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Authentication of Natural Vanilla Flavorings: Isotopic Characterization Using Degradation of Vanillin into Guaiacol

FABIENNE F. BENSAID,* KARINE WIETZERBIN, AND GÉRARD J. MARTIN

Eurofins Scientific Laboratories, rue Pierre Adolphe Bobierre, BP 42301, 44323 Nantes Cedex 03, France

The isotopic investigation of vanillin has been extended to the new sources of natural precursors of vanillin recently introduced with a view of obtaining natural vanillin by biotechnological processes. To check the consistency of the isotopic composition of vanillin with that of the corresponding aromatic fragment, a selective degradation reaction into guaiacol was carried out. The reaction was shown to proceed without significant isotopic fractionation at the sites of interest, and an optimized procedure was defined from the results of an experimental design involving the quantity of reagent and the temperature and duration of the experiment. Guaiacol, which can be easily obtained in a reasonable time, is an interesting isotopic probe for carbon- and oxygen-isotope ratio mass spectrometry (IRMS) determinations. It provides ^{13}C information specific to the aromatic fragment and, combined with $\delta^{13}C$ values measured on vanillin itself, it improves the authentication potential of carbon-IRMS. Thus, the natural status of ferulic acid may be characterized by significant ^{13}C depletion at the formyl site. Similarly, the oxygen-18 content of guaiacol is a better authentication tool than $\delta^{18}O$ of vanillin because it does not suffer the drawback of being altered by chemical exchange of the sp_2 oxygen atom with water in industrial or laboratory procedures. Although collaborative studies are still necessary to improve the interlaboratory reproducibility of the $\delta^{18}O$ parameters, consistent results can be obtained in an intralaboratory context. It is shown in particular that chemical oxidation of ferulic acid is characterized by a relative enrichment of the aromatic moiety of vanillin.

KEYWORDS: Vanillin; guaiacol; isotopic fractionation; authentication; carbon-13; oxygen-18

INTRODUCTION

A number of studies have been devoted to the authentication of vanilla as the natural product extracted from beans of *Vanilla planifolia* is worth a lot more than its synthetic or semisynthetic counterpart. Stable isotope analysis (*1*), carried out by isotope ratio mass spectrometry (IRMS) (*2*−*5*) and by nuclear magnetic resonance (SNIF-NMR) (*6*−*8*), provides very powerful tools for distinguishing vanillin ex-beans from the two other important origins, guaiacol and lignin (**Figure 1**). However, the increased demand for vanilla and the relative shortage of supply of natural extract from beans have encouraged the development of new sources of vanillin, the major flavoring component, on the market. These sources are mainly biotechnologically produced from natural precursors, which have a molecular structure as described in **Figure 2**, where R = CH_3 (isoeugenol, **1a**, from eugenol of clove oil), R = COOH (ferulic acid, **1b**, from rice bran or sugar beet pulp), or R = $CO-CH_2-R'$ (curcumin, **1c**, from the roots of Zingiberaceae). The vanillin obtained from these molecules involves an oxidative cleavage of the lateral double bond (*9*, *10*) (**Figure 3**).

The authentication problem is therefore twofold. First, the natural status of the precursor needs to be authenticated and, second, the process used to transform the precursor into vanillin must be guaranteed free from any chemical step.

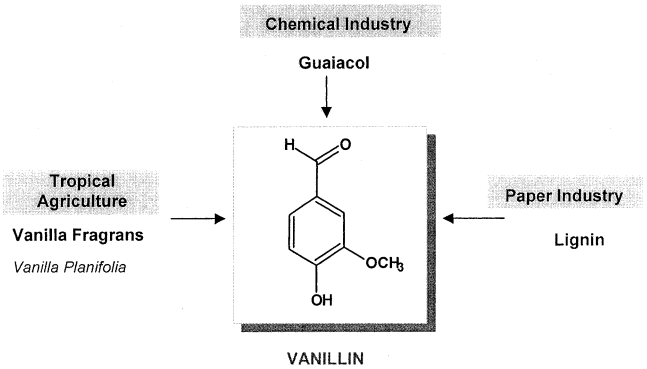

Figure 1. Three principal origins of vanillin.

Previous studies have been carried out to determine whether any unusual ^{13}C or ^{2}H enrichment of the formyl or methoxy group may have occurred as a consequence of illegal manipulation of the vanillin molecule (*11*, *12*). More recently, the oxygen isotope ratios of vanillin, $\delta^{18}O$, were also proposed as efficient additional parameters for distinguishing the natural samples from the guaiacol and lignin origins (*13*). However, it has been shown that the oxygen atom of the formyl group of vanillin undergoes easily a chemical exchange with that of water during any

* Author to whom correspondence should be addressed (e-mail fabiennebensaid@eurofins.com; telephone +33 2 51 83 21 00; fax +33 2 51 83 21 11).

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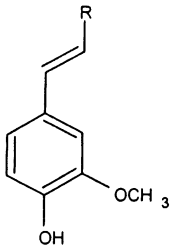

Figure 2. Molecular structure of vanillin precursors: **1a**, R = CH_3 (isoeugenol); **1b**, R = COOH (ferulic acid); **1c**, R = COCH2R′ (curcumin). **1a**–**c** are detailed in **Figure 3**.

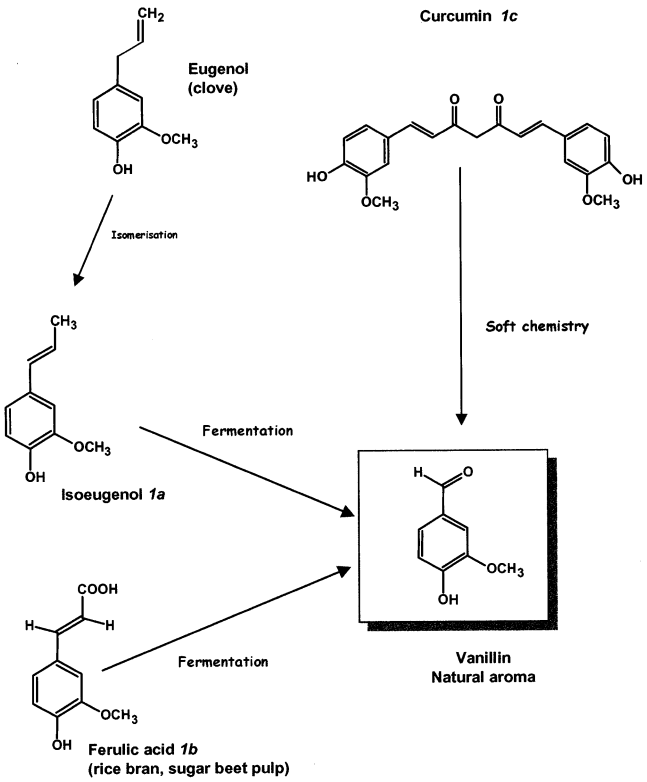

Figure 3. New sources of vanillin starting from ferulic acid, curcumin, or isoeugenol.

preparation and purification steps (*14*, *15*). It was then necessary to develop a new tool that can overcome this drawback of the ^{18}O procedure, and the degradation of vanillin into guaiacol was considered to be free from this disadvantage.

Taking into account the need for identification criteria including the new biotechnological sources of vanillin, the purpose of this work is also to determine the confidence domains of vanillin samples from different precursors based on both carbon and oxygen isotopic data and to investigate the influence of the oxidative cleavage on the isotopic parameters of the resulting molecule.

MATERIALS AND METHODS

Fourteen samples of vanillin from various origins were investigated: two samples were extracted from beans (1a,b); two were produced synthetically from lignin (2a,b); three were obtained synthetically from guaiacol (3a−c); one sample was produced by biotechnology from natural ferulic acid (*4*); one sample was produced by biotechnology from synthetic ferulic acid (*5*); three samples ex-natural ferulic acid were produced according to three different synthetic procedures: ozone treatment (6a), ozone with water (6b), and osmium tetraoxide (6c); two samples ex-natural isoeugenol were obtained by chemical synthesis involving either ozone with water (7a) or osmium tetraoxide (7b).

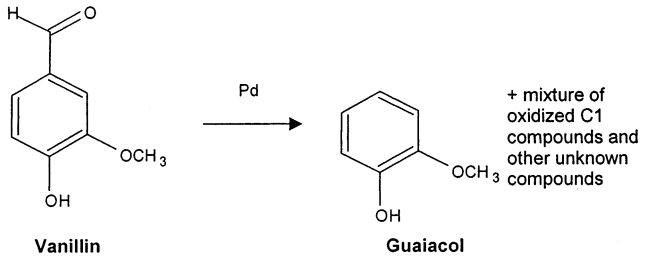

Figure 4. Reaction of degradation of vanillin into guaiacol.

Samples 1a and 1b were extracted according to the procedure described by Remaud (*12*). Samples 2a−3c were purchased directly from the manufacturers (Rhône-Poulenc, Eurovanillin, Aldrich, Lancaster), and vanillins 4−7b were kindly provided by Rhodia.

All of the samples were purified according to the procedure described by Remaud (*12*).

Transformation of Vanillin into Guaiacol. The reaction involves a catalytic cleavage of the benzene−formyl bond with palladium (*16*) (**Figure 4**). A 250 mL round-bottom flask fitted with a condenser is charged with the same quantity of vanillin (at least 200 mg) and 1% w/w palladium deposited on activated carbon. The mixture is manually stirred, and the solution is refluxed in an oil bath for 2 h at 210 °C (*17*). The mixture is then cooled to room temperature for 30 min, and the resulting guaiacol is dissolved into 30−40 mL of dichloromethane. It is then filtered under vacuum through a fritted glass funnel (no. 4).

Extraction and Purification. The last step of the procedure is the purification of the solution, which is a mixture of guaiacol and other oxidized compounds from vanillin.

Because of the small quantities of guaiacol involved in the reaction, purification on a silica column appears to be the best procedure to achieve separation with minimum isotopic fractionation. The solution to be separated is concentrated by removal of solvent on a rotary evaporator and then loaded uniformly onto the column. To purify guaiacol obtained from 1000 mg of vanillin, 100 mL (measured in a beaker) of silica gel (35−70 μm) is necessary in a 2 cm o.d. glass column equipped with a fritted disk (no. 4). The silica gel is first conditioned with pentane. The column is initially eluted with 100% pentane until guaiacol appears in a collected fraction. The gradient is then increased in CH_2Cl_2. Each fraction is controlled by thin layer chromatography (TLC). All of the fractions are gathered in a 2 L round-bottom flask, and the purity is checked by gas chromatography (GC). The solvent is removed using a rotary evaporator. The $\delta^{13}C$ and $\delta^{18}O$ ratios are then measured by IRMS on the purified guaiacol.

Isotopic Determinations. The mass spectrometric determinations of the carbon isotope ratios were carried out by on-line analysis using a Carlo-Erba NA 1500 II elemental analyzer fitted to a Finnigan MAT DELTA S mass spectrometer. Samples placed in tin containers were submitted to a flash combustion in a stream of helium enriched with pure hydrogen. The results are expressed on the δ ‰ scale with respect to the international standard VPDB according to the relation

$$\delta \text{‰} = 1000[(R_{\text{product}}/R_{\text{standard}}) - 1] \text{ where } R = {}^{13}C/{}^{12}C$$

The precision of the method may be estimated at 0.2‰ (*18*).

The mass spectrometric determinations of the oxygen isotope ratios were carried out by on-line analysis using a Carlo-Erba NA 1500 II elemental analyzer fitted to a Micromass mass spectrometer. The samples are first introduced into silver containers as carefully as possible to avoid contamination. They are then dropped into the microanalyzer, where pyrolysis takes place at 1060 °C. All of the organic matter is degraded into carbon monoxide gas. A helium flow carries the pyrolysis gas into a GC column to separate carbon monoxide from other degradation compounds. Carbon monoxide is then brought by the helium flow into the mass spectrometer. The results are expressed in δ ‰ with respect to the international standard V.SMOW. The precision of the method carried out in our laboratory is estimated at 0.8‰.

Optimization of the Method. Three factors are expected to influence the conversion rate of vanillin into guaiacol: the reaction time, the temperature, and the quantity of reactants used. An experimental design,

Table 1. Isotopic and Quantitative Results of the Experimental Design[a]

	reaction temp		initial quantity of vanillin			
expt	°C	level	mg	level	δ^{13}C guaiacol (‰)	yield (%)
1	180	–	250	–	–29.21	17.7
2	230	+	250	–	–28.37	22.1
3	180	-	1000	+	–29.94	37.3
4	230	+	1000	+	–28.93	31.9

[a] Two parameters are involved: the reaction temperature and initial quantity of vanillin. The high level is 230 °C and 1000 mg, and the low level is 180 °C and 250 mg, respectively.

Table 2. Influence of the Reaction Time on the Isotopic Values of Guaiacol Obtained from Vanillin

	guaiacol molecule	
reaction time (min)	δ^{13}C (‰)	δ^{18}O (‰)
30	–29.8	4.6
	–29.5	5.2
60	–29.5	5.3
	–29.8	5.4
90	–29.4	6.1
	–30.4	4.6
120	–29.0	5.0
	–29.4	4.7
240	–29.2	5.2
	–29.0	4.5
repeatability[a]	1.2	1.3

[a] Repeatability is expressed in ‰ according to the ISO Norm 5725 (*22*).

consisting of two steps, was defined to optimize the procedure. In the first step the effect of temperature and initial quantity of vanillin on the final yield of guaiacol was evaluated using a two-factor experimental design as described by Goupy (*19*). The different levels and the results of this two-parameter study are given in **Table 1**. The low and high levels of the temperature and vanillin quantity variables were, respectively, 180 °C and 250 mg and 230 °C and 1000 mg. Reaction time was 120 min. The yield is expressed in terms of pure guaiacol recovered but does not represent the rate of conversion of vanillin, which is nearly quantitative. The different levels for both parameters were chosen to take into account the commercial availability of pure vanillin from several sources (1 g of pure vanillin is a substantial quantity) and previous observations by Nicol (*17*).

In another series of experiments, the effect of the reaction time was investigated on a sample of ex-guaiacol vanillin purchased from Aldrich. The experimental conditions were defined according to the results of the previous experimental design, and five different periods of time were selected: 30, 60, 90, 120, and 240 min. The reaction was repeated twice for each selected time using ~1000 mg of vanillin at 210 °C (**Table 2**).

RESULTS AND DISCUSSION

Samples from 14 different natural chemical and biotechnological origins (described under Materials and Methods) were compared. A preliminary authentication step was carried out by the ^{2}H-SNIF-NMR method. The site-specific hydrogen isotope parameters were able to unambiguously distinguish the natural and synthetic origins of the aromatic ring (*8*). However, in the present state of its development the method does not provide clear information on the nature of the double-bond cleavage.

To obtain more selective carbon and oxygen isotopic parameters by IRMS, the formyl group of vanillin was eliminated through the transformation of vanillin into guaiacol, which represents the aromatic moiety of vanillin (**Figure 4**).

Optimization of the Chemical Transformation. Any physical or chemical treatment may be responsible for undesirable isotope fractionation effects, so it was necessary to investigate the influence of each experimental step on the measured isotope ratios. To this aim, the purification procedure developed for guaiacol was checked by loading pure commercial guaiacol of known ^{13}C and ^{18}O isotope ratios on the top of a column. The differences between initial and final values were, respectively, equal to 0.1‰ for δ^{13}C and 0.3‰ for δ^{18}O. Therefore, it may be concluded that the purification step leads to negligible isotopic fractionation. Similarly, it is immediately obvious from the results of **Table 2** that the reaction time has no effect on the δ^{13}C and δ^{18}O values of guaiacol. Therefore, a maximum level of uncertainty of the method may be calculated from these results, and the repeatability is found to be equal to 1.2‰ for ^{13}C and 1.3‰ for ^{18}O. In conditions of incomplete chemical transformation of vanillin into guaiacol, kinetic isotope effects occurring in the course of the transformation may result in significant fractionation effects, especially at sites directly involved in competitive reactions. Because the GC purity check revealed no residual vanillin in the reaction products, a quantitative conversion rate may be assumed. However, in the reaction conditions used, several other degradation products are observed, such as vanillic acid. Given that the extraction and purification steps lead to a decreased amount of recoverable pure guaiacol, we first tried to optimize the values of different factors directly involved in the yield of the transformation. Therefore, several experiments defined on the basis of a two-parameter experimental design, as described under Materials and Methods, were carried out to estimate the influence of the two variables, temperature and quantity of vanillin (**Table 1**). The results were analyzed by multiplying the yield values by the sign of the corresponding factors and then summing and dividing by the number of experiments (*19*). When this rule was applied, the effects due to the temperature and quantity variables were found to be equal to 0.25 and 7.32, respectively. On this basis, the experimental conditions selected for the transformation were the following: for a reaction time of 2 h, a temperature of ~200 °C and a quantity of pure vanillin as high as possible.

Precision and Accuracy of the Isotopic Results. Taking into account the precision of the IRMS determinations (Materials and Methods), the isotopic values given in **Table 1** show that the carbon isotope ratio of guaiacol does not depend significantly on the experimental conditions. To further estimate the possible occurrence of fractionation effects in the course of the transformation, three experiments were carried out, in the experimental conditions defined above, using three ex-guaiacol vanillin samples from the same production line kindly provided by Rhodia. Having also their guaiacol precursor, we could measure the target isotopic values: $\delta^{13}C = -30.0$‰ and $\delta^{18}O = 7.3$‰. The following average values were calculated from the results of the three transformations performed on the three vanillin samples: $\delta^{13}C = -30.2$‰ ($\sigma = 0.09$‰) and $\delta^{18}O = 8.9$‰ ($\sigma = 0.34$‰). These results are very gratifying, especially in the case of carbon, and they prove that the method is both reliable and accurate.

Therefore, the optimized procedure has been used in strictly identical experimental conditions for investigating 14 vanillin samples from different origins (**Table 3**).

Because satisfactory reproducibility is obtained in an intralaboratory context, the present results may be safely interpreted on a relative basis. However, it should be emphasized that, whereas the interlaboratory reproducibility of δ^{13}C IRMS values

Table 3. Values of the Isotope Ratios $\delta^{13}C$ and $\delta^{18}O$ (in ‰) of 14 Vanillin Samples from Different Origins and of the Corresponding Guaiacol Molecules Obtained by Chemical Degradation (Computed Values of the Isotope Ratios $\delta^{13}C$)

	sample		vanillin molecule		formyl group	guaiacol molecule		
case	origin	method	^{13}C	^{18}O	^{13}C	^{13}C	^{18}O	yield (%)
1a	beans	extraction	–20.4	12.2	–21.8	–20.2	8.7	26.1
1b	beans	extraction	–20.2	14.0	–9.9	–21.7	10.3	29.5
2a	lignin	semisynthesis	–27.2	8.8	–27.3	–27.2	8.2	19.7
2b	lignin	semisynthesis	–27.4	8.7	–30.1	–27.0	8.5	23.4
3a	guaiacol	synthesis[c]	–29.7	10.1	–18.6	–31.3	8.9	29.5
3b	guaiacol	synthesis[c]	–28.5	9.1	–21.6	–29.5	7.0	28.7
3c	guaiacol	synthesis[c]	–28.1	8.0	–18.3	–29.5	5.0	36.3
4	ferulic acid[a]	biotechnology	–36.1	13.2	–38.2	–35.8	9.4	34.0
5	ferulic acid[b]	biotechnology	–27.1	12.4	–26.4	–27.2	9.2	45.9
6a	ferulic acid[a]	ozone + water	–36.9	15.5	–63.7	–33.0	11.2	16.5
6b	ferulic acid[a]	OsO_4	–37.9	16.3	–67.5	–33.7	12.6	17.5
6c	ferulic acid[a]	ozone	–36.7	16.7	–48.6	–35.0	13.1	28.3
7a	isoeugenol	ozone + water	–30.9	13.3	–36.5	–30.1	7.5	28.8
7b	isoeugenol	OsO_4	–31.3	11.8	–31.8	–31.2	8.4	16.8

[a] Natural precursor (rice bran). [b] Fossil precursor. [c] Obtained from glyoxylic acid from Hoechst (*7*).

determined in collaborative studies is now very good (*18*), the situation is less favorable for the $\delta^{18}O$ determinations. Due probably to referencing problems and the use of different pyrolytic systems, a relatively high interlaboratory variability has been observed in preliminary collaborative studies. Unfortunately, this situation imposes strong limitations on the comparison of results obtained with different equipments.

Isotopic Characterization of the Formyl Fragment of Vanillin. In principle, the results given in **Table 3** may be used for estimating the isotopic contents in the formyl fragment of vanillin from the isotopic balance involving vanillin and guaiacol according to the relation

$$8\,\delta^{13}C(\text{vanillin}) = 7\,\delta^{13}C(\text{guaiacol}) + \delta^{13}C(\text{CHO}) \quad (1)$$

Unfortunately, the experimental error has a strong influence on the $\delta^{13}C$ value of the CHO fragment computed in this way, because the overall relative error $\Delta\delta/\delta$ on $\delta^{13}C(CHO)$ is given by

$$\Delta\delta/\delta(\text{CHO}) < 8\,\Delta\delta/\delta(\text{vani}) + 7\,\Delta\delta/\delta(\text{guai}) \quad (2)$$

Taking into account the repeatability determined under Materials and Methods, $\Delta\delta(\text{vani}) = 0.2‰$ and $\Delta\delta(\text{guai}) = 0.4‰$, the maximum error on $\Delta\delta C(CHO)$ should be of the order of 4‰. From this point of view it would be preferable to measure the $\delta^{13}C(CHO)$ value on carbon dioxide resulting from the decarboxylation of vanillic acid obtained by oxidation of vanillin following the procedure described by Krueger (*11*). Unfortunately, the procedure is not sufficiently reproducible.

In the same respect, it has been shown that $\delta^{13}C(CHO)$ of vanillin can also be determined directly by the ^{13}C-SNIF-NMR method, which gives precise relative values consistent with those measured by IRMS (*20, 21*). For example, the value calculated for the formyl group according to eq 1 using ^{13}C data of **Table 3** (−18.5‰, cases 3a−c) is relatively close to the $\delta^{13}C$ value [−21.5‰ (±1)] determined by NMR on a vanillin ex-guaiacol sample synthesized via the mandelic acid process (*7*). This reasonable agreement further supports the reliability of the present method.

Influence of the Chemical or Biochemical Origin on the Isotopic Parameters. Some trends in the isotopic distribution of guaiacol, in relation to its vanillin source, can be drawn from the results of **Table 3**.

Carbon Isotope Ratios. In the case of vanillin from natural precursors (beans, ferulic acid, lignin, and isoeugenol), the guaiacol molecule is slightly enriched in ^{13}C (with the exception of sample 1b). A mean value of 1.3‰ is computed. In contrast, a slight depletion (≈ −1‰) is observed for the fossil precursors. This behavior suggests that the formyl group of vanillin is enriched in ^{13}C in the synthetic product with respect to the same fragment from a natural origin. This observation, which parallels a typical behavior of the hydrogen isotopic parameter (*7*), is consistent with the high ^{13}C content (∼ −20‰) of glyoxylic acid used in the synthesis of vanillin (*7*). Furthermore, it is in agreement with the results of Krueger and Nicol (*11*, *17*). Despite the restricted precision of the $\delta^{13}C(CHO)$ values computed from eq 1, it may be concluded that the depletion of the formyl group is particularly high for vanillin from ex-natural ferulic acid ($\delta^{13}C$ values between −38‰ and −67‰ for samples 4 and 6a−c). These results strongly support the exploitation of the $\delta^{13}C$ value of vanillin from natural ferulic acid (mean $\delta^{13}C = -36.9‰$, samples 4 and 6a−c) for ascertaining this natural status. Moreover, the mean ^{13}C content of guaiacol obtained from the degradation of vanillin ex-natural ferulic acid ($\delta^{13}C = -34.4‰$ samples 4 and 6a−c) mirrors nicely that of ferulic acid from rice bran ($\delta^{13}C = -35.5‰$).

Oxygen Isotope Ratios. In practice, oxygen isotope ratios do not play an important role in authentication procedures. As discussed in the previous section, the low level of interlaboratory reproducibility complicates the comparison of absolute values, and the results must usually be interpreted on a relative basis. In addition, the risk of a loss of selectivity due to oxygen exchange must be taken into account. Thus, in the case of vanillin, exchange of the sp_2 oxygen atom with water is likely to occur in the course of extraction and preparation procedures of the sample (*15*), and vanillin itself is not a convenient general probe for ^{18}O-IRMS. In principle, guaiacol derived from vanillin is better preserved from the risk of exchange. In addition, its $\delta^{18}O$ value, which is the average over two positions only, exhibits a higher selectivity. Still more selective parameters can be determined but at the price of further degradation reactions of vanillin. Fronza et al. (*9*) have recently published selective $\delta^{18}O$ values for the three precursors beans, lignin, and guaiacol. Their results show that the oxygen isotope profile provides interesting mechanistic information on the origin of the three oxygen atoms. The authentication potential of guaiacol as an ^{18}O-IRMS probe for characterizing, in a reasonable time, the whole set of natural, chemical, and biotechnological origins can be evaluated from the results of **Table 3**. It is observed that the ^{18}O content directly measured on vanillin is generally lower

for synthetic and semisynthetic vanillin ($\delta^{18}O = 8.9‰$, samples 2a,b and 3a−c) than for vanillin extracted from beans or derived from natural precursors ($\delta^{18}O = 14.1‰$, samples 1a,b, 4, 6a−c, and 7a,b). This difference is probably not linked to specific properties of the precursors but rather to the industrial or laboratory nature of the extraction process because samples 2 and 3 were commercial industrial products, whereas the others were prepared and extracted on the laboratory scale. Guaiacol obtained in strictly standardized conditions is expected to provide an oxygen isotopic probe representative of the sole non-exchangeable atoms and, therefore, be less dependent on sample treatment. Although the range of variation of $\delta^{18}O$ is rather limited, it may be concluded that chemical oxidation of the double bond of ferulic acid increases slightly the $\delta^{18}O$ value of vanillin as compared to the product obtained from the enzymatic reaction in aqueous media.

Conclusion. Taking into account the economic importance of vanillin and the introduction of new biotechnological sources, there is a continued interest in the development of authentication criteria. The SNIF-NMR method is particularly efficient for distinguishing the main origins of vanillin when sufficient quantities of sample can be extracted. However, to face the increased sophistication of frauds and the need for investigating diluted media, it is useful to also resort to IRMS determination of carbon and oxygen isotope ratios. The present results, which extend previous investigations, show that guaiacol, which can be prepared from vanillin in reproducible isotopic conditions, provides a new probe free from perturbations due to exchange of the formyl oxygen, for the determination of both carbon and oxygen isotope ratios. Information on the isotopic content of the formyl group can also be obtained, when the guaiacol results are combined with isotope ratios measured on vanillin. It is shown that the experimental method developed for degrading vanillin into guaiacol is reliable and accurate. Thus, the mean square deviation computed between the $\delta^{13}C$ values of vanillin and guaiacol (MSD = 1.77) shows that guaiacol is a good complementary indicator for the authentication of vanillin precursors.

ABBREVIATIONS USED

VPDB, Vienna Pee Dee Belemnite; V.SMOW, Vienna Standard Mean Ocean Water; IRMS, isotope ratio mass spectrometry; SNIF-NMR, site-specific natural isotope fractionation studied by nuclear magnetic resonance.

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