Determination of the Authenticity of Vanilla Extracts by Stable Isotope Ratio Analysis and Component Analysis by HPLC

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The examination of vanilla extracts by stable isotope ratio analysis and chemical component analysis is described: Vanillin was extracted and purified by semipreparative high-performance liquid chromatography (HPLC), and the $^{13}C/^{12}C$ isotope ratio was determined by mass spectrometry. The concentrations of the main flavoring components relative to vanillin were determined by HPLC. Authentic extracts, prepared in the laboratory from vanilla pods, as well as commercially available extracts were examined. The authenticity and the degree of adulteration in blended samples were determined with both methods. The results are in good agreement.

Keywords: *Vanilla extracts; vanillin; stable isotope analysis; liquid chromatography; preparative liquid chromatography; pattern recognition*

INTRODUCTION

Vanilla extracts contain the sapid and odorous principles of the matured pods and are one of the highest priced and most widely used flavoring aromas. The fragrance of vanilla pods is developed during the fermentation process after picking. Vanillin and aromatic acids, aldehydes, and alcohols are liberated by cleavage of glycosidic bonds. The quality is greatly influenced by the source of origin, climatic conditions, and curing procedures.

Essences may be prepared either by direct extraction of the pods with aqueous ethanol or by dilution of concentrated extracts.

The high demands and the high price for authentic vanilla extracts lead to numerous efforts of blending and adulteration (Thompson and Hoffman, 1988). Extracts have been altered by addition of flavoring substances like the naturally occurring vanillin, isolated from lignin, which is cheaper, or the more intense tasting ethyl vanillin. Other examples are the addition of coumarin, another naturally occurring substance, mainly found in tonka pods and in low amounts in vanilla pods and piperonal. It is therefore necessary to control the quality of the vanilla extracts declared to be authentic.

The discrimination between authentic samples of vanilla pods and altered samples is not easy, because the composition of the latter can be adjusted to mimic an authenic sample. Several methods have been developed to verify the authenticity of vanilla extracts. The AOAC methods are widely used. They apply chromatography [gas chromatography (GC), thin-layer chromatography (TLC), and paper chromatography], but some of the methods are time-consuming and laborious.

Concerning the photosynthetical pathway, the Crassulacean acid metabolic pathway is a characteristic of the *Vanilla planifolia* and *Vanilla tahitensis* (Schmidt, 1986). As a consequence, natural vanillin is enriched in deuterium (Bricout et al., 1974) and carbon-13 (Winkler and Schmidt, 1980) as compared to synthetic vanillin. Therefore, one way to verify the authenticity is the determination of the stable isotope ratio of vanillin by mass spectrometry (MS) (Culp and Noakes, 1992; Hoffman and Salb, 1979). The only limitation of this method is given by the addition of ^{13}C-enriched synthetic vanillin, containing ^{13}C in certain molecule positions (Winkler and Schmidt, 1980). To solve this analytical problem, highly sophisticated degradation methods for the determination of the stable isotope ratio in certain molecule positions have been developed. The determination of the stable isotope ratio of other compounds (e.g., 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid) or the measurement of the site-specific natural isotope ratio of deuterium/hydrogen by nuclear magnetic resonance (SNIF-NMR) (Martin and Martin, 1981; Martin et al., 1985; Martin and Remaud, 1991) may be regarded as alternative.

Because vanilla extracts are very complex in their composition, the analysis of characteristic accompanying components, occurring in authentic vanilla extracts at certain concentrations, is another way to characterize vanilla extracts (Herrmann and Stöckli, 1982; Archer, 1989). In most cases this includes the determination of 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, and vanillic acid, whereby their concentration ratios in relation to vanillin are more significant than absolute concentration values (Archer, 1989).

The separation of vanilla extracts is achieved chromatographically by numerous methods such as GC, gas–liquid chromatography (GLC) (Martin et al.,1973; Schlack and Diccero, 1974), and HPLC (Alfons et al., 1980; Guarino and Brown, 1985; Archer, 1989). Limitations of these methods are due to the fact that the main components can be added to allegedly authentic extracts. To overcome this problem, the analysis may be extended to the determination of other characteristic components of vanilla extracts, e.g., vanillyl alcohol.

The aim of this work is to characterize vanilla extracts by two independent methods, stable isotope ratio analysis (SIRA) and chemical component analysis, and to estimate the authenticity or the possible degree of adulteration in extracts.

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EXPERIMENTAL PROCEDURES

Materials. *Vanilla Extracts.* Vanilla pods from several importers were purchased in local food shops. Extracts were prepared in the laboratory according to the National Formulary (NF) method for 1-fold extracts (Williams, 1984). Samples of vanilla extracts and authentic extracts were obtained from Steirerobst (Styria, Austria). These included 1−10-fold extracts and extracts of unknown fold.

Reagents. Chloroform, diethyl ether, methanol, and hydrochloric acid of analytical grade were purchased from E. Merck (Darmstadt, Germany). Water was bidistilled by a quartz distillation apparatus (Model Destamat, Heraeus, Oosterode, Germany). Vanillin of analytical grade was obtained from Fluka (Buchs, Switzerland). Vanillin isolated from lignin and guaiacol was obtained from Steirerobst. For liquid chromatography the standards 3,5-dihydroxybenzoic acid, piperonal, syringaldehyde, 4-methoxybenzylic alcohol, and 4-hydroxybenzaldehyde were purchased from Janssen (Brüggen, Germany). All others were from Sigma (Deisenhofen, Germany).

Methods. *Preparative Part. (a) Preparation of Reference Vanilla Extracts.* As reference for stable isotope ratio analysis and for component analysis, five different charges of Bourbon vanilla pods were extracted according to the following procedure: Two grams of vanilla pods was cut into small pieces and macerated for 16 h at 30 °C with 4 mL of water in a closed glass vial. After addition of 4 mL of ethanol, the mixture was mixed thoroughly and allowed to stand for a further 3 days with occasional shaking of the extract. The mixture was then filtered through a paper filter, and the filter cake was pressed and washed with 6 mL of ethanol/water (4:1 v/v). The solution was used for analysis.

(b) Isolation of Vanillin from Extracts. Vanilla extracts containing approximately 0.1 g of vanillin were diluted with water or methanol/water (1:1 v/v) depending on their sugar and alcohol content, respectively, and extracted three times with 25 mL of diethyl ether. The combined organic extracts were concentrated on a rotating evaporator to a viscous liquid. The residue was taken up in ethanol and diluted with water to an alcohol content of less than 30%. Final volume was 7−10 mL. The solution was cleared by filtering through a paper filter.

(c) Preparative Liquid Chromatography. Prior to isotope analysis, it is necessary to purify the crude extracts of vanillin. This is generally achieved by chromatographic methods such as GC (Hoffman and Salb, 1979), TLC (Martin et al., 1981), and liquid chromatography (LC) (Bricout et al., 1974). In this investigation semipreparative HPLC was applied, using octadecyl silica as adsorbent and an acidified mixture of water/methanol as eluent. Under these conditions an efficient separation of vanillin from 4-hydroxybenzaldehyde is achieved.

(1) Apparatus. A high-pressure liquid chromatograph (Model L-6200, E. Merck) equipped with an injection valve (Model 7120, Rheodyne, Berkeley, CA) and a 1-mL sample loop was used.

Separation was carried out on a 25 × 1 cm i.d. stainless steel column packed with octadecyl silica (LiChrospher 100 RP18, E. Merck) of 5-μm particle size.

Detection was performed by a diode array detector (Model L-3000, E. Merck). Diode array data were stored and processed on a personal computer (Model Vectra, Hewlett-Packard, Meyrin, Switzerland). Fractions of the effluent were collected by a programmable fraction collector (Model Foxy, Isco, Lincoln, NE).

(2) Operation. The mobile phase consisted of water, acidified with hydrochloric acid to pH 2.8 (A), and methanol (B). Isocratic elution was performed at 70% A and 30% B; the flow rate was set to 2.7 mL/min. After 25 min, cleaning of the column was started with a linear gradient to 100% B within 7 min; the column was washed for a further 12 min with methanol, followed by reequilibration of the column.

The injection volume depends on the concentration of crude vanillin and accompanying substances. Volumes up to 1 mL containing 7−10 mg of vanillin were injected. The column was operated below overloading conditions, which were observed at approximately 12 mg of vanillin in the samples. Working at overloading conditions would require additional runs for further purification of the vanillin-containing fractions. Collection of the fractions was initiated by monitoring the absorption signal at 340 nm and a signal level of 2.0 absorbance units (AU). Fraction size was set to 2.7 mL. After each run, diode array data were examined, and only those fractions containing vanillin and no 4-hydroxybenzaldehyde were pooled. In most cases two to three runs were necessary to obtain 10 mg of purified vanillin. The pooled fractions were extracted three times with 10 mL of chloroform. The volume of the combined organic layers was reduced by distillation, transferred to a 5 mL pointed flask, and evaporated to dryness. The residue was dissolved in diethyl ether, transferred to a weighed sample vial, evaporated to dryness, and held at water pump vacuum at 40 °C for 10 min. After the sample was weighed, the vial was sealed and sent for SIRA to the Austrian Research Center in Seibersdorf.

Analytical Part. (a) Stable Isotope Ratio Analysis. Stable isotope analysis was performed with continuous flow mass spectrometry. In principle, the isotope ratio MS (Mat 251, Finnigan, Bremen, Germany) was coupled to the output of a modified elemental analyzer (EA 1108, Carlo Erba Strumentatione, Milano, Italy) by a homemade splitting device, which branches off about one per mil of the effluent gas mixture for mass spectrometry (Pichlmayer and Blochberger, 1988). The main part of the EA is the quartz combustion reactor, heated to 1000 °C. A defined oxygen volume of 5 mL was added to the helium flow by means of an injection valve. A tin crucible, containing the weighed sample, was introduced at this moment into the quartz furnace and flash combusted by exothermic reaction. Superfluous oxygen was retained by copper, and the reaction-formed water was trapped subsequently. The different combustion gases, carried by the helium, were separated on a GC column.

For the ^{13}C determinations the effluent carbon dioxide was used, and therefore the ion currents of the masses 44, 45, and 46 were measured. Ion beam registration was done by two dual channel integrators (Model SP 4270, Spectra-Physics, San Jose, CA).

Vanillin (0.1−0.3 mg) was used for MS analysis, and at least 3 repetitions per sample were made.

The ^{13}C abundances are expressed in the δ-notation

$$\delta^{13}C_{PDB} = (R_{sample}/R_{standard} - 1) \times 1000\ (‰)$$

whereby isotopic ratio (R) = abundance of ^{13}C/abundance of ^{12}C. The results are oxygen corrected (Craig, 1957) and related to the international PDB carbon standard.

(b) Sample Preparation for Component Analysis. Two milliliters of pure vanilla extracts was diluted with 8 mL of water/methanol (1:1 v/v). Possible precipitations were dissolved by addition of methanol. The samples were further diluted by factors of 5 and 40 with water/methanol. All dilutions were used for chromatography.

(c) Component Analysis by HPLC. The separation for component analysis of vanilla extracts was performed by reversed-phase HPLC applying a gradient of acidified water/methanol.

(1) Apparatus. The chromatographic system used for separation of vanilla extracts consisted of a high-pressure pump (Model L-6200, E. Merck) with a low-pressure gradient former. Samples were injected by an intelligent autosampler (Model AS-4000, E. Merck). Separation was carried out on a 250 × 4 mm octadecyl silica column (LiChroCart Superspher 100 RP18, E. Merck) packed with 4-μm particles and protected by a 35 × 4 mm precolumn packed with LiChrospher 100 RP18 of 5-μm particle size. Both columns were thermostated to 35 °C by a column thermostat (Model T-6300, E. Merck).

Detection was performed by UV and fluorometric detectors coupled in series. Spectra of the effluent were recorded by a diode array detector (Model L-3000, E. Merck) with a 5-μL flow cell. Fluorescence detection was performed by a fluorescence spectrophotometer (Model F-1000, E. Merck) with a 12-μL flow

Table 1. Performance of Different Octadecyl Silicas[a] for Preparative Separation of 4-Hydroxybenzaldehyde from Vanillin

packing material	peak shape	resolution of 4-hydroxybenzaldehyde/ vanillin
LiChroprep RP18, 40–63 μm	asymmetric	<0.3
LiChrosorb RP18, 10 μm	asymmetric	<0.3
LiChroprep RP18, 25–40 μm	symmetric	0.55
LiChrospher RP18, 5 μm	symmetric	2.3

[a] Conditions: eluent, water/HCl (pH 2.7)–methanol 7:3 (v/v); column, 25 × 1 cm i.d.; flow rate, 3.0 mL/min; injection volume, 1.0 mL (300 μg of vanillin, 280 μg of 4-hydroxybenzaldehyde).

cell. The detector signal of the fluorescence detector was digitized by an intelligent data sampling module (Model 763, Nelson Analytical, Cupertino, CA). Data storage and data processing of both detectors were carried out on a personal computer (Model Vectra, Hewlett-Packard) by use of chromatographic software (E. Merck and Nelson Analytical).

(2) Operation. A binary eluent consisting of an aequous solution of 0.01 M sodium acetate adjusted to pH 4.0 with hydrochloric acid (A) and methanol (B) was used. Flow rate was set to 0.8 mL/min. Elution of the components was achieved by a linear gradient, starting with 15% B and reaching 85% B after 25 min and 100% B after 5 more min. Each cycle was finished by cleaning of the column with methanol for 10 min, followed by reequilibration of the column.

Injection volume was set to 30 μL.

Spectra of the effluent were recorded in the wavelength range 200–360 nm every 1.1 s. For fluorescence detection, excitation wavelength was set to 280 nm and emission wavelength to 350 nm.

The components of the extracts were identified by measuring their retention times and identity was verified by comparison of their UV spectra with the spectra of the standard compounds recorded under the same conditions. Quantitation was performed by calculation of the peak area in the absorption maximum of each compound or by evaluation of the area of the fluorescence signal if signal response was better and not disturbed by other components.

Calibration of the chromatographic system was obtained by standard mixtures in methanol/water (1:2 v/v) containing 50, 15, and 12.5 ppm of each compound.

RESULTS AND DISCUSSION

Preparative HPLC. For selection of the proper stationary phase system, octadecyl silicas of different particle sizes and shapes was tested for their suitability for preparative work (Table 1). For the separation of vanillin from 4-hydroxybenzaldehyde, only octadecyl silica of type Lichrospher RP18 with 5-μm particle size provided sufficient resolution capacity and produced symmetric peaks even at higher column loading, keeping the number of preparative runs and the time requirement for purification low. All other tested materials with greater particle size were not suitable for this separation problem, even at the expense of additional runs for purifying vanillin.

To avoid contamination of the purified vanillin, only volatile organic modifiers and no organic buffer salts were used. A better separation of vanillin from 4-hydroxybenzaldehyde was achieved when methanol was used instead of acetonitrile in the eluent.

SIRA. Samples of vanilla extracts and the pooled extracts prepared in the laboratory were purified according to the procedure described above. Nearly all extracts were known to be obtained from Bourbon vanilla pods. One extract was prepared from pods of the Comoro Islands, which fell into the same range. Results of the stable isotope ratio analysis and values found by other groups are listed in Table 2. In cases where more than one determination was carried out, purified vanillin from different preparative runs was used. Published values for authentic extracts from Madagascar pods range from $\delta_{PDB}{}^{13}C = -18.7$ to -21.5. Extracts with $\delta_{PDB}{}^{13}C$ values more negativ than -21.5 were therefore considered to be adulterated.

Table 2. SIRA of Carbon in Vanillin

sample	vanilla extract[a] (fold)	$\delta_{PDB}{}^{13}C$[b] (‰)
1	Madagascar (1×)	−20.8[c]
2	Madagascar (7×)	−20.2[c]
3	Madagascar (10×)	−21.4
4	Comoro (10×)	−20.6
5	provenience unknown	−20.4
6	Madagascar (fold unknown)	−25.0
7	provenience unknown	−27.7[d]
8	pooled laboratory prepared extracts	−21.5
	Published Values for Authentic Samples	
Bricout et al.	Madagascar	−20.5
	Comoro	−20.8
Hoffman and Salb	Madagascar	−18.7 to −20.4
Hubner	Java	−18.7
	Tahiti	−16.8
	Mexiko	−20.5
Martin et al.		−18.7 to −20.9
Schmidt		−19.5 to −21.5
	Synthetic Vanillins	
vanillin chem. pure	(this work)	−27.6
vanillin p.a.	(this work)	−28.2
Hoffman and Salb		−27.0
vanillin ex guaiacol	(this work)	−29.1
Hoffman and Salb		−32.7
Schmidt		−29 to −36
synthetic lignin vanillin	(this work)	−26.8
Hoffman and Salb		−27.0
Martin et al.		−26.9
Bricout et al.		−26.9 to −28.7
Schmidt		−28.0

[a] 1-fold extract = 100 g of vanilla pods for 1 L of vanilla extract. [b] Precision: ±1‰. [c] Mean of two independent preparations. [d] Mean of three independent preparations.

In addition, the stable isotope ratio of synthetic vanillins from different sources was determined (Table 2). The values were in agreement with results reported in the literature (Hoffman and Salb, 1979; Martin et al., 1981; Bricout et al., 1974; Schmidt, 1986).

The analysis of the samples suggests that one extract contains entirely synthetic vanillin (sample 7) and another extract (sample 6) has been altered by addition of synthetic vanillin.

Component Analysis. A chromatogram of a mixture of standards of the chemical analysis is shown in Figure 1. All investigated components except coumarin and piperonal were well resolved from each other, including the separation of vanillin from isovanillin and syringaldehyde.

The concentration ratios of the main components are related to vanillin and expressed in micrograms of compound per milligram of vanillin per unit of volume. The results are shown in Table 3. The analysis was also extended to components that were not or seldom determined (e.g., vanillyl alcohol, anisic acid, and

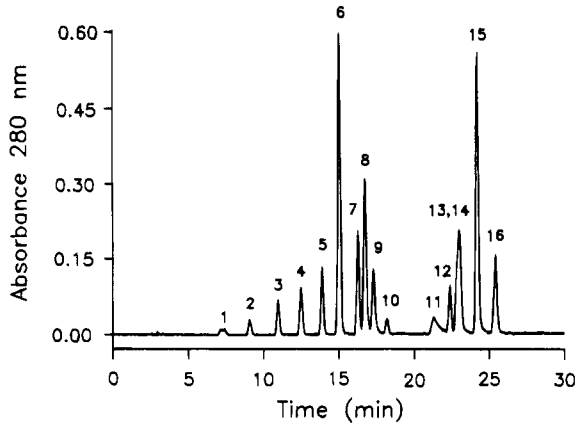

Figure 1. Chromatogram of a standard mixture. Conditions are as described under Experimental Procedures. Samples: 1, 3,5-dihydroxybenzoic acid; 2, 4-hydroxybenzyl alcohol; 3, 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol); 4, 4-hydroxybenzoic acid; 5, 4-hydroxy-3-methoxybenzoic acid (vanillic acid); 6, 4-hydroxybenzaldehyde; 7, 3-hydroxy-4-methoxybenzaldehyde (isovanillin); 8, 4-hydroxy-3-methoxybenzaldehyde (vanillin); 9, 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde); 10, 4-methoxybenzyl alcohol (anise alcohol); 11, 2-hydroxy-3-methoxybenzaldehyde (*o*-vanillin); 12, 4-methoxybenzoic acid (anisic acid); 13, 3,4-(methylenedioxy)benzaldehyde (piperonal); 14, 2*H*-1-benzopyran-2-one (coumarin); 15, 4-methoxybenzaldehyde (anisaldehyde); 16, 4-hydroxy-3-ethoxybenzaldehyde (ethylvanillin).

Table 3. Component Analysis of Vanilla Extracts

	ratio (mg of compound/g of vanillin)			
	vanillic acid	4-hydroxy-benzaldehyde	4-hydroxy-benzoic acid	vanillyl alcohol
laboratory-prepared extract				
1	28	65	8.8	25
2	26	54	6.4	3.4
3	39	50	10	16
4	71	86	21	52
5	45	88	15	36
6	23	41	8.5	4.8
variability	23–71	41–88	6–21	4–52
sample				
1	28	53	10	26
2	55	69	6.7	14
3	40	45	12	7.1
4	42	43	11	4.0
5	55	67	42	19
6	9.5	50	4.5	1.5
7	128	3	23	<0.1

anisic aldehyde). For anisic acid and anisic aldehyde a high variation of the concentration was found in the laboratory-prepeared extracts (results not shown). They were therefore not used for the characterization of vanilla extracts. In all extracts neither ethylvanillin nor piperonal was found.

A chromatogram of an authentic vanilla extract is depicted in Figure 2. The stable isotope ratio of vanillin and the concentration of the main components in relation to vanillin were found to be within the limits of variety.

Chromatograms of authentic extracts are marked by substances eluting before vanillin, which are absent in simple and clumsy adulterations as can be seen in Figure 3A: This extract (sample 7) is characterized by a very low concentration of vanillin (5–10% of a 1-fold extract) and low component concentrations in relation to vanillin. An exception is vanillic acid, which may be

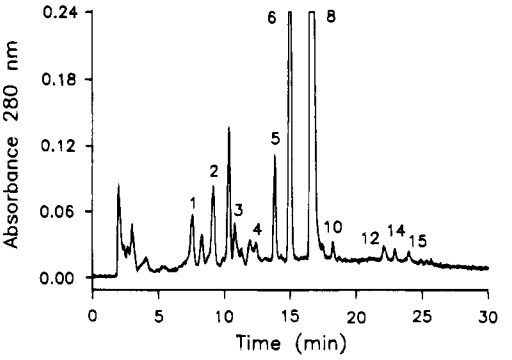

Figure 2. Chromatogram of an authentic vanilla extract. Conditions and legend are as in Figure 1.

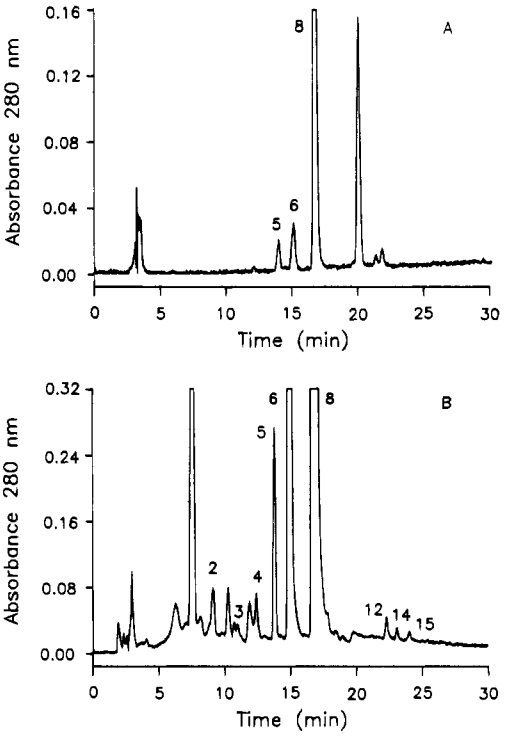

Figure 3. Chromatograms of adultered vanilla extracts: (A) synthetic mixture; (B) blended vanilla extract. Conditions and legend are as in Figure 1.

due to some extent to aging of the sample, but vanillyl alcohol—stable toward oxidation—was also present at very low concentrations. The chromatogram is further characterized by an unidentified compound, which was not found in authentic vanilla extracts. It elutes at 20.8 min, showing an absorption maximum at 263 nm and strong fluorescence (excitation at 280 nm, emisson at 360 nm). Both analytical results, SIRA ($\delta_{PDB}{}^{13}C = -27.7$) as well as component analysis, indicate a synthetic sample containing possibly a few percent of vanilla extract.

Figure 3B shows the chromatogram of an extract (sample 6) identified as a blended extract by SIRA ($\delta_{PDB}{}^{13}C = -25.0$). From component analysis it was found that the concentration of 4-hydroxybenzaldehyde, generally also used for the validation of authentic vanilla extracts, was within the range of variety, whereas vanillic acid, 4-hydroxybenzoic acid, and vanillyl alcohol were present in very low concentrations, which leads to the conclusion that vanillin and 4-hydroxybenzaldehyde have been added. By comparison of the elution pattern of this sample extract with that of the extracts prepared in the laboratory, very similar

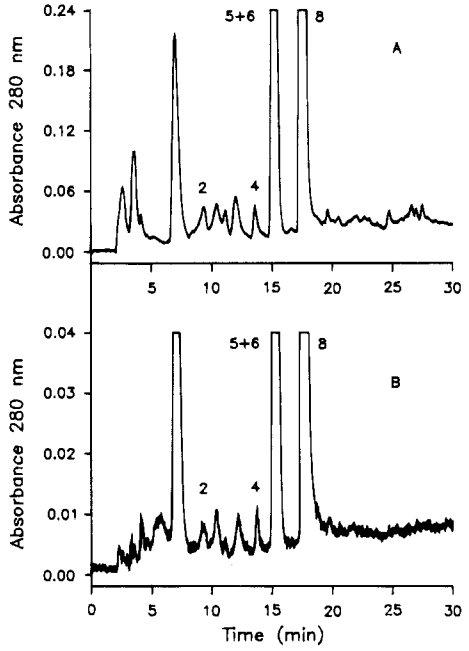

Figure 4. Elution pattern of (A) laboratory-prepared extract 6 and (B) sample extract 6. Conditions: eluent A = water/acetic acid (pH 3.4); eluent B = MeOH; linear gradient, 0–30 min, 15–75% B, flow rate 0.8 mL/min; column, Hibar LiChrospher 100 RP18, 250 × 4 mm; particle size, 5 μm; injection volume, 20 μL.

Table 4. Amount of Vanillin Added to Sample Extract 6 Calculated from the Results of Component Analysis

	amount of vanillin added[a] (%)		
reference extract	vanillic acid	4-hydroxybenzoic acid	vanillyl alcohol
2	63	30	56
6	59	47	69

[a] Formula for calculation: excess vanillin (%) = $100(\text{ratio}_{ref} - \text{ratio}_{sample})/\text{ratio}_{ref}$.

patterns were found for extracts 2 and 6. Nearly an exact match was found for extract 6 (Figure 4), indicating the same origin of the vanilla pods. Taking their ratios as reference, the amount of added vanillin can be calculated (Table 4). The least variation for both reference extracts was found for vanillyl alcohol and vanillic acid, for which the addition of 62% vanillin (mean value) can be estimated in sample 6.

The amount of added synthetic vanillin can also be calculated from the results of the isotope analysis similar to that done by Hoffman and Salb. Under the assumption that lignin vanillin has been added ($\delta_{PDB}{}^{13}C = -27.0$) and vanillin of authentic extracts of Madagascar pods has a mean $\delta_{PDB}{}^{13}C = -20.8$, a content of approximately 68% artificial vanillin is calculated.

Conclusions. The analytical data demonstrate that SIRA and component analysis show comparable results; SIRA detects the amount of added synthetic vanillin much more precisely and is therefore taken for quantitation of adulterations.

On the other hand, component analysis of vanilla extracts has the advantage that component concentrations and added substances, which do not occur in authentic vanilla extracts, can be determined. Simple adulterations can easily be recognized by component analysis, whereas the addition of flavoring compounds, which occur in vanilla extracts (mostly vanillin), is much more difficult to detect. Because of the relatively wide concentration range in which the components occur, the estimation of the degree of adulteration is hardly possible. Nevertheless, an estimation by component analysis is feasible, if analytical data of vanilla pods of the same origin are available. Their chromatograms, recorded under the same conditions, show almost identical elution patterns. The application of pattern recognition is therefore an appropriate method for the determination of the origin and crop of vanilla pods, reducing further the range of variability of the components in vanilla pods and allowing statements of their authenticity by component analysis.

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