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Improved rapid authentication of vanillin using $\delta^{13}C$ and δ^2H values

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Abstract Vanilla still remains one of the most important and widely used flavours in the food industry and is also extensively employed by fragrance and pharmaceutical manufacturing companies. Natural vanilla flavour, extracted from the pods of the tropic orchid *vanilla*, is considerably more expensive than synthetic vanillin. The disparity of prices between natural vanillin and that derived from other sources has given rise to many cases of fraudulent adulteration, and for more than 30 years, strenuous efforts have been made to authenticate sources of vanillin. Stable isotope analysis is one of the most powerful analytical tools to distinguish between natural vanillin and that originating from other sources. Recently, a rapid and precise method for analysis of both $\delta^{13}C$ and δ^2H values of plant methoxyl groups has been published. Here, we report an application of the method for the control of authenticity of vanillin. Carbon and hydrogen stable isotope values of the vanillin molecule and vanillin methoxyl groups of vanillin samples of different origins including authentic and synthetic samples were measured. The results clearly show that use of this approach provides a rapid and reliable authenticity assessment of vanillin. The technique used for these studies is robust and rapid, involves minimum sample preparation and requires only a small amount of vanillin sample, usually 1 mg for stable carbon and 4 mg for stable hydrogen analysis.

Introduction

Vanilla still remains one of the most important and widely used flavours in the food industry and is also extensively employed by fragrance and pharmaceutical manufacturing companies. Natural vanilla flavour, extracted from the pods of the tropic orchid *vanilla*, is considerably more expensive than synthetic vanillin. For example a few years ago, when natural vanillin was costing between $1,200 and $4,000/kg, its synthetic equivalent was only around $15/kg [1–3]. The yearly global demand of vanillin is more than 12,000 tons per annum, but only 120 tons per annum of natural vanillin are extracted, which corresponds with less than 1% of the yearly global demand [1]. The deficit is chiefly covered by chemical synthesis with lignin or guaiacol used for starting material.

The reason why natural flavours attract premium prices is because they are very highly sought-after by the consumer. In the jurisdiction of the European Union, flavours which are produced biotechnologically from natural starting materials must *de jure* be considered natural. Thus, great efforts have been made to produce vanillin employing microbial techniques using natural starting materials such as eugenol or ferulic acid. However, even vanillin produced biosynthetically is not economically competitive when compared to synthetic vanillin. The disparity of

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prices between natural vanillin and that derived from other sources has given rise to many cases of fraudulent adulteration, and for more than 30 years, strenuous efforts have been made to authenticate sources of vanillin. This necessitated development of analytical methods to determine the origin of vanillin, and these techniques still require further refinement and advancement.

Stable isotope analysis is one of the most powerful analytical tools to distinguish between natural vanillin and that originating from other sources. Initially, $\delta^{13}C$ analysis of the vanillin molecule was used to differentiate natural vanillin from vanillin produced from lignin of conifer wood. Here, advantage was taken in view from the fact that the vanilla orchid is a CAM-plant which produces $\delta^{13}C$ biomass values in a characteristic range (around $-21.0‰$) different from plant biomass where CO_2 is fixed by the C_3-(wood) or the C_4-pathway of photosynthesis. This method was also suitable for detection of vanillin synthesised using guaiacol or eugenol from C_3 or C_4 plants [4–7].

However, measurement of the $\delta^{13}C$ values of vanillin was no longer sufficient for authenticity purposes when it became possible to enrich synthetic vanillin in ^{13}C on both the methoxyl and aldehydic groups and thereby produce synthetic material with the same $\delta^{13}C$ value as that of natural vanillin. New techniques were therefore needed to detect this type of sophisticated adulteration. One approach was the specific stable isotope analysis of the methoxyl group of vanillin which required cleavage of methoxyl groups by hydriodic acid (HI) [11–13] and employment of pyrolytic techniques [14]. Further progress was achieved by analysis of $\delta^{18}O$ values of vanillin [8] and by quantification and $\delta^{13}C_{V-PDB}$ correlations of characteristic components of natural vanilla like vanillin and 4-hydroxybenzaldehyde [9, 10]. Subsequently, multielement stable isotope analysis became popular, and several studies reported the $\delta^{13}C$ and δ^2H values of all carbon and hydrogen atoms of the vanillin molecule. This was done using a site-specific natural isotope fractionation–nuclear magnetic resonance (SNIF–NMR) technique [15–18]. However, there are drawbacks to this procedure in that a long time interval is required for measurement, several lengthy procedures are necessary for extraction and purification of vanillin as well as a large sample amount is needed to conduct the analysis (∼500 mg).

Recently, a rapid and precise method for analysis of both $\delta^{13}C$ and δ^2H values of plant methoxyl groups without any measurable fractionation has been published [19, 20], which is based on the 'Zeisel method' [21], where methoxyl groups are transformed to methyl iodide (CH_3I) using HI (Fig. 1). The reaction was first employed together with continuous flow isotope ratio mass spectrometry by Keppler et al. [22]. Here, we report an application of the
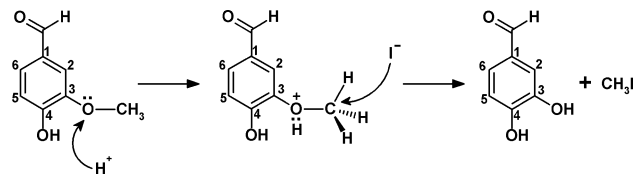

Fig. 1 Reaction between vanillin and hydriodic acid

method for the authenticity control of vanillin. Carbon and hydrogen stable isotope values of the vanillin molecule and vanillin methoxyl groups of vanillin samples of different origins (including authentic and synthetic samples) were measured and then compared graphically in a number of different permutations. The results clearly show that use of this approach provides a rapid and reliable authenticity assessment of vanillin. Moreover, only a minimal amount of sample (1–4 mg) is required to perform the analysis.

Materials and methods

Chemicals

Hydriodic acid (55–58%) was obtained from Fluka (Buchs, Switzerland), Methyl *tert*-butyl ether (MTBE, 99.5%) from Roth (Karlsruhe, Germany). Methyl iodide (99.5%) sourced from Sigma–Aldrich (Seelze, Germany) was used as a reference standard. Acetonitrile and hexafluorobenzene for NMR sample preparation were purchased from Merck (Darmstadt, Germany) and Fluka, respectively.

Vanillin samples

Commercially available synthetic vanillin samples were purchased from different suppliers: Roth (Karlsruhe, Germany; >99%), Merck (Darmstadt, Germany; Reag. Ph. Eur.), Sigma–Aldrich (Schnelldorf, Germany; 99%) and Fluka (Buchs, Switzerland; ≥98%). Semisynthetic vanillin ("vanillin *ex* lignin"; spruce wood; Eurovanillin supreme) and synthetic vanillin ("vanillin *ex* guaiacol"; Eurovanillin regular) were provided by Borregaard Synthesis (Sarpsborg, Norway).

Authentic vanillin ("vanillin ex vanilla") was isolated from vanilla pods of three vanilla species (*V. planifolia, V. tahitensis* and *V. pompona*), according to the official method of AOAC [23], at the Institute for Food Chemistry of the University of Frankfurt/Main.

The samples declared as "natural" and that as "ex lignin" were obtained from Haarmann & Reimer, Holzminden, Germany, and two samples were of unknown origin (provided by the Institute for Food Chemistry of the University of Frankfurt/Main). A detailed overview of all samples investigated in this study is presented in Table 1.

Table 1 Stable carbon and hydrogen isotope values of vanillin bulk and vanillin methoxyl groups

No.	Sample identification	Sample origin	Vanillin bulk values		Vanillin methoxyl groups	
			$\delta^{13}C_{V-PDB}$ [‰][a]	$\delta^{2}H_{V-SMOW}$ [‰][b]	$\delta^{13}C_{V-PDB}$ [‰][a]	$\delta^{2}H_{V-SMOW}$ [‰][b]
1	Vanillin Merck	Synthetic. commercial	−28.45	72.9	−41.73	−111.1
2	Vanillin Aldrich	Synthetic. commercial	−28.89	60.9	−49.36	−157.9
3	Vanillin Fluka	Synthetic. commercial	−29.06	79.7	−42.91	−84.5
4	Vanillin Roth	Synthetic. commercial	−29.74	54.6	−52.24	−169.4
5	Eurovanillin regular	Synthetic ex guaiacol	−28.17	74.6	−29.73	−121.4
6	Vanillin (B) H&R[c]	Unknown. declared as "ex lignin"	−32.46	−61.7	−37.15	−235.6
7	Eurovanillin supreme	Synthetic ex spruce wood	−28.08	−155.0	−19.63	−313.2
8	Vanillin nat. H&R[c]	Unknown. declared as "natural"	−19.65	−55.7	−19.03	−173.1
9	Vanillin ex *V. planifolia*	Authentic. self-extracted	−18.52	−55.9	−24.08	−171.5
10	Vanillin ex *V. tahitensis* 1	Authentic. self-extracted	−14.58	−58.5	−8.43	−181.6
11	Vanillin ex *V. tahitensis* 2	Authentic. self-extracted	−14.63	−50.2	−7.08	−175.9
12	Vanillin ex *V. pompona* 1	Authentic. self-extracted	−17.13	−47.4	−19.43	−149.0
13	Vanillin ex *V. pompona* 2	Authentic. self-extracted	−18.02	−50.6	−19.46	−157.8
14	Unknown origin 1	Unknown	−36.47	−150.3	−49.52	−183.7
15	Unknown origin 2	Unknown	−28.54	80.5	−43.60	−107.7

[a] Standard deviation over five measurements was better than ±0.27‰

[b] Standard deviation over five measurements was better than ±2.8‰

[c] Haarmann and Reimer

Sample preparation

Vanillin

Vanillin samples (0.5–1 mg for carbon, 3–4 mg for hydrogen analysis) were dissolved in methyl *tert*-butyl ether (1 mL) and then a portion (1–2 μl) was automatically injected into the GC-C/P-IRMS system.

Vanillin methoxyl group

Hydriodic acid (0.5 mL, 55–58%) was added to vanillin samples (1–2 mg for carbon, 4–7 mg for hydrogen analysis) in crimp glass vials (1.5 mL), which were then sealed with crimp caps containing PTFE lined butyl rubber septa (thickness 0.9 mm). The vials were placed in an oven and heated for 30 min at 130 °C. At the end of the heating period, the vials were equilibrated at room temperature (22 ± 0.5 °C, air conditioned room) for at least 30 min. Following the equilibration period, a sample of vial headspace gas (30–90 μL) was automatically injected into the GC-C/P-IRMS system.

CH₃I reference standards were prepared by injection of liquid CH₃I standard (1 μL) through the septum of sealed vials. The standards were then treated in a similar fashion to the samples.

$\delta^{13}C$ sample analysis using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS)

Determinations of $^{13}C/^{12}C$-isotope ratios were performed using an HP 6890N gas chromatograph (Agilent, Santa Clara, USA) equipped with an auto sampler A200S (CTC Analytics, Zwingen, Switzerland), coupled to a Delta-^{PLUS}XL isotope ratio mass spectrometer (ThermoQuest Finnigan, Bremen, Germany) via an oxidation reactor [ceramic tube (Al₂O₃), length 320 mm, 0.5 mm i.d., with Cu/Ni/Pt wires inside (activated by oxygen), reactor temperature 960 °C] and a GC Combustion III Interface (ThermoQuest Finnigan, Bremen, Germany). The gas chromatograph (GC) was fitted with a ZB-5 ms capillary column (Phenomenex, Torrance, USA) (30 m × 0.25 mm i.d., d_f 1.0 μm).

GC conditions for vanillin bulk ^{13}C values were split injection (ratio 10:1), injector temperature 200 °C; initial oven temperature at 150 °C for 0 min, ramp at 4 °C/min to 190 °C and ramp at 15 °C/min to 230 °C for 1 min. Helium was used as carrier gas at a flow of 1.8 mL/min constant flow. For analysis of methoxyl groups, only the GC oven temperature program was altered: initial oven temperature at 30 °C for 3.8 min, ramp at 30 °C/min to 100 °C.

All $^{13}C/^{12}C$-isotope ratios are expressed in the conventional δ notation in per mil versus V-PDB, defined as:

$$\delta^{13}C = \left(\left(^{13}C/^{12}C_{sample}\right) / \left(^{13}C/^{12}C_{standard}\right)\right) - 1.$$

A tank of high purity carbon dioxide (carbon dioxide 4.5, Messer Griesheim, Frankfurt, Germany) with a known $\delta^{13}C$ value of $-23.6‰$ (VPDB) was used as the working reference gas. Samples were analysed five times ($n = 5$), and the average standard deviations of the GC-C-IRMS measurements were in the range of 0.02–0.27‰. This meets the internal precision for $\delta^{13}C$ measurements (less than 0.3‰) specified by the manufacturer for the IRMS instrument.

All $\delta^{13}C$ values obtained from analysis of methoxyl groups were corrected using the CH_3I working standard. The $\delta^{13}C$ value of CH_3I working standard was calibrated against international reference substances (IAEA-CH6, IAEA-CH-7, NBS-22) using an offline EA-IRMS (elemental analyser-isotopic ratio mass spectrometer, Iso-Analytical Ltd, Sandbach, UK). The calibrated $\delta^{13}C$ value in ‰ versus V-PDB for CH_3I was $-69.27 \pm 0.05‰$ ($n = 15$, 1σ). CH_3I standard measurements were performed after every fifth sample injection. Correction of the vanillin bulk $\delta^{13}C$ values were made against a vanillin working standard ($\delta^{13}C_{V\text{-}PDB} = -29.6‰$) supplied by the Institute for Food Chemistry of the University of Frankfurt.

δ^2H sample analysis using gas chromatography pyrolysis isotope ratio mass spectrometry (GC-P-IRMS)

Determinations of D/H-isotope ratios were performed using an HP 6890N gas chromatograph (Agilent, Santa Clara, USA) equipped with an auto sampler A200S (CTC Analytics, Zwingen, Switzerland), coupled to a Delta-^{PLUS}XL isotope ratio mass spectrometer (ThermoQuest Finnigan, Bremen, Germany) via a pyrolysis reactor [ceramic tube (Al_2O_3), length 320 mm, 0.5 mm i.d., reactor temperature 1,450 °C] and a GC Combustion III Interface (ThermoQuest Finnigan, Bremen, Germany). The GC column was identical to that used for carbon isotope measurements.

GC conditions for vanillin bulk D/H values were split-less injection, injector temperature 200 °C; initial oven temperature at 100 °C, ramp at 5 °C/min to 240 °C and hold for 2 min. Carrier gas flow (helium) was 0.8 mL/min, constant flow.

For methoxyl group analysis, the following GC conditions were employed: splitless injection, initial oven temperature at 30 °C for 10 min, ramp at 20 °C/min to 105 °C and hold for 1 min. Helium was used as carrier gas at a flow of 0.6 mL/min constant flow.

All D/H-isotope ratios are expressed in the conventional δ notation in per mil versus V-SMOW, defined as:

$$\delta^2H = \left(\left(^2H/^1H_{sample}\right) / \left(^2H/^1H_{standard}\right)\right) - 1.$$

A tank of high purity hydrogen (hydrogen 5.0, Linde, Höllriegelskreuth, Germany) with a known δ^2H value of $-295‰$ (VSMOW) was used as the working reference gas. The H_3^+ factor, determined daily during the measurement period, was in the range 3–4. Samples were analysed five times ($n = 5$), and the average standard deviations of the GC-P-IRMS measurements were in the range of 0.4–2.6‰. This meets the internal precision for δ^2H measurements (less than 3‰) specified by the manufacturer for the IRMS instrument.

All δ^2H methoxyl group values were corrected relative to VSMOW using the CH_3I working standard. The δ^2H value of CH_3I was calibrated against international reference substances (IA-R002, IAEA-CH-7, NBS-22) using an offline EA-IRMS (elemental analyser-isotopic ratio mass spectrometer, Iso-Analytical Ltd, Sandbach, UK). The calibrated δ^2H value in ‰ versus V-SMOW for CH_3I was $-179.0 \pm 2.9‰$ ($n = 15$, 1σ). The standard was measured after every fifth sample injection. Correction of the vanillin bulk δ^2H values was made using the vanillin working standard as described above ($\delta^2H_{V\text{-}SMOW} = 79.7‰$).

Results and discussion

The results of the stable carbon and hydrogen isotope measurements for both the vanillin bulk and methoxyl group of all samples are presented in Table 1. To enable simple visual identification, the six possible 2D permutations of the isotope values were plotted for all samples. Four of these ($\delta^{13}C_{Bulk}$ vs. δ^2H_{Bulk}, $\delta^{13}C_{Methoxyl}$ vs. $\delta^2H_{Methoxyl}$, $\delta^{13}C_{Bulk}$ vs. $\delta^{13}C_{Methoxyl}$ and δ^2H_{Bulk} vs. $\delta^2H_{Methoxyl}$) are displayed in Fig. 2a–d. The other two 2D combinations (δ^2H_{Bulk} vs. $\delta^{13}C_{Methoxyl}$ and $\delta^{13}C_{Bulk}$ vs. $\delta^2H_{Methoxyl}$) are not shown here because the pattern of isotope information was almost identical to that presented in Fig. 2a, d and thus did not assist with interpretation of data in this instance.

Comparison of the measured $\delta^{13}C$ and δ^2H bulk values of vanillin with other published isotope data for vanillin (see for example Schmidt et al. [24]) reveals good agreement. Literature values for $\delta^{13}C_{Bulk}$ are in the range of -16.8 to $-21.5‰$ for natural vanillin from vanilla pods and -24.9 to $-36.2‰$ for semisynthetic vanillin from wood lignin, guaiacol and eugenol. In this study, natural vanillin varied from -14.6 to $-19.7‰$, whilst synthetic material was in the range -28.1 to $-36.5‰$. Reported δ^2H_{Bulk} values for vanillin from vanilla pods ranged from -52 to $-115‰$, whereas in comparison, wood-derived

Fig. 2 Stable carbon and hydrogen isotope values of vanillin bulk and vanillin methoxyl groups of different origins. (a) $\delta^{13}C_{Bulk}$ versus δ^2H_{Bulk} (b) $\delta^{13}C_{Methoxyl}$ versus $\delta^2H_{Methoxyl}$ (c) $\delta^{13}C_{Bulk}$ versus $\delta^{13}C_{Methoxyl}$ (d) δ^2H_{Bulk} vs. $\delta^2H_{Methoxyl}$. The ellipses drawn correspond to the isotope values measured in this study (no statistical information)
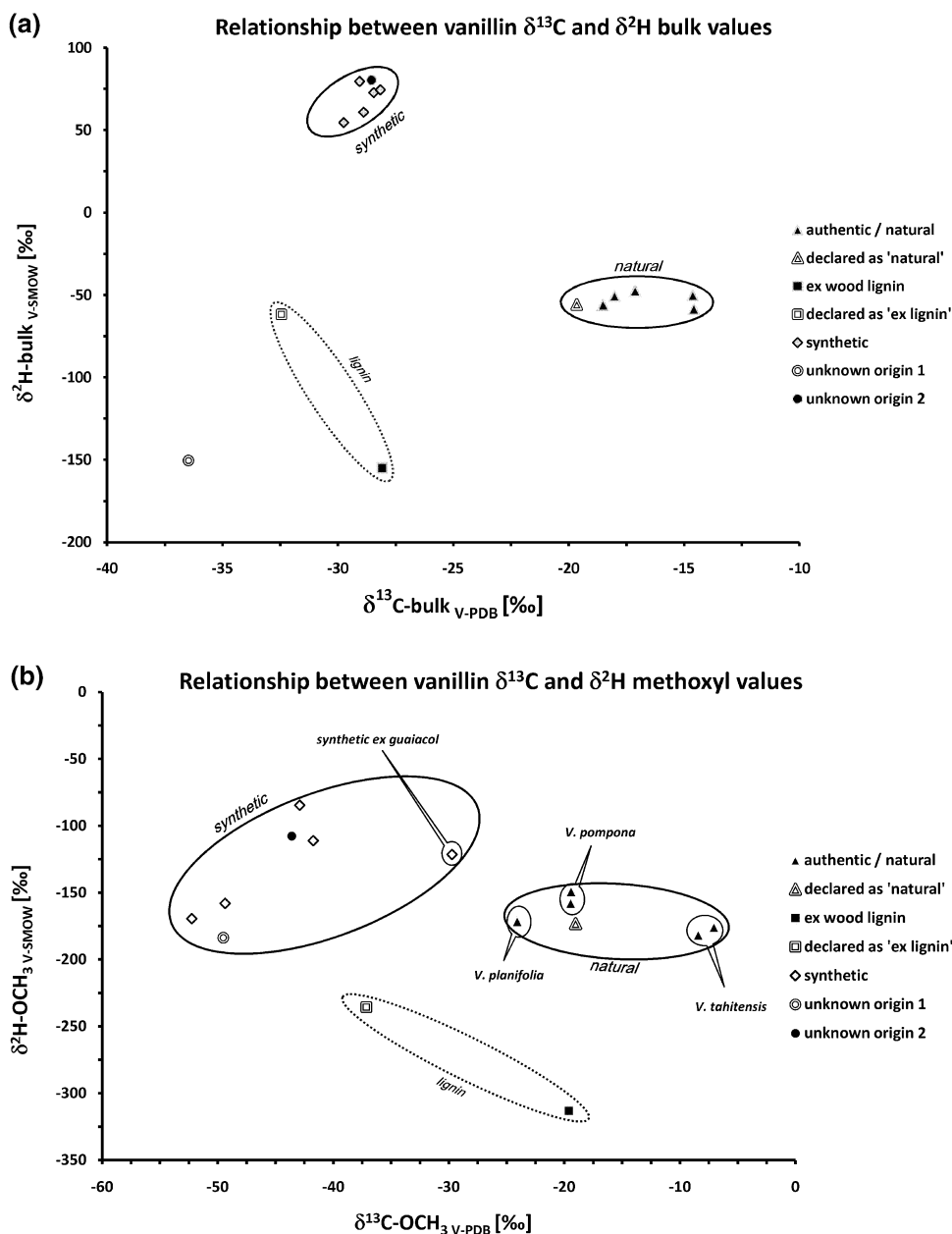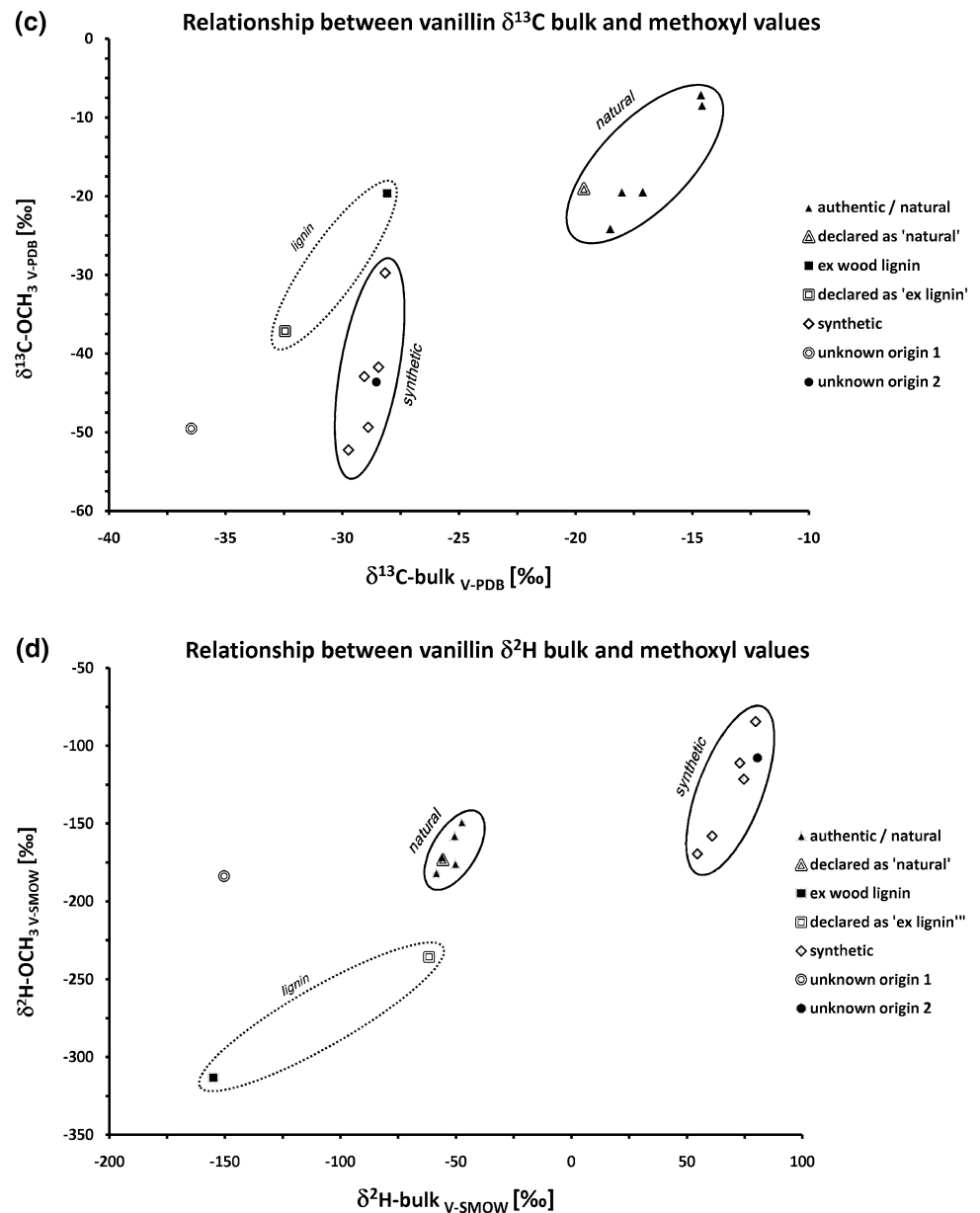

vanillins were considerably more depleted (−170 to −204‰) and synthetic vanillins from guaiacol slightly enriched (−17 to −23‰) in their ^2H content. The δ^2H_{Bulk} values of vanillin from vanilla pods measured in this study all fell within a narrow range (−47.4 to −58.5‰), whilst the synthetic samples were more enriched in ^2H (+54.6 to +80.5‰). Whenever the $\delta^{13}C$ and δ^2H vanillin bulk values are plotted against each other (Fig. 2a), authentic vanillin samples extracted from vanilla pods can be clearly distinguished from all other vanillin samples including synthetic, lignin-derived and that derived from ferulic acid. Thus, from Fig. 2a, it would appear that determination of $\delta^{13}C$ and δ^2H vanillin bulk values would be sufficient to distinguish between authentic vanillin and vanillin arising from

other sources. However, using advanced adulteration techniques including ^{13}C and ^2H enrichment of vanillin [12, 13, 25], it is possible to change the bulk carbon and hydrogen isotope composition of vanillin samples. To police this type of fraudulent adulteration, improved techniques such as the SNIF–NMR were developed [15–18]. Here, we show that measurement of $\delta^{13}C$ and δ^2H values of the methoxyl group of vanillin would greatly assist with identification of the origin of vanillin and thus aid with the efforts countering fraud.

Up to now, only scant data is available on isotope measurements of methoxyl groups in vanillin. The first publication on this topic was presented by Galimov et al. [11], but the $\delta^{13}C$ isotopic values measured in that study

Fig. 2 continued

(c)

Relationship between vanillin $\delta^{13}C$ bulk and methoxyl values

(d)

Relationship between vanillin δ^2H bulk and methoxyl values

are not easy to correlate with the V-PDB scale in common use today. Nevertheless, Galimov and co-workers reported that the methoxyl group carbon is isotopically lighter than any of the other carbon atoms in the vanillin molecule. A few years later, Krueger et al. [12, 13] reported on the detection of fraudulent adulteration of vanillin by the use of synthetic vanillin with ^{13}C-enriched methoxyl groups. In those studies, the reported $\delta^{13}C_{Methoxyl}$ values of natural vanillin and lignin vanillin were in the range of -23.3 to $-27.4‰$ and -28.2 to $-28.8‰$, respectively. Several SNIF–NMR studies provide D/H ratios of vanillin methoxyl groups [15–17]. John et al. [16] reported $\delta^2H_{Methoxyl}$ values for vanillin from vanilla pods ranging from 127.5 to 123.5 ppm, the equivalent of -181.5 to $-207.2‰$ when

converted to the V-SMOW scale (1 ppm ^2H corresponds to roughly 6.42‰ in δ^2H-units; reference point: V-SMOW = 155.76 ± 0.5 ppm [26]). Comparable to these values, Remaud et al. [17] found a mean $\delta^2H_{Methoxyl}$ value for natural vanillin of $-187.3‰$ (126.6 ppm) as well as $-320.2‰$ (105.9 ppm) for vanillin derived from lignin and $-107.0‰$ (139.1 ppm) for synthetic vanillin from guaiacol.

Measurements performed in this study reveal $\delta^{13}C_{Methoxyl}$ values (Table 1) more enriched in ^{13}C than those reported in the literature for natural vanillin (-7.1 to $-24.1‰$) and "lignin-vanillin" ($-19.6‰$). Commercially available synthetic vanillin had very light $\delta^{13}C_{Methoxyl}$ values (-41.7 to $-52.2‰$) compared to those for natural vanillin.

Results from $\delta^2 H_{Methoxyl}$ measurements (Table 1) show no significant trends in the values for natural and synthetic samples (range -84.5 to -183.7‰) except for the synthetic vanillin which had originated from spruce wood (-313.2‰). This strongly depleted value is presumably solely due to ^2H depletion of the methoxyl groups of the wood lignin itself, an observation previously reported by Keppler et al. [27]. Thus, analysis of $\delta^2 H_{Methoxyl}$ would be a powerful tool to validate a vanillin as derived from wood lignin, although it must be borne in mind that the $\delta^2 H_{Methoxyl}$ value will be strongly dependent on the geographical region where the wood was grown. $\delta^2 H_{Methoxyl}$ values for wood lignin from central Europe and Scandinavia have been found to be in the range -260 to -330‰ [27]. The latter more negative value fits well with the value measured here for the vanillin sample produced from spruce wood. However, if vanillin were to be produced from wood originating in more southern latitudes or equatorial regions, the vanillin methoxyl group would be enriched in ^2H with $\delta^2 H_{Methoxyl}$ values up to -200‰ expected. However, even these values would still be easily distinguishable from the values measured for the synthetic and natural vanillin in this study (see Table 1 and Figures with $\delta^2 H_{Methoxyl}$). Moreover, in tropical regions, coniferous wood is not as widespread as in colder regions, an important consideration in this context since vanillin cannot be synthesized from wood of deciduous trees. Unlike coniferous wood, lignin from deciduous wood consists of sinapyl alcohol which has two methoxyl groups on the aromatic moiety.

In Table 1, there is a sample declared as "ex lignin", which, as can be seen in each of Fig. 2a–d is well separated from all other samples (isotope signature area of "vanillin ex lignin" is marked with dashed lines). Based upon the measured isotope signatures, this declaration is most certainly true since such a negative $\delta^2 H_{Methoxyl}$ value (-235.6‰, Table 1) can only originate for vanillin derived from plant lignin. The relatively large difference between the two lignin sample isotope signatures (~ 78‰) might be either due to different geographical origin of the woods (see above) or alternatively the vanillin was produced from non-wood lignin-rich plant material such as straw or brown coal. Such materials would be expected to exhibit relatively negative $\delta^2 H_{Methoxyl}$ values with a much less negative $\delta^2 H_{Bulk}$ value than that measured for the "ex wood lignin" sample.

The isotopic data of the methoxyl groups as illustrated in Fig. 2b clearly make it possible to distinguish between natural and synthetic vanillin samples. Additionally, the $\delta^{13} C_{Methoxyl}$ values may provide even more information and allow differentiation of vanillins on a species basis since the signatures measured for samples from V. planifolia,

V. tahitensis and V. pompona are clearly different. However, a much larger sample set of the investigated natural vanilla samples would require investigation to confirm this contention.

Interestingly, the methoxyl group carbon isotope signature for the synthetic sample originating from guaiacol (-29.7‰, see Table 1) is quite different when compared to the other synthetic samples (-41.7 to -52.2‰). In spite of this, whenever comparisons between $\delta^{13} C_{Bulk}$ and $\delta^{13} C_{Methoxyl}$ (Fig. 2c) and $\delta^2 H_{Bulk}$ and $\delta^2 H_{Methoxyl}$ (Fig. 2d) are considered, it becomes very evident that all synthetic samples are based on non-natural precursors. Quite simply, such positive $\delta^2 H_{Bulk}$ values ($+54.6$ to $+79.7$‰) are not found in vanillin prepared from natural substances.

In Fig. 2a, c and d, the sample labelled "unknown origin 1" can be clearly separated from all other samples, mainly due to its light $\delta^{13} C$ values ($\delta^{13} C_{Bulk}$: -36.47‰, $\delta^{13} C_{Methoxyl}$: -49.52‰). Tenailleau et al. using a $^{13} C$ NMR technique [18] reported that the $\delta^{13} C_{Methoxyl}$ of vanillin produced from guaiacol was clearly heavier (-36.8‰) than that from ferulic acid (synthetic: -46.1‰, biotechnological: -46.4‰). These workers also reported a $\delta^{13} C_{Bulk}$ value of vanillin from guaiacol to be over 6‰ more positive (-29.2‰) than that from ferulic acid (synthetic: -35.3‰, biotechnological: -36.4‰). The isotopic signature of the sample labelled "unknown origin 1" is directly comparable with that for vanillin derived from ferulic acid.

Up to now, ferulic acid used to produce vanillin biotechnologically is obtained from rice or corn bran [28–32]. Thus, biotechnologically produced vanillin must de jure be considered as natural as vanillin extracted from vanilla pods. Nevertheless, a differentiation between "biotechnological vanillin" and vanillin from vanilla pods is still required, since flavourings with a certificate of origin like "bourbon vanilla" must originate from vanilla pods. From the above results, it would appear reasonable to assume that this isotope method could provide such authentication. Unfortunately, however, authentic vanillin produced biotechnologically was not available to enable absolute confirmation of the origin of sample "unknown origin 1" and thus corroborate the above assertion. It is also interesting to point out that if a vanillin sample were of biotechnological origin, this method will indicate the substrate used for its production since the C_4-plant corn shows 10–20‰ less negative $\delta^{13} C$ biomass values than the C_3-rice plant.

The sample labelled "unknown origin 2" (Table 1) can be unambiguously identified as a synthetic sample. Visually, all the plotted isotope combinations (Fig. 2a–d) show this sample to very closely match with all known synthetic samples. Similarly, the sample "declared as natural" shows an isotope signature well matched to that of the authentic

vanillin samples extracted from vanilla pods, thus clearly verifying it to be of natural origin. Furthermore, from the graphical data, especially Fig. 2b, it is tempting to further speculate that this vanillin was produced by plants of the *pompona* species.

Conclusions

The technique used for these studies is robust and rapid, involves minimum sample preparation and requires only a small amount of sample, usually 1 mg for stable carbon and 4 mg for stable hydrogen analysis.

Our results show that determination of $\delta^{13}C$ and δ^2H values of both vanillin bulk and vanillin methoxyl groups and simple graphical correlations between these values can be used to fully differentiate between natural vanillin extracted from pods of the tropical orchid *vanilla* and vanillin from synthetic (e.g. guaiacol) or semisynthetic (e.g. lignin) sources. Indeed, even if only bulk $\delta^{13}C$ and δ^2H values of vanillin or δ^2H of the bulk and methoxyl groups are determined, then it should be possible to decide the origin of a vanillin sample (see Fig. 2a, d). However, the information gained from the 2D plots makes it virtually impossible for adulterated vanillins to remain undetected, even when sophisticated techniques that change the isotopic content of the vanillin are employed.

All samples with unknown or high uncertainties in origin could be clearly identified in this study. Thus, sample number 8 (Table 1, "declared as natural") was indeed confirmed as vanillin from vanilla pods, sample number 14 (Table 1, "unknown origin 1") was recognised as non-natural vanillin based on ferulic acid, whilst sample number 15 (Table 1, "unknown origin 2") was clearly identified as synthetic vanillin most likely derived from guaiacol. Furthermore, it appears that the method may also have the potential to differentiate between vanilla species, such as *V. planifolia*, *V. tahitensis* and *V. pompona*. It is important to point out that this study was conducted with a restricted sample set and thus has its limitations. Hence, more studies involving a larger number of vanillin samples from each of the different sources will be necessary so as to fully establish the potential of this method. However, despite the aforementioned limitations of the study, it would seem that the method would be ideally suited to the food industry and be of major assistance for the detection of adulteration of vanilla flavourings and foodstuff containing vanillin.

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