Progress in the Authenticity Assessment of Vanilla. 1. Initiation of Authenticity Profiles

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The genuineness of vanilla is discussed by integral authenticity evaluation of vanillin and some characteristic minor compounds such as 4-hydroxybenzyl alcohol, vanillic acid, 4-hydroxybenzaldehyde, anisic alcohol, anisic acid, and 4-hydroxybenzoic acid, including isotopic data as well as gas chromatographic quantification of compounds analyzed. Such integral authenticity profiles are new and promising perspectives in the authenticity evaluation of genuine vanilla. Data-banks with two parameter series (isotopic data *and* quantification using internal standards) are suggested to be realized by interlaboratory ring tests.

Keywords: *Genuine vanilla; authenticity assessment; authenticitiy profiles; isotope analysis; multicomponent cGC-IRMS analysis*

INTRODUCTION

Because of its outstanding and famous flavor quality, vanilla is highly appreciated by the consumer and most important from a commercial point of view. *Vanilla* plants belong to the orchid family, existing in more than 100 species, but only three species, *Vanilla planifolia*, *V. tahitensis*, and *V. pompona*, are of practical relevance. By far the most important is *V. planifolia*.

Continuously increasing demand for genuine vanilla (>1800 tons per year), limited natural resources, and high price levels may serve as stimulating motivations for fraudulent addition of synthetic or semisynthetic vanillin. Also, manipulation of the ^{13}C content is a well-known practice of natural vanillin imitation. Against this background, reliable analytical methods for the origin assessment of vanilla flavors are of fundamental interest.

In recent years isotope discrimination during biosynthesis has been used as an endogenous parameter in the authenticity control of natural flavor and fragrance compounds. Special techniques of nuclear magnetic resonance as well as mass spectrometry have proved to be reliable methods for measuring these isotopic discrimination effects.

Using ^{2}H NMR (SNIF-NMR), it is possible to measure the ^{2}H content even at individual atomic sites of molecules. Regarding the molecule vanillin, five different monodeuterated structures are used for comparing samples. When the values of these monodeuterated compounds are known, a unique isotopic fingerprint results, closely related to the origin of the molecule and inimitable by synthetic copies (Martin and Remaud, 1993). Therefore, a real guarantee of the natural origin of vanilla is possible, but, in practice, for quality assurance and control, this method is limited by extremely high investment as well as operating costs, as it requires high amounts of (single) substances and cumbersome processes of concentration and purification. Therefore, this procedure is not normally practical for the judgment of single components from complex mixtures.

In recent years isotope ratio mass spectrometry has found widespread application in food science, in particular in the origin-specific analysis of vanillin (Koziet, 1992; Fayet et al., 1995; Bréas et al., 1994; Lamprecht et al., 1994).

Very recently cGC coupled online via combustion interface with isotope mass spectrometry (IRMS) has been realized successfully in the case of $^{13}C/^{12}C$ ratios. The substances eluting from the cGC column are converted into carbon dioxide in a combustion oven and then directly analyzed in the isotope mass spectrometer, adjusted for the simultaneous recording of masses 44, 45, and 46 in the nanomole range with high precision. The instrumental configuration of cGC-IRMS combines the precision of IRMS with the high purification effect of cGC separation, with large savings on laborious sample cleanup procedures. This method will become more and more important in authenticity control of vanilla as a sophisticated technique, which can be used for the direct $\delta^{13}C$ analysis of vanillin as well as for the characteristic minor components in vanilla extracts. These data are correlated with the amounts of compounds analyzed, yielding characteristic authenticity profiles of vanilla.

EXPERIMENTAL PROCEDURES

1. Preparation of Samples and References. *1.1. Vanilla Extraction*. As a reference for a typical vanilla composition, the AOAC method is applied (AOAC, 1980), using different samples of vanilla: 25 g of vanilla pods was finely chopped and macerated for 12 h at 40 °C with 20 mL of water in a closed vessel. Ethanol (20 mL) was added, and the mixture was thoroughly mixed and macerated for 3 days. Subsequently, the mixture was poured through a sintered filter and the filtrate collected in a 100 mL flask. The filter cake was pressed and washed with ethanol until the total volume of filtrate and washings came up to 100 mL. For GC analysis 50 mL of the ethanolic solution was extracted exhaustively with diethyl ether/pentane (1:1; total volume = 100 mL) and dried over anhydrous sodium sulfate.

1.2. V. planifolia. At least three different samples of ≥500 g were purchased from Madagascar, Comores, Indonesia, Tonga, and Mexico (harvest 1993) and from Madagascar and Comores (harvest 1994). n (samples investigated) = 21.

1.3. V. tahitensis. At least three different samples of ≥400 g were obtained from Tahiti and Moorea (harvest 1994). n = 6.

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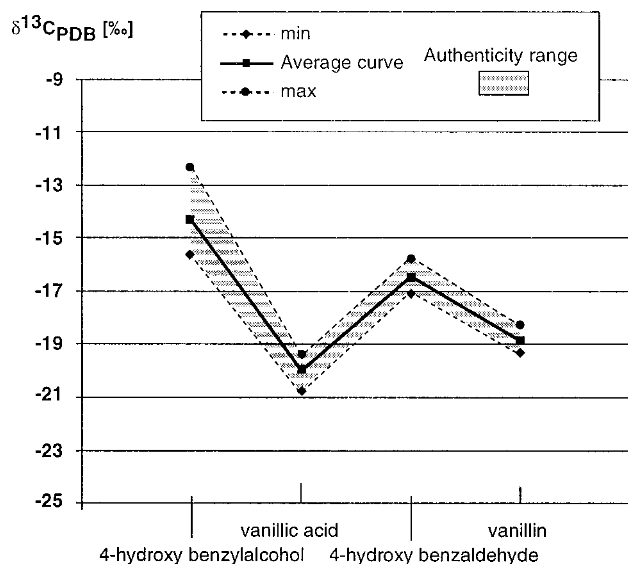


Figure 1. Isotopic fingerprint of *V. planifolia*: average curve of authentic samples ($n = 21$) of different geographical origin (see Experimental Procedures). The average curve represents a mean value of all compounds of any sample (triple measurements each, using column system I and column system II alternatively). Standard deviations for any compound ≤0.3‰.

Each sample was subjected to triple measurement using column systems I *and* II (section 2.1.).

2. Gas Chromatography Online Coupled with Isotope Ratio Mass Spectrometry (cGC-IRMS). *2.1. Instrumentation.* A Varian 3400 GC is connected to a Finnigan MAT isotope mass spectrometer delta S via the combustion interface II: injector, 220 °C; split, 20 mL/min; carrier gas, helium at 1.90 mL/min. Alternative I: HP-5 (Ultra 2) fused silica capillary column, 50 m × 0.32 mm, df, 0.25 μm; temperature program, 80 °C isothermal for 5 min, then raised at 5 °C/min to 300 °C. Alternative II: fused silica capillary column coated with heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin 33% in PS 268, 25 m × 0.32 mm, df, 0.5 μm; temperature program, 100 °C isothermal for 5 min, then raised at 3 °C/min to 230 °C; oxidation reactor (Cu/Ni/Pt), 1000 °C, hot ion source.

2.2. GC-IRMS Technical Adjustment. Besides phenolic aldehydes, phenolic alcohols and acids, such as 4-hydroxybenzyl alcohol, 4-hydroxybenzoic acid, vanillyl alcohol, vanillic acid, anisic alcohol, and anisic acid, are known as characteristic minor compounds of vanilla extracts. Exact GC-IRMS measurements of these compounds failed if the delta S machine was used in its conventional technical layout; this was due to extreme peak broadening and discrimination of compounds analyzed, which in turn was caused by Al_2O_3 absorption effects within the first part (2 cm) of the combustion device.

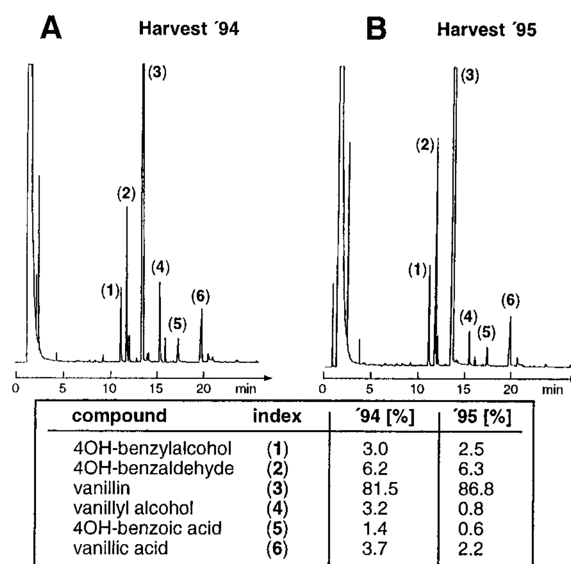
To overcome this technical disadvantage, the connection device, commonly used between gas chromatograph and combustion oven, was pierced (0.5 mm diameter) to pass through the fused silica coupling capillary (0.32 mm i.d., corresponding with 0.42 mm o.d.).

2.3. Calculation. Isotope ratios are expressed in δ notation versus the PDB standard:

$$\delta^{13}C_{PDB} = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{PDB}}{(^{13}C/^{12}C)_{PDB}} \times 1000\ (‰)$$

The isotope mass spectrometer was calibrated against CO_2 gas with a defined $^{13}C/^{12}C$ content relative to the PDB standard, defined as the $^{13}C/^{12}C$ isotope ratio for CO_2 gas yielded by the reaction of *Belemnitilla americana*, from the Peedee Formation of South Carolina with 100% phosphoric acid; it is commonly used for $^{13}C/^{12}C$ IRMS (Craig, 1957).

The system performance was checked by introducing a mixture of reference compounds with well-known $\delta^{13}C_{PDB}$ values: 5-nonanone (−27.24‰), thymol (−26.37‰). Average curves are generated by mean values of all compounds of any



compound	index	´94 [%]	´95 [%]
4OH-benzylalcohol	(**1**)	3.0	2.5
4OH-benzaldehyde	(**2**)	6.2	6.3
vanillin	(**3**)	81.5	86.8
vanillyl alcohol	(**4**)	3.2	0.8
4OH-benzoic acid	(**5**)	1.4	0.6
vanillic acid	(**6**)	3.7	2.2

Figure 2. Annual influences on $\delta^{13}C$ values of *V. planifolia* compounds: (A) FID chromatogram of *V. planifolia*, extracted beans from Comores (1994); [compounds analyzed: 4-hydroxybenzyl alcohol (**1**), 4-hydroxybenzaldehyde (**2**), vanillin (**3**), vanillyl alcohol (**4**), 4-hydroxybenzoic acid (**5**), vanillic acid (**6**)]; (B) FID chromatogram of *V. planifolia*, extracted beans from Comores (1995) [concentrations were evaluated by integration (area %) (for chromatographic conditions see Experimental Procedures 3.2)]; (C) isotopic fingerprints of *V. planifolia* samples from Comores.

sample (triple measurements each); standard deviation for any compound was ≤0.3‰.

3. Quantititive Analysis. *3.1. Instrumentation.* A Fisons GC Vega Series II, equipped with a FID, was used.

3.2. GC Conditions: injector, 220 °C; split, 30 mL/min; detector, 300 °C; carrier gas, helium at 1.9 mL/min; HP-5 (Ultra 2) fused silica capillary column, 50 m × 0.32 mm, df, 0.25 μm; temperature program, 120 °C isothermal for 5 min, then raised at 3 °C/min to 300 °C.

3.3. Calculation. The peaks were evaluated by integration (area %).

4. Separation of Vanillin (Unsuccessful Experiments). *4.1. HPLC.* Columns used were a Superspher RP18, a Lichrospher RP18, and Lichrospher RP8 (5 and 3 μm): methanol/acidified water; methanol/buffer; gradient programs [also under conditions according to those of Lamprecht (1994) and Fraisse et al. (1984)], various temperatures, 5−40 °C; detection, UV, 270 (275) nm.

Different fractions were extracted with diethyl ether, dichloromethane, pentane, or mixtures of these solvents, dried over anhydrous sodium sulfate, and proved by GC analysis.

Annotation: Polar (SiO_2) as well as semipolar (amino-, cyano-) stationary phases were checked for LC separation of vanillin. However, these materials cannot be recommended.

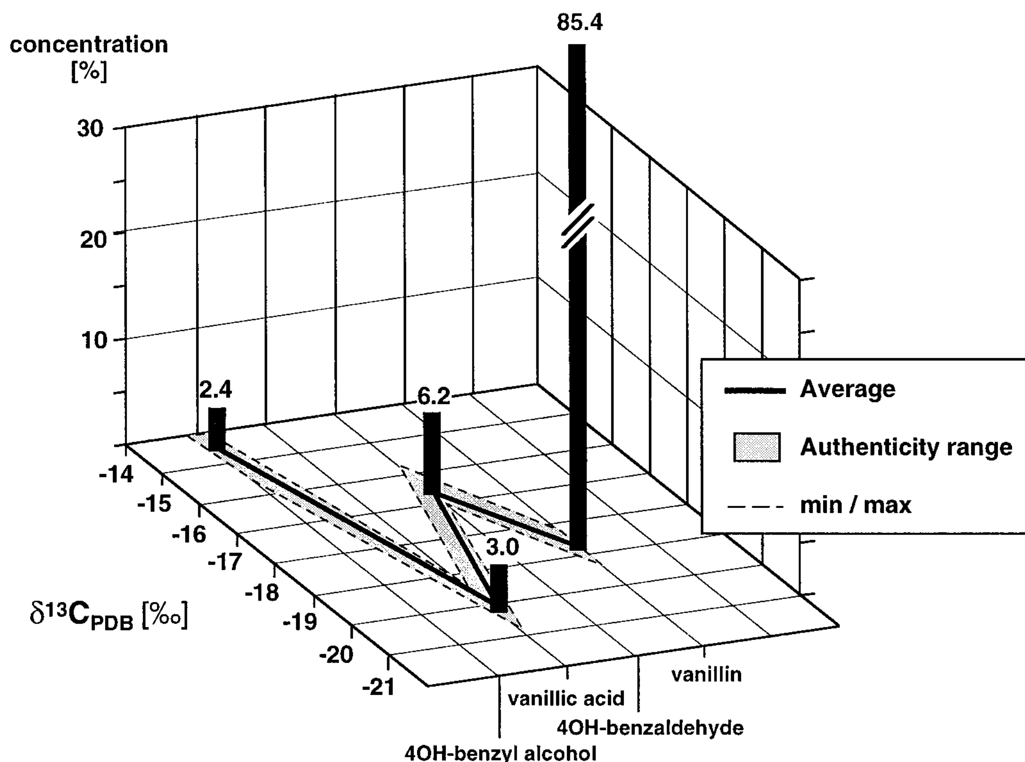


Figure 3. Authenticity profile of *V. planifolia*; integral consideration of isotopic and quantitative data (3D plot): *x*-axis, $\delta^{13}C$ values (‰); *y*-axis, compounds analyzed; *z*-axis; concentrations (area %).

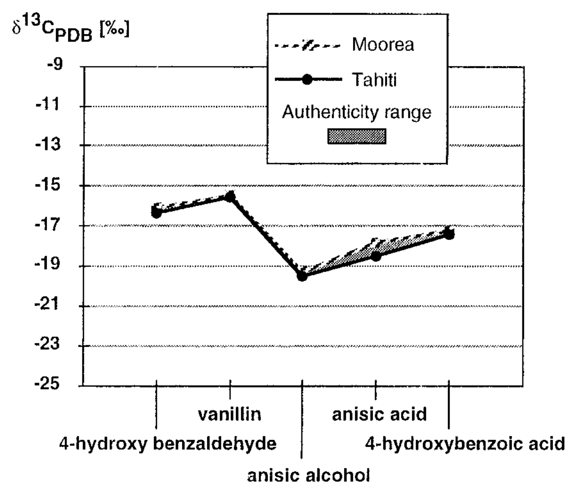


Figure 4. Isotopic fingerprint of *V. tahitensis*: *x*-axis, $\delta^{13}C$ values (‰); *y*-axis, compounds analyzed (for conditions see Figure 1).

4.2. Preparative High-Resolution Segment Chromatography (PHS). A Bakerbond PHS was used (Kraft and Strömmer, 1989): solvent 1, dichloromethane/diethyl ether (95:5; 200 mL); solvent 2, dichloromethane/diethyl ether/pentane (8:1:1; 200 mL); each fragment was eluted by 10 mL of diethyl ether, concentrated, and investigated by GC analysis (for conditions see section 3.2).

4.3. Preparative Thin Layer Chromatography. Silica gel plates were used with dichloromethane/ethanol (95:5) as a solvent; detection was by UV at 254 nm.

4.4. Crystallization. The procedure used was taken from Guarino et al. (1982).

4.5. Derivatization Procedures. Hydrazones and semicarbazones were generated according to the method of Hünig et al. (1979). Water soluble Girard hydrazones were obtained according to the method of Armour et al. (1959).

4.6. Purification by Carrez Filtration. To get purified vanilla extracts, filtration by Carrez I and Carrez II was applied according to the procedure of Dalang et al. (1982); unfortunately, the minor compounds of vanilla extracts suffer from isotopic discrimination effects caused by Carrez filtration.

RESULTS AND DISCUSSION

Vanilla is accepted as the most important flavor worldwide. Its fine unique taste and odor and its soothing effect make this aroma essential for many different flavor and fragrance compositions. Additionally, synthetic vanillin is needed for flavors and perfumery oils. In recent years, considerable progress in the synthetic reconstitution of flavors has occurred to satisfy the requirements of flavoring materials worldwide. On the other hand, naturalness of foods and beverages is in high demand by the customer. Therefore, authenticity evaluation of vanilla has become more and more important in quality assurance of vanilla flavors.

Stable isotope measurements have been reported as efficient methods in the authenticity control of natural flavors and fragrances. Capillary gas chromatography, coupled on-line with IRMS, is the method of choice in the direct analysis of vanillin as well as for the characteristic minor components in vanilla extracts. Because of the well-known fact that mainly vanillin is present in vanilla extracts, it seemed to be reasonable to separate or isolate this compound to enrich the minor components. In this investigation many methods were tested, as outlined (see sections 4.1−4.6 under Experimental Procedures). Unfortunately, each method applied leads to an isotope discrimination effect relative to the minor components. As published by Braunsdorf et al. (1993) and also discussed by George et al. (1992), it is imperative especially for vanillin to keep each substance quantitatively for the $\delta^{13}C$ measurement, because chromatographic discrimination of isotopomers is detectable.

The minor components in vanilla extracts are members of different classes of substances. Therefore, different retention and adsorption effects result. Moreover, one detection wavelength cannot represent the absorption maximum for any compound. Thus, one should keep in mind there are a lot of pitfalls in view of

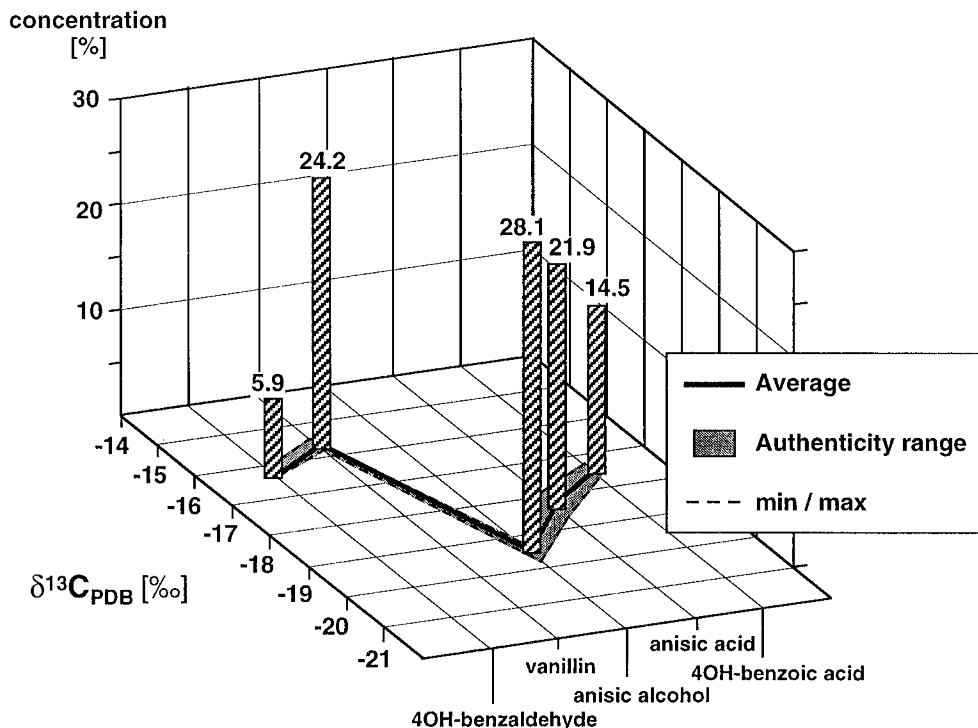


Figure 5. Authenticity profile of *V. tahitensis* ($n = 6$). Integral consideration of isotopic and quantitative data (3D plot) results in an authenticity profile characteristic for *V. tahitensis*; *x*-axis, $\delta^{13}C$ values (‰); *y*-axis, compounds analyzed; *z*-axis, concentrations (area %).

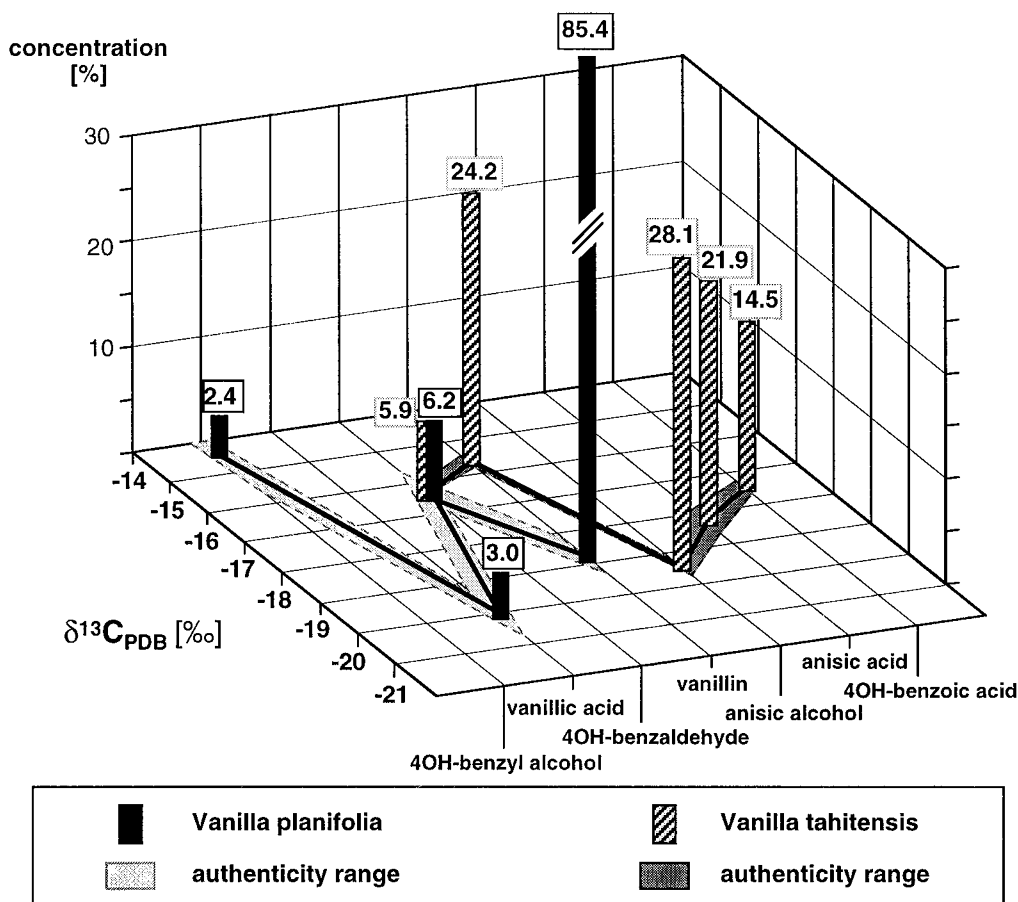


Figure 6. Authenticity profile of vanilla, obtained by summarizing Figures 3 and 5 (3D plot): *x*-axis, $\delta^{13}C$ values (‰); *y*-axis, compounds analyzed; *z*-axis, concentrations (area %) (for conditions see Figure 1).

correct $\delta^{13}C$ evaluation. The need for quantitative registration of each compound is absolutely necessary. In 1987, Fayet et al. published $\delta^{13}C$ values for 4-hydroxybenzaldehyde ($\delta^{13}C$ values from −18.8‰ to −21.6‰ for ex vanilla), obtained by isolation using preparative thin layer chromatography with double extrication. In contrast to these results, we detected 4-hydroxybenzaldehyde with considerably higher values from any vanilla extract. As can be seen from Figure 2, $\delta^{13}C$ values are independent of annual influences. The divergences

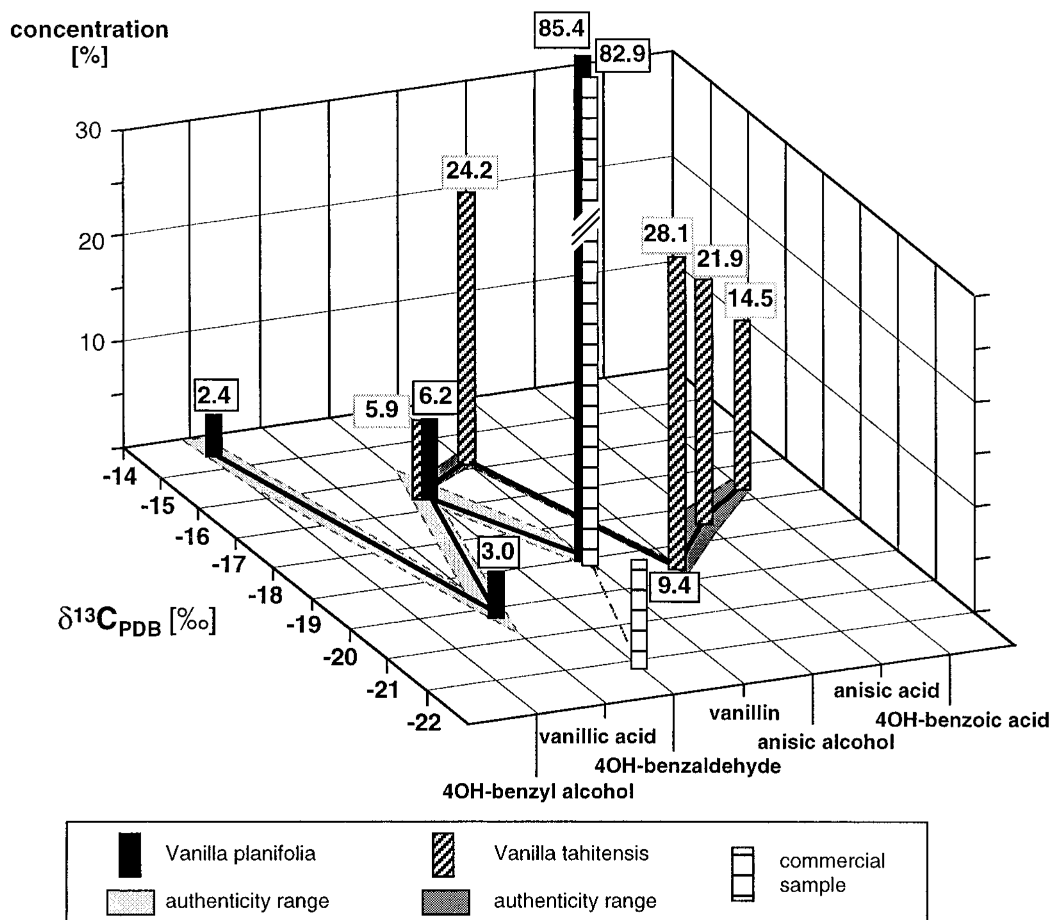


Figure 7. Authenticity profile of vanilla (see Figure 6) including integral consideration of isotopic and quantitative data of a commercial vanilla extract of unknown origin (3D plot): *x*-axis, $\delta^{13}C$ values (‰); *y* axis, compounds analyzed; *z*-axis, concentrations (area %).

reported by Fayet et al. (1987) may be explicable due to an isotopic discrimination during chromatographic purification.

In this investigation the $\delta^{13}C$ values of the minor compounds were obtained by cutoff of the vanillin (backflush mode). Additionally, all $\delta^{13}C$ values were ascertained by a second stationary phase [column alternatives I (II), see section 2.1, Experimental Procedures].

From comprehensive isotopic data of genuine vanilla extracts of defined botanical variety (*V. planifolia*), characteristic isotope "fingerprints" were detected, irrespective of geographical origin. An authenticity range was defined, arising from an average curve and minimum as well as maximum values (Figure 1).

Figure 2 demonstrates the annual influence. Whereas the concentrations of the components investigated may vary from year to year (Figure 2A,B), the $\delta^{13}C$ values seem to be constant (Figure 2C). To confirm these findings, further investigations should be done in view of annual influences on $\delta^{13}C$ values.

By correlating isotopic data with concentrations of compounds analyzed, a three-dimensional profile results as a characteristic for *V. planifolia* (Figure 3). Analogously, an authenticity profile is obtained (Figures 4 and 5) for *V. tahitensis*. In this case, besides vanillin and 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, anisic acid, and anisic alcohol were also measured.

By summarizing Figures 3 and 5, an authenticity profile for vanilla is obtained.

Figure 6 outlines the integral consideration of $\delta^{13}C$ values as well as quantitative data as a three-dimensional plot, characteristic for vanilla.

The $\delta^{13}C$ values of vanillin from different *Vanilla* varieties differ remarkably. The $\delta^{13}C$ value for *V. tahitensis* is −15.5‰ and rather high, compared with the corresponding $\delta^{13}C$ value of −19.0‰ of *V. planifolia*, whereas the $\delta^{13}C$ values for 4-hydroxybenzaldehyde are quite similar.

Furthermore, the first results from industrial aromas and commercial extracts are available now. One should keep in mind, however, that individual amounts of vanilla components in relation to each other may differ enormously. Therefore, some minor compounds of genuine vanilla remain undetectable in vanilla flavor formulations. Also, carrier compounds such as di- or triacetine are interferring agents. Nevertheless, our investigations clearly define the 4-hydroxybenzaldehyde/vanillin concentration ratios in connection with their $\delta^{13}C$ values as characteristic parameters of genuine vanilla.

As an example, a commercial ethanol extract of unknown origin for (probably) industrial use is compared with the authentic extracts (Figure 7). This presentation clearly reveals the presence of a strange 4-hydroxybenzaldehyde compound in an ethanolic vanilla extract (commercial sample), whereas the concentration ratio of 4-hydroxybenzaldehyde and vanillin seems to be in the natural range and inconspicuous.

CONCLUSIONS AND PERSPECTIVES

According to the recommendations of the International Organization of the Flavor Industry (IOFI), the authenticity of vanilla flavors is currently judged by $\delta^{13}C$ measurements of vanillin (−21 ± 0.5‰) and

p-hydroxybenzaldehyde (−22 ± 0.5‰). As further analytical criteria the so-called IOFI comparative figures are used.

The following IOFI ratios are given as guide values for vanilla beans and pure extracts: vanillin/*p*-hydroxybenzaldehyde, 10−20; vanillin/*p*-hydroxybenzoic acid, 53−110; vanillin/vanillic acid, 15−29; *p*-hydroxybenzoic acid/aldehyde, 0.15−0.35; vanillic acid/*p*-hydroxybenzaldehyde, 0.53−1.00.

The range of these ratios is set so that the growth-conditioned variation spectrum of the named substances can be recognized. It should, however, be borne in mind that both the $\delta^{13}C$ single measurements of vanillin and 4-hydroxybenzaldehyde and their quantity ratio can easily be manipulated.

In this paper cGC on-line coupled with IRMS is used for the first time for the direct $\delta^{13}C$ analysis of vanillin and for characteristic minor components in vanilla extracts, yielding authenticity profiles. This integral consideration including $\delta^{13}C$ values in connection with quantitative data of components analyzed is a new and promising perspective in comprehensive authenticity assessment of natural vanilla.

We therefore suggest to set up data-banks with two parameter series ($\delta^{13}C$ values *and* quantification using internal standards) for vanillla substances to establish authenticity profiles. This, however, is a very sizeable and time-consuming undertaking that should be realized by interlaboratory ring tests.

Current activities are focused on further systematic investigations with authentic vanilla samples (different varieties, geographic origins, and age groups) and commercially available vanilla extracts.

ABBREVIATIONS USED

cGC-IRMS, capillary gas chromatography on-line coupled with isotope ratio mass spectrometry; SNIF-NMR, site-specific natural isotope fractionation measured by nuclear magnetic resonance; HPLC, high-performance liquid chromatography; PHS, preparative high-resolution segment chromatography; IOFI, International Organization of the Flavor Industry.

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