1 and from only site 8 (66%) for CP2. Other sites contributed <13% each. These results are consistent with observation of **Figure 4a** where the biggest differences between origins are in sites 1 and 8.

Similarly, PCA was used to analyze the 84-data-point set in the space of the eight specific isotopic deviations $\delta_1-\delta_8$, calculated from the ^{13}C NMR spectra acquired with $D = 131$ s (**Figure 5b**). A variance analysis reveals sites 1 and 8 as the most discriminating. Significant contributions came from sites 1 (20%) and 8 (52%) for CP1 and from sites 1, 5, and 8 (31, 15, and 20%, respectively) for CP2. These results are consistent with the observation of **Figure 4b**, where the biggest differences between origins are in sites 1 and 8.

A comparison of **Figure 3b** with **Figure 5** shows broad consistency between the groupings in the PCA plane. Thus, as found with the partially reduced molar fractions ($D = 21$ s), guaiacol-derived samples are well separated from those from other sources. However, the introduction of the data for the three additional sites (2−4) has critically influenced the spread of the grouping of the data on the PCA plane, even though they contribute only ≤13% toward CP1 and CP2. As is now evident (**Figure 5**), ex-lignin samples are efficiently distinguished from natural vanillin (ex-beans). Similarly, the groupings of biotechnology-derived and ex-ferulic acid samples are distinct, although still relatively close.

Hence, by acquiring spectra under fully quantitative conditions for those sites with the longer relaxation times (experimental time of 3.5 h per sample), additional discriminatory power is obtained. Of particular importance is the greatly improved capacity to differentiate ex-lignin and ex-pod vanillins on the basis of the total reduced molar fractions. Thus, it is apparent that this parameter is sufficient as a suitable criterion for vanillin authentication, which removes the need to make additional IRMS measurements.

However, although partial molar fractions are a more accessible parameter to differentiate vanillin samples than isotopic deviations, they remain a purely relative parameter and as such risk being susceptible to inter-instrument variation. However, as specific isotopic deviations δ_i are related to the actual site-specific ^{13}C contents, their inclusion in the analysis should add to the robustness and improve inter-laboratory variation. To compare data from different analytical contexts and to use this to examine the biosynthesis of vanillin, eight specific isotopic deviations δ_i need to be determined.

Until now only very few site-specific ^{13}C data for vanillin have been reported in the scientific literature: Caer et al. from NMR (*11*) and Krueger and Krueger from IRMS (*15*, *18*). The fraudulent addition of [*methyl*-^{13}C]vanillin to adjust the overall $\delta^{13}C$ value was highlighted as early as 1981, and methods of chemical degradation were introduced to release and measure the C8 (*15*, *22*). It was shown that partial degradation effectively detected fraudulent addition of lignin-derived vanillin. In these cases, the $\delta^{13}C$ was measured both on the residual aromatic moiety and on the released methyl group. Similarly, the addition of [*carbonyl*-^{13}C]vanillin could be detected by lengthy degradation with the release of the C1 as CO_2 (*18*). This approach was taken by Bensaid et al. (*9*), who describe conditions to degrade vanillin to guaiacol, although these authors determined the $\delta^{13}C_1$ only indirectly, an approach strongly influenced by small experimental errors.

^{13}C NMR has the advantage that it not only efficiently and effectively detects the ^{13}C content simultaneously at both the carbonyl (C1) and methyl (C8) positions—previously indicated to be the most influential carbon atoms for providing discriminatory power—but simultaneously provides additional information on the six aromatic positions. Sample preparation is simple, and chemical treatment that could introduce fractionation is avoided. Moreover, it readily measures the relative molar fractions between these three parts of the molecule, making it much easier to detect fraudulent enrichment due to the addition of either [*methyl*-^{13}C]- or [*carbonyl*-^{13}C]vanillin.

Otherwise, only 500 mg of vanillin is needed for ^{13}C NMR analysis instead of 1 g for ^{2}H NMR. It could be very useful in food analysis, where very small quantities of vanillin are present in the product (e.g., yoghurt or ice cream).

Moreover, eight rather than four data points are determined by ^{13}C NMR compared with ^{2}H NMR. Critically, from the total reduced molar fractions and the global deviation $\delta^{13}C$, it is possible to calculate a value for each of the sites $\delta^{13}C_1-\delta^{13}C_8$ in the same molecule under the same analytical conditions (**Table 2**). These can be compared to the values related to the actual site-specific ^{13}C contents of $\delta^{13}C_{OCH3}$, $\delta^{13}C_{CHO}$, and $\delta^{13}C_{Ar}$ obtained by IRMS after degradation by different approaches. It is found that in natural vanillin the $\delta^{13}C_{OCH3}$ and $\delta^{13}C_{CHO}$ thus estimated are richer in ^{13}C than is the global $\delta^{13}C$, in contrast to the 12 samples analyzed by Krueger (*15*, *18*) or one of the two samples analyzed by Bensaid et al. (*9*). This could indicate that the total reduced molar fraction may not give the correct absolute $^{13}C/^{12}C$ ratios. As previously shown (*19*), the absolute values are very sensitive to the decoupling conditions (power and sequence) used. Optimization of these to give absolute values is in progress.

Critically, however, these values of total reduced molar fractions allow the value at each of the eight positions obtained

under identical conditions to be compared directly between samples. Thus, apparent $\delta^{13}C_{OCH3}$, $\delta^{13}C_{CHO}$, and $\delta^{13}C_{Ar}$ values can be calculated and compared directly, which is difficult to achieve by degradation and estimation of $\delta^{13}C_i$ indirectly from IRMS (*9*, *15*, *18*).

Furthermore, the long-term repeatability of the relative data provides high discriminatory capacity, enabling the isotopic signature of all samples analyzed to be correlated satisfactorily with their origin. In previous work (*19*), we have studied the stability in time of the measurement of molar fractions by ^{13}C NMR. The results obtained demonstrate that the successive acquisitions from a single sample vary $<0.3\%$ of the mean. The intersample repeatability within a batch from the same source is also good, varying $\leq 0.3\%$. Furthermore, the repetition of measurements over a 15-month period produced a scatter within the sample set of results that is no larger than that obtained with the measurements of several samples at the same time.

It is thus apparent that the precision of the method is quite sufficient to differentiate the $^{13}C/^{12}C$ ratios, first, between the different carbon atoms within a given molecule and, second, between molecules of the same chemical species obtained from different sources. The intramolecular $^{13}C/^{12}C$ ratios are found to be highly nonstatistical for all sources of vanillin examined. This is particularly important, as a more sophisticated approach to fraud will use blending of vanillin from different origins to provide a synthetic product with characteristics close to that of natural vanillin. A major strength of the approach reported here is that eight parameters are determined for each sample and that these parameters can be seen to bear a consistent relationship representative of their origin (**Figures 3a** and **4**). Blended samples will show deviations from these established patterns, readily detectable by ^{13}C NMR.

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