The Positional $\delta(^{18}O)$ Values of Extracted and Synthetic Vanillin

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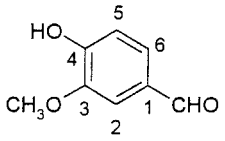
The positional $\delta(^{18}O)$ values of vanillin (**1**) of different origins have been determined from the global values of 2-methoxy-4-methylphenol (**4**), obtained from **1** upon *Clemensen* reduction, and of 3-methylanisole (**5**), obtained from **4** by removal of the phenolic O-atom. By these means, it is possible to differentiate samples of **1** of synthetic origin from those extracted from *Vanilla* plants or produced from lignin by chemical oxidation. The main difference between the samples derived from guaiacol and those possessing the aromatic moiety of natural origin is in the enrichment values of the O-atoms at C(3) and C(4), while the extractive materials from the pods are distinguished from the product from lignin on the basis of the carbonyl oxygen $\delta(^{18}O)$ values, ranging from $+25.5$ and $+26.2$ in the natural material to $+19.7$‰ in the lignan-based sample. The values for the phenolic O-atom vary from $+8.9$ and $+12$‰ of the synthetic materials to $+6.5$, $+5.3$, and $+6.3$‰, respectively, of the sample from lignin and the two samples from *Vanilla* pods,whereas the MeO O-atoms show the following values for the same compounds: -2.9, -3.2, $+3.5$, $+3.1$, and $+2.3$‰, respectively. This study indicates the significance of the positional $\delta(^{18}O)$ values of polyoxygenated compounds for the definition of their origin.

Introduction. – The distribution of stable isotopes within the atomic species constituting an organic molecule is not statistical but rather depends upon the way in which it was formed [1]. A relevant practical analytical application of this principle deals with the determination of the origin of food components and the authentication of the *natural* rather than *natural-identical* status of flavor materials [2][3]. Most of the measurements performed to this end have, until now, concerned the quantification of ^{13}C and ^{2}H, and comparatively little attention has been dedicated to the pattern of O-isotopes in an organic molecule. This is most likely due to experimental difficulties in the analytical determinations. Indeed, a great deal of information [4] can be drawn from the O-isotope content of a molecule, since the three primary sources for the O-atoms incorporated into an organic material, *i.e.*, CO_2, atmospheric O_2 gas, and ground water, possess quite different isotope enrichments. The O-isotope enrichment of a molecule is expressed as the $\delta(^{18}O)$ value, which is the relative difference of the isotope ratio of a compound to that of an international standard (*i.e.*, ocean water) in ‰. Accordingly, the $\delta(^{18}O)$ values of CO_2, atmospheric O_2, and ground water, the infinite reservoirs that supply oxygen to organic compounds, are: $+40.3 - +42.5$‰, $+23.5 - +23.8$‰ and $-10 - 0$‰, respectively [4].

Vanillin (**1**), perhaps, is the organic molecule whose isotopic content has been most extensively investigated [5][6], mainly because there are two sources for this industrially important phenolic aldehyde. The first is supplied from the costly material

extracted from the cured pods of *Vanilla planifolia* and *V. tahitensis*, while the second includes the material obtained both by chemical oxidation of the lignin present in waste sulphite liquors and by oxidative decarboxylation of the substituted mandelic acid produced in the condensation of guaiacol with glyoxylic acid [7]. Recognition of fraudulent dilution of extracted material with the chemically identical product of synthetic origin is still a problem in the food industry. The origin of vanillin can be determined through the quantification of the total carbon and positional H-isotopes [5][6]. The global $\delta(^{18}O)$ value of samples of extracted vanillin has also been measured and found to fall in the range $+8.2 - +15.2$‰, whereas the figures for the synthetic products from lignin and guaiacol are in the range of $+6.5 - -2.8$‰ [8–11]. The presence in **1** of three different oxygen functionalities and the large range of the values measured indicate that clear diagnostic information on the origin of different vanillin samples can be drawn from the positional $\delta(^{18}O)$ values.



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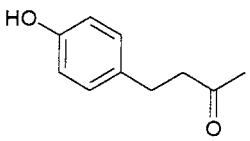

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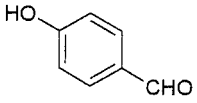

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Additional interest in this kind of determination relative to vanillin (**1**) arises from the results of recent studies on the origin of C_6-C_4 4-(4-hydroxyphenyl)butan-2-one (raspberry ketone; **2**) [12][13]. The $\delta(^{18}O)$ values of the phenol O-atom of **2** from *Taxus baccata* and from the hydrolysis of the glucoside lindleyine, extracted from *Aeonium lindleyi*, are -0.8‰ and $+0.6$‰, respectively, whereas that of the phenol O-atom present at C(4) of the C_6-C_3 moiety of naringin from *Citrus*, determined through degradation to 4-methylphenol *via* the intermediate 4-hydroxybenzaldehyde (**3**), ranks at $+11.0$‰. The different isotopic enrichments of the phenol moieties of natural raspberry ketone **2** and of naringin observed induced us to extend such studies to vanillin **1**, with which it shares the common biosynthetic phenylpropanoid derivation from C_6-C_3 *p*-coumaric acid **8**. With respect to **2** and naringin, vanillin (**1**) possesses the additional feature of bearing, adjacent to the O-atom originally present at C(4) of **8**, an additional O-atom at C(3), conceivably introduced into the aromatic moiety *via* a different activation mechanism [14]. The present study includes, in addition to extracted vanillin samples, synthetic samples from lignin and guaiacol and from an undefined method of preparation. It should provide, through the determination of the positional $\delta(^{18}O)$ values, not only information on the mode of introduction of O-atoms into the aromatic ring but also an additional analytical tool for the definition of the origin of different samples.

Results and Discussion. – The samples of vanillin (**1**) examined included: *Sample 1* (synthetic, of undefined origin (*Merck*)); *Sample 2* (synthetic, from guaiacol (*Rhone Poulenc*)); *Sample 3* (synthetic, from lignin (Eurovanillin)); *Sample 4* (natural, extracted from *V. planifolia* of Madagascar); *Sample 5* (natural, extracted from *Vanilla* pods). The global $\delta(^{18}O)$ values of these five samples, surprisingly enough [9][10],

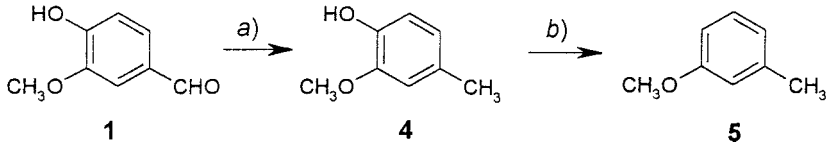
showed quite similar values in the range +9.9–11.9‰ (*Table*). However, the latter products are dramatically different when the positional $\delta(^{18}O)$ values are considered. These were drawn from the global $\delta(^{18}O)$ values of products derived from **1** by selective and progressive removal of the O-functionalities (*Scheme 1*). Vanillin (**1**) was converted first to 2-methoxy-4-methylphenol (**4**), which retains the two O-functionalities of the aromatic ring. In a subsequent step, **4** is transformed into 3-methylanisole (**5**), keeping only the O-atom originally present at C(3) of **1**. The $\delta(^{18}O)$ values of **4** and **5** obtained for the five vanillin samples were thus measured. The numerical figures accounting for the $\delta(^{18}O)$ values of the phenolic O-atom at C(4), of the ethereal O-atom at C(3), and, finally, of the carbonyl O-atom of vanillin (**1**) were thus calculated from the $\delta(^{18}O)$ values of **1**, **4**, and **5** (*Table*).

Table. *Positional and Global* $\delta(^{18}O)$ [‰] *Values for Samples of Vanillin* (**1**) *of Different Origin*[a])

Sample	$\delta(^{18}O)$ (OH)[b])	$\delta(^{18}O)$ (MeO)[b])	$\delta(^{18}O)$ (CO)[b])	$\delta(^{18}O)$ (total)[c])
1	8.9	−2.9	28.8	11.6
2	12.0	−3.2	26.9	11.9
3	6.5	3.5	19.7	9.9
4	5.3	3.1	25.5	11.3
5	6.3	2.3	26.2	11.6

[a]) *Sample 1* (synthetic of undefined origin); *Sample 2* (synthetic, from guaiacol); *Sample 3* (synthetic, from lignin); *Samples 4* and *5* (natural, from *Vanilla* pods). [b]) Standard deviations ±0.7‰. [c]) Standard deviations ±0.4‰.

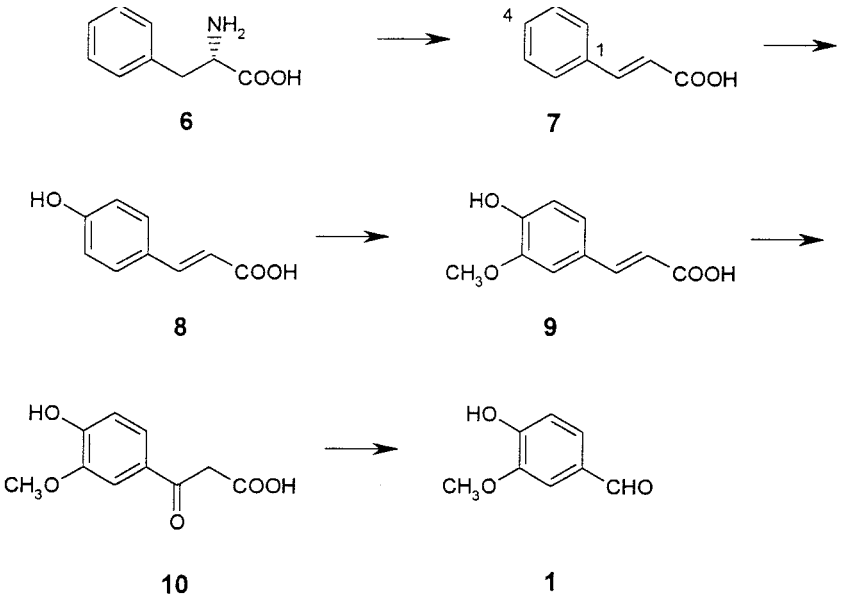
Scheme 1. *Synthesis of the Deoxygenated Vanillin Derivatives* **4** *and* **5**



a) Zn(Hg)/aq. HCl, EtOH, reflux. *b*) NaH, $(EtO)_2POCl$, THF, then Li, $NH_3(l)$, −78°.

Inspection of the positional $\delta(^{18}O)$ values (*Table*) allows the identification of three distinct sets represented by *Samples 1* and *2* of synthetic origin, *Sample 3* from lignin, and *Samples 4* and *5* from *Vanilla* pods. Thus, vanillin samples showing similar global $\delta(^{18}O)$ values (see last column of the *Table*), are clearly differentiated when considering the positional $\delta(^{18}O)$ figures. In the first instance, synthetic *Sample 1* (*Merck*) of unspecified mode of preparation appears to be quite similar to *Sample 2* (*Rhone Poulenc*) from guaiacol, thus suggesting identical modes of production. The differentiation of *Samples 1* and *2* (synthetic) from those possessing an aromatic moiety of natural derivation, *i.e.*, *Samples 3*, *4* and *5*, stands on the $\delta(^{18}O)$ values relative to positions 3 and 4. For the synthetic set, these range between −2.9– −3.2‰ and +8.9–+12.0‰, respectively, whereas, among the natural samples, these fall in the ranges +2.3–+3.5‰ and +5.3–+6.5‰, respectively. However, *Sample 3*, produced by oxidative chemical fragmentation of the carbon skeleton of lignin, may be differentiated from *Samples 4* and *5*, extracted from *Vanilla* plants, by the carbonyl $\delta(^{18}O)$

Scheme 2. *Steps in the Biosynthesis of Vanillin* (**1**)

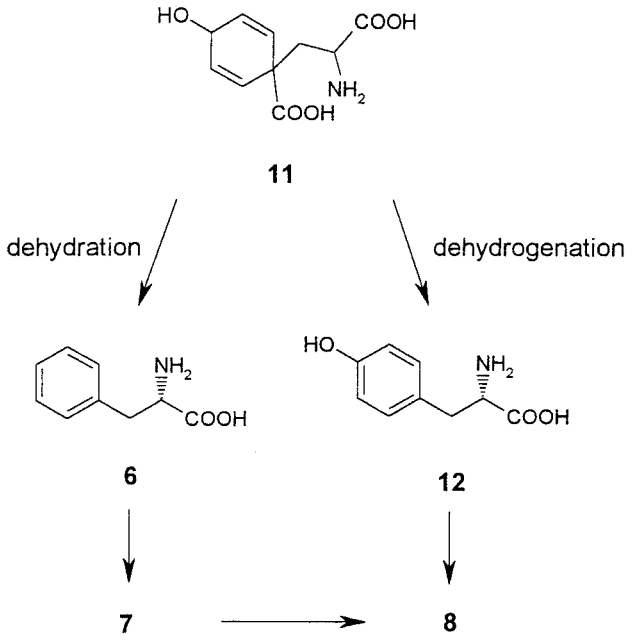


values of +19.7‰ (*Sample 3*) compared to +25.5‰ and 26.2‰ (*Samples 4* and *5*), respectively.

These data, which are significant for authentication studies, can perhaps provide also subtle insights into the mode of formation of oxygenated organic compounds. The biosynthetic aspects are first considered. C_6-C_1 Vanillin is supposedly derived in nature (*Scheme 2*) [15] by C(2) degradation of the oxo acid **10**, formed, in turn, from C_6-C_3 ferulic acid (**9**). Ferulic acid (**9**) is formed by regioselective methylation of caffeic acid, which is derived from *p*-coumaric acid (**8**), the key intermediate in the generation of plant phenol compounds. However, *p*-coumaric acid (**8**) is apparently accessible in nature from carbohydrates *via* two pathways. The first is based on L-phenylalanine **6** as first aromatic intermediate and involves cinnamic acid (**7**), formed from **6** by the action of phenylalanine-ammonia lyase. The subsequent conversion of **7** to **8** requires formal attachment of an O-atom to the sp^2-C-atoms at position 4 of **7**, which is assumed to occur *via* rearrangement of the corresponding 3,4-arene oxide [16]. Alternatively, *p*-coumaric acid (**8**) may be formed from L-tyrosine (**12**) by elimination of ammonia (*Scheme 3*). The enzymic activity presiding over the latter transformation is not widespread in nature. However, the switching point for the generation of L-phenylalanine and L-tyrosine from carbohydrates is arogenic acid (**11**), which provides L-phenylalanine on dehydration and L-tyrosine on dehydrogenation, respectively (*Scheme 3*). To differentiate between the two pathways of formation of **8** (arogenic acid → L-tyrosine and elimination of ammonia *vs.* arogenic acid → L-phenylalanine, elimination of ammonia, cinnamic acid, and aromatic hydroxylation) seems possible on the basis of the isotopic oxygen values of the phenolic O-atom at C(4) of the derived aromatic compounds. In the case of the direct aromatic hydroxylation (*i.e.*, **7** → **8**), the

O-atom is introduced from O_2 through the action of the membrane-bound cytochrome P-450 enzyme cynnamate-4-hydroxylase [EC 1.14.13.11] [14][16]. Theoretical calculations on the isotope effects associated with the atmospheric O_2 activation [4] indicate that, starting from an infinite atmospheric O_2 pool with $\delta(^{18}O)$ value of *ca.* +22.5–23.5‰, a phenolic O-atom with values in the range 0– +7‰ should be obtained. This result is expected to be the consequence of the combined action of isotope fractionations induced by oxygenases during the binding, activation, and transfer of the O-atom and of isotope discrimination occurring during O_2 diffusion in the plant [17]. Conversely, the direct pathway from **11** to **8** would imply conservation in **8** of the O-atom originally present at C(4) of arogenic acid (**11**), derived from CO_2, without involvement of atmospheric O_2 sugar. In this event, a $\delta(^{18}O)$ value of *ca.* +30‰ is expected, as was determined for quinic acid [4].

Scheme 3. *Biosynthetic Scheme for the Conversion of Arogenic Acid* (**11**) *to* p-*Coumaric Acid* (**8**)



The present results, showing $\delta(^{18}O)$ values for the phenolic O-atom of *Samples 3–5* of between +5.3 and 6.5‰, fit perfectly with the proposal that cinnamic acid (**7**) is intermediate in the biosynthesis of **1**, and are in line with those reported for monooxygenated natural aromatic compounds like anethol (+6– +8‰) [18], eugenol (+5‰) [18], and safrole (+6.9‰) [4]. The values found for the phenolic O-atom of raspberry ketone (**2**; −0.8‰ [12] and +0.6‰ [13]) are at the lower limit of the calculated range [4], while the +11.0‰ value found for the phenolic O-atom of naringin seems to be outside the range of expected values. Direct feeding experiments in *Vanilla* plants that would indicate derivation from cinnamic acid are apparently lacking. However, *Kirby* and co-workers [19] have shown that, in *Capsicum annuum*, regiospecifically [^{3}H,^{14}C]-labelled cinnamic acid (**7**) is incorporated into the C_6–C_1, vanillin-derived

moiety of capsaicin in a mode indicating that the introduction of an O-atom into the *para*-position of the aromatic moiety occurs without shifting the H-atom at C(4) to the adjacent *meta*-position (*i.e.*, without the so-called NIH-shift) [16].

The $\delta(^{18}O)$ values relative to the ethereal O-atom present at C(3) of the vanillin framework (+2.3 – +3.5‰) of *Samples 3–5* are, to some extent, lower than those of the O-atom first introduced into the aromatic moiety (*i.e.*, that at C(4)). The O-atom enzymically introduced in the position adjacent to the phenol originates from atmospheric O_2, as shown by *Mason et al.* in his pioneering work on phenolase [20]. The modest, but definite differences in the $\delta(^{18}O)$ values at the two positions suggest that the kinetic isotope effect on O_2 in the reaction catalyzed by the cynnamate-4-hydroxylase is slightly lower than that in the reaction catalyzed by the *p*-coumarate-*o*-hydroxylase.

It is worth noting that the ^{18}O-labelling pattern of the aromatic moiety of the vanillin sample derived from lignin obtained from trees grown in Norway (*Sample 3*) is quite similar to that of the extractive materials from *V. planifolia*, one of which (*Sample 4*) originates from Madagascar. Vanillin and lignin share the same biosynthetic precursors, since one of the monomeric components incorporated into the polymeric matrix of lignin is coniferyl alcohol, the reduction product of ferulic acid (**9**) [21]. Vanillin is extruded from this moiety of lignin in the oxidative treatment that induces fragmentation of the C-framework. The coincidence of the isotopic oxygen values within this set of materials thus confirms the view that identical oxygenated compounds derived from atmospheric O_2 show a composition not influenced by climatic or latitudinal effects, since O_2 gas shows a constant $\delta(^{18}O)$ value all over the world.

However, the *extracted* vanillin (*Samples 4* and *5*) is differentiated from the partially *synthetic* material from lignin (*Sample 3*) on the basis of the carbonyl $\delta(^{18}O)$ values (+25.5 and +26.2‰ *vs.* +19.7‰, resp.). The former values seem in line with those predicted [4] on the basis of partial equilibration of the carbonyl carbon of **1** with the leaf water in hot climates. Conversely, the figure found for the material derived from lignin by chemical oxidation is coincident with the +19‰ observed when acetone is equilibrated with tap water [22].

At present, the interpretation of the positional $\delta(^{18}O)$ values found for the synthetic vanillin produced from phenol [7] is almost impossible, since the actual starting material and the intermediates of *Samples 1* and *2* were not known. However, the O-atom of synthetic phenol supposedly derives from the atmospheric O_2 incorporated into cumene hydroperoxide. Similarly, the O-atoms of H_2O_2, from which the O-atom introduced in the *ortho*-position to the existing OH function in the conversion of phenol to benzene-1,2-diol originates, arises from atmospheric O_2 [23]. Irrespective of the ^{18}O enrichments associated with these two reactions, the benzene-1,2-diol becomes a symmetrical substrate for the methylation to guaiacol. The reported $\delta(^{18}O)$ values for 'synthetic' phenol fall in the range +13.4 – 18.5‰ [24], whereas a synthetic sample of guaiacol in our hands showed a $\delta(^{18}O)$ value of +15‰. In the case of the synthetic *Samples 1* and *2*, the $\delta(^{18}O)$ values are smaller than that reported above. The values found for the OH O-atom at C(4) are +8.9 and +12.0‰, respectively, while those for the ethereal O-atom at C(3) are even negative (−2.9 and −3.2‰, resp.). The marked difference in ^{18}O enrichment between the phenolic O-atoms of synthetic vanillin, both probably originating from atmospheric O_2, indicates that a strong kinetic effect may

occur in the selective methylation reaction of benzene-1,2-diol to guaiacol. However, the complete understanding of the observed ^{18}O-isotope distribution requires the isotopic determination of the starting material and of the compounds at each step of the synthetic process.

The present study on vanillin (**1**) thus demonstrates the great potential of information associated with the determination of the positional $\delta(^{18}O)$ values of chemically identical materials of different origin. We will report in due course work designed to clarify the origin of the observed isotopic oxygen fractionation found for the synthetic vanillin under examination.

Experimental Part

General. The selective deoxygenations of vanillin (**1**) to **4** and of the latter to **5** were based on reported methodologies. In particular, the removal of the carbonyl O-atom of **1** to provide **4** was straightforwardly achieved through a modified *Clemensen* reduction [25]. The subsequent conversion of **4** to **5** by hydrogenolysis of the corresponding 5-phenyltetrazolyl ether, according to *Musliner* and *Gates* [26], gave poor results, even in the presence of a large amount of catalyst. Conversely, **4** was smoothly deoxygenated to **5** *via* Li/NH_3 treatment of the corresponding phosphate ester [27][28]. All the reactions proceeded in almost quant. yields without the formation of by-products. To minimize isotope fractionation by physical processes, identical fractions from parallel column chromatographies were pooled [6]. Finally, all products were submitted to bulb-to-bulb vacuum distillation prior to the ^{18}O measurements.

Preparation of Creosol **4** *from Vanillin Samples*. A soln. of vanillin (15.2 g, 0.1 mol) in EtOH (50 ml) and conc. HCl (150 ml) were added dropwise to a refluxed and well-stirred mixture of amalgamated Zn (150 g) in conc. HCl (80 ml). After the addition was complete, the mixture was stirred and refluxed for 1 h more. The mixture was cooled, diluted with Et_2O (300 ml), and the liquid phase was decanted from the Zn. The aq. layer was removed and washed three times with Et_2O (3×100 ml). The combined org. extracts were washed with brine (2×100 ml), dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by chromatography, eluting with hexane/AcOEt (95:5 to 80:20) to obtain pure creosol (**4**) (12.4 g, 89.8 mmol; 90%).

Preparation of **5** *from the Samples of* **4**. A soln. of **4** (6 g, 43 mmol) in dry THF (50 ml) was added dropwise under N_2 to a stirred suspension of NaH (1.9 g of a 60% dispersion in mineral oil; 47 mmol), in dry THF (50 ml), with the temp. kept below 20° by external cooling. After the addition was complete, the mixture was stirred for 30 min more, and $(EtO)_2POCl$ (6.5 ml, 45 mmol) was added dropwise. The mixture was stirred at r.t. for 1 h and then poured on crushed ice. The mixture was acidified with 5% aq. HCl (100 ml) and extracted with AcOEt (3×100 ml). The combined org. phases were washed with brine (2×100 ml), dried (Na_2SO_4), and concentrated under reduced pressure. The resulting crude phosphate was dissolved in dry Et_2O (30 ml) and added dropwise under stirring to cool (−78°) liq. NH_3 (60 ml). To the resulting soln., Li powder (0.6 g, 86 mmol) was added in small portions until a deep blue color persisted for at least 2 min. After 15 min, the reaction was quenched by adding solid NH_4Cl (8 g, 150 mmol). The NH_3 was distilled off by warming at r.t., and the residue was diluted with Et_2O (200 ml) and H_2O (100 ml). The layers were separated, and the org. phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by chromatography, eluting with hexane/AcOEt (95:5 to 90:10), and bulb-to-bulb distillation (oven temp. 80°/0.2 Torr) to afford pure **5** (3.9 g, 32 mmol; 74%).

Isotopic Determination. The $^{18}O/^{16}O$ ratio was determined by pyrolysis of the samples over nickelized carbon under He flow (80 ml/min) at 1070° in a *Carlo Erba CHN 1108* elemental analyzer [29]. For the pyrolysis, 0.2 mg of sample was placed into a silver capsule, then thoroughly sealed and dropped into the elemental analyzer. The conversion of the org. compound into CO was based on the *Unterzaucher* reaction [30]. The isotope-ratio measurements were carried out on a *ThermoQuest Delta Plus* mass spectrometer, directly interfaced with the elemental analyzer, which performs the simultaneous determination of the abundance of each isotopomer of CO, such as $^{12}C^{16}O$ (m/z 28), $^{13}C^{16}O$ and $^{12}C^{17}O$ (m/z 29), and $^{12}C^{18}O$ (m/z 30).

The values are expressed as δ per mil (‰) scale *vs.* the working standard used ($\delta(^{18}O_{S/WS})$), according to the formula:

$$\delta(^{18}O_{S/WS}) = (R_S/R_{WS} - 1) \times 1000$$

where R_S and R_{WS} are the oxygen stable-isotope ratios of the sample and of the working standard, respectively. These results are reported *vs.* the international primary reference standard V-SMOW (Vienna Standard Mean Ocean Water) [31] according to the formula:

$$\delta(^{18}O_{S/V\text{-}SMOW}) = \delta(^{18}O_{S/WS}) + \delta(^{18}O_{WS/V\text{-}SMOW}) + (\delta(^{18}O_{S/WS}) \times \delta(^{18}O_{WS/V\text{-}SMOW}))/1000$$

A sucrose sample ($\delta = +23‰$) provided by *Werner et al.* [32] was used as the working standard. The $^{18}O/^{16}O$ ratio of the working standard was determined according to the classical method described by *Rittenberg* and *Ponticorvo* [33] based on the pyrolysis of sample at 500° in sealed quartz tubes with $HgCl_2$ as catalyst.

The determination of the $\delta(^{18}O)$ value over four repetitions gave a standard deviation of $\pm$ 0.4‰ for the samples of vanillin (solid samples) and of $\pm$ 0.7‰ for the degradation products (liq. samples).

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