Analytical Methods

Protocol for isolation of vanillin from ice cream and yoghurt to confirm the vanilla beans origin by $^{13}$C-EA-IRMS

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ABSTRACT

An analytical method for the stable isotope ratio analysis of vanillin in ice cream and yoghurt is described. The milk proteins were removed by precipitation and vanillin was extracted by liquid–liquid extraction. Several solvents were tested to achieve maximum recovery for vanillin. Separation from accompanying components was performed by semipreparative HPLC. After chromatography several sample preparation techniques for the off-line transfer of the vanillin fractions were tested and optimised to minimise losses of vanillin. The $^{13}$C/$^{12}$C ratio of the fractions was determined by $^{13}$C-EA-IRMS. The method was applied to samples obtained from local food stores. The majority of the samples contained authentic vanillin.

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1. Introduction

One of the most common and widely used flavouring aromas is vanillin, which is added to many foodstuffs and beverages to enhance their taste and quality. It can be added either as authentic vanillin in form of an extract of vanilla beans, which resembles the most palatable but also expensive form, or pure as synthetic vanillin. The determination of the origin of vanillin in food products is a difficult problem, because sample matrix is complex requiring selective sample preparation techniques coupled with an efficient separation system. The origin of vanillin in food products can be determined by different approaches.

The analysis of the main components of vanilla extracts such as vanillin, vanillic acid, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid, expressed by their ratios, allow statements about the origin of vanillin. Component analysis is performed by chromatographic methods such as high-performance liquid chromatography (Ehlers, 1999; Kahan, Krueger, & Dana, 1997; Scharrer & Mosandl, 2001), high-performance thin layer chromatography (Belay & Poole, 1993; Poole, Daly, & Poole, 1993), micellar electrokinetic chromatography (Boyce, Haddad, & Sostaric, 2003; Pyell, Pletsch-Viehmann, & Ramus, 2002) and gas chromatography (Scharrer & Mosandl, 2001; Sostaric, Boyce, & Spickett, 2000). In a chemometric approach the chromatograms were treated as fingerprints to distinguish vanillin from different sources (van-Nederkassel et al., 2006). In milk products, however, the enzymatic activity may alter the characteristic ratios obtained from authentic vanilla extracts (Kempe & Kohnen, 1999; Littmann-Nienstedt & Ehlers, 2005).

The determination of the stable isotope ratio of carbon is a reliable method for discrimination between natural and synthetic products (Rossmann, 2001; Schmidt, 1986; Winkler & Schmidt, 1980). The main part of synthetic vanillin is produced from guaiacol and lignin. Vanilla pods are still the preferred source for natural vanillin, but biotechnological production methods are gaining increased importance (Priefert, Rabenhorst, & Steinbüchel, 2001; Ramachandra & Ravishankar, 2000). Because natural vanillin is enriched in $^{13}$C, it can be distinguished from synthetic vanillin produced from different sources. This is accomplished by mass spectrometry such as isotope ratio mass spectrometry (Bensaid, Wietzerbin, & Martin, 2002; Culp, Legat, & Otero, 1998; Fayet, Saltron, Tisse, & Guerre, 1999; Fellous, George, & Schippa, 1992) and site-specific natural isotopic fractionation (SNIF) NMR (John & Jamin, 2004; Martin, Hanmeguelle, & Remaud, 1993; Remaud, Martin, Martin, & Martin, 1997; Tenailleau, Lancelin, Robins, & Akoka, 2004).

The application of these methods to isotope analysis of vanillin in milk products requires complex sample preparation procedures for isolation of vanillin from accompanying compounds such as proteins, amino acids and other interfering components. Especially ice cream requires extensive sample clean-up techniques. Reports about that field of work are rare (Fayet et al., 1995).
The following method describes the isolation of vanillin from ice cream and miscellaneous dairy products for the analysis by $^{13}$C-EA-IRMS.

2. Materials and methods

2.1. Chemicals

Ethanol and analytical grade methylisobutyl ketone (hexone) were purchased from Roth (Karlsruhe, Germany). Chemicals of analytical grade used for sample preparation and methanol of HPLC grade were purchased from VWR (Darmstadt, Germany). Double distilled water was further purified by an Elgastat water purification system (Waters, Eschborn, Germany).

2.2. Sample preparation

A scheme, showing the individual steps for sample preparation, is shown in Fig. 1.

Depending on the amount of vanillin in the samples, sample amounts of 10 g up to 500 g were used for analysis. Some samples contained macerated vanilla beans as additional vanillin source. Vanillin can be released by alkaline extraction. A portion of 200 mL of 20% (%v/v) ethanol and 10 mL of 15% (%w/v) aqueous sodium carbonate were added to 100 g sample. After stirring for 1 h, the solution was acidified with 10 mL of 6 M hydrochloric acid. Proteins were precipitated by addition of 25 mL 0.5 M potassium hexacyanoferate(II) (reagent Carrez I) and 25 mL of 0.5 M zinc sulphate (reagent Carrez II). The solution was stirred for another 30 min and then filtered.

The filtrate of the ice cream samples was treated with 10 mL 15% (w/v) aqueous sodium carbonate, extracted once with 50 mL hexane and acidified with 10 mL 6 M hydrochloric acid. This acidic solution and the filtrate of the yoghurt samples were extracted with three portions of 50 mL methylisobutyl ketone. The organic extracts were combined and vanillin was extracted into aqueous medium with two portions of 5 mL 0.1 M sodium hydroxide. The volume of the combined aqueous extracts was reduced to 1 mL by evaporation on a rotary evaporator (Büchi, Buchs, Switzerland), followed by acidification with 100 μL acetic acid.

2.3. Preparative liquid chromatography

Chromatography was performed on a 250 mm × 10 mm i.d. stainless steel column packed with Kromasil RP 18 (Ekachemicals, Bohus, Sweden) of 7 μm particle size. The column was protected by a 30 mm × 8 mm i.d. precolumn packed with 40–65 μm Lichroprep RP 18. The sample (1.0 mL) was injected into a Rhodyne injection valve (Berkeley, CA, USA) fitted with a 1.4 mL sample loop.

The mobile phase was delivered by a Merck/Hitachi model L-6200 gradient pump. For evaporation of the eluent after fractionation, only volatile eluent components were used. The constituents were separated by a binary linear gradient consisting of water, adjusted to pH 3.0 with hydrochloric acid (eluent A), and methanol (eluent B) at a flow rate of 4.0 mL/min. The gradient started with 10% B, reaching 30% B after 5 min and 40% B after further 15 min. The columns were cleaned with 100% B for 7 min and then reequilibrated with 10% B.

Detection was performed by a Merck/Hitachi model L-4500 diode array detector and fractions were collected with an Isco model Foxy fraction collector (Lincoln, NE, USA) at 0.6 min intervals.

2.4. Analysis of the fractions

After preparative chromatography, side fractions of the vanillin peak were analysed for their vanillin content. The HPLC system for analysis of the fractions consisted of a Merck model L-6000 isocratic pump. Isocratic elution was performed with an aqueous solution of 20% (v/v) methanol acidified with 1.0 mL acetic acid per litre at 1.0 mL/min.

Samples of 20 μL were injected by a Merck/Hitachi model AS-4000 auto sampler and separated on a 125 × 4 mm i.d. LiChrospher RP 18 (VWR) column of 5 μm particle size. Detection was performed by a Merck/Hitachi model L-4200 UV detector at 280 nm.

All fractions containing vanillin were pooled and the overall vanillin concentration was determined.

2.5. Preparation of the fractions for stable isotope analysis

The volume of the pooled vanillin fractions was reduced by evaporation on a rotary evaporator. Vanillin was extracted from the aqueous residue with three portions of 1.0 mL diethylether, the organic extracts were combined and dried over sodium sulphate. Silica of type LiChrosorb Si 100 with a carbon content lower
than 1 mg/g was heated at 110 °C for 2 h. An aliquot (20 mg) was transferred into a 2 mL glass vial, followed by a 500–700 μL portion of diethyl ether extract. Diethyl ether was evaporated by gently heating the vial and after evaporation of the last portion, the vial was hermetically sealed.

For vanillin amounts above 2 mg, the addition of silica can be omitted. After evaporation of diethyl ether, the amount of vanillin in the residue was found to be sufficient for stable isotope ratio analysis.

2.6. Stable isotope ratio analysis

Stable isotope analysis was performed with continuous flow mass spectrometry. A modified element analyzer was coupled to an isotope ratio mass spectrometer. The quartz combustion reactor was heated to 1000 °C and a tin crucible, containing the weighed sample, was introduced into the quartz furnace. The combustion gases were formed in an atmosphere of Helium and oxygen and separated on a GC column. The $^{13}$C/$^{12}$C ratio was measured in the effluent carbon dioxide at the ion currents of the masses 44, 45 and 46.

The $^{13}$C abundances are expressed in the δ-notation (Eq. (1))

$$\delta^{13}C_{PDB} = \left( \frac{^{13}C_{sample}}{^{12}C_{standard}} - 1 \right) \times 1000 \%$$

The values were oxygen corrected (Craig, 1957) and related to the international V-PDB carbon standard. About 200–300 μg vanillin was required for analysis. Depending on the actual amount, samples were divided into 3–6 portions and their $^{13}$C/$^{12}$C ratio was determined. From these results, mean value and standard deviation were calculated.

3. Results and discussion

3.1. Sample preparation – extraction

Although ice cream consists mainly of water (about 60% w/w), further components such as milkfat (10–16% w/w), proteins and carbohydrates (12–16% w/w), sweeteners, sucrose and glucose based corn syrup (12–16% w/w) add up to a quite complex mixture. The isolation of vanillin, which was found in most samples in the mg/kg concentration range, is therefore a challenging task for the analytical chemist. The sample preparation of ice cream turned out to be more difficult than the preparation of yoghurt products. An overview of the complete method is shown in Fig. 1.

Vanillin can be added to ice cream as synthetic vanillin or authentic vanillin, mostly in the form of vanilla extracts or macerated vanilla beans. In the latter case, it can be released by alkaline extraction. The used Carrez reagent is a convenient reagent for precipitation of proteins (Fayet et al., 1995) and after filtration of the proteins, vanillin was enriched by liquid–liquid extraction. Sample size and volume of the filtrate depended on the vanillin concentration in the samples. Filtrate volumes of 0.5–2.0 L were obtained in some cases, so that the use of an efficient solvent was aspired for extraction.

Several organic solvents were tested to determine their extraction efficiency for vanillin from aqueous solution (Table 1). It was found that the extraction efficiency of organic solvents containing polar substituents such as ketones, halogenated hydrocarbons or alcohols was better than for hydrocarbons or ethers. The best results were achieved with methylisobutyl ketone. The extraction efficiency of diethyl ether, which is a preferred solvent because of its ease of evaporation, was only moderate.

3.2. Chromatography

Separation of the extracted components was achieved by reversed-phase HPLC. Eluents consisted of vaporizable components to allow evaporation of the solvent of the collected fractions. The usage of a diode array detector simplified the detection and identification of vanillin and many other compounds occurring in vanilla extracts such as 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid and aromatic hydroxy acids. The occurrence or absence of these components was an indication for the addition of vanilla extracts or synthetic vanillin, respectively.

Representative chromatograms for the preparative separation of the extracts are shown in Fig. 2. The most complex

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<th>Table 1</th>
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<td>Solvent</td>
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<td>Methylisobutylketone</td>
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<tr>
<td>Ethylacetate</td>
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<tr>
<td>1-Pentanol</td>
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<tr>
<td>Trichloromethane</td>
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<td>Dichloromethane</td>
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<tr>
<td>1-Butanol</td>
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<td>1,2-Dichloroethane</td>
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<td>Diisopropylether</td>
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<td>Methylisobutylketone</td>
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$^a K_d = \frac{\text{Vanillin}}{\text{Water}} \cdot \frac{\text{Solvent}}{\text{Water}}$

$^b$ Extraction at pH 11.

Fig. 2. Representative chromatogram of the preparative separation of an ice cream extract containing authentic vanillin (A) and synthetic vanillin (B).
chromatograms were obtained for ice cream samples containing vanilla extract (Fig. 2A), whereas complexity is reduced in samples containing only synthetic vanillin (Fig. 2B). Similar chromatograms were obtained for yoghurt samples.

The isotopic composition of a pure chromatographic peak is different at each location of the peak (Caimi & Brenna, 1997; Lamprecht & Haberhauer, 2001). Therefore, it was necessary to collect the complete peak of vanillin for correct isotope analysis. In addition, side fractions were analysed for their vanillin content and impure fractions were subjected to further chromatographic separation (Fig. 1).

Vanillin was isolated from most of the extracts in a single chromatographic run. For some samples separation efficiency was not sufficient and further chromatographic separation was necessary. This was performed on a separation system of different selectivity such as a strong basic anion-exchanger at pH 7.0. After separation, the pure fractions were desalted by reversed-phase chromatography.

3.3. Sample preparation for stable isotope ratio analysis

The vanillin fractions were pooled and concentrated for analysis by IRMS. The low amount of vanillin found in some ice cream samples forced us to elaborate a procedure that avoided losses of vanillin and incorporation of additional, non-volatile carbon. In this respect, several procedures were tested for their practicability.

Freeze-drying is a gentle method for concentration of susceptible compounds coupled with the ease of dissolution of the residue. After dilution of the fractions with water and deep freezing, they were freeze-dried. It was found, however, that recovery of vanillin was often low and not reproducible.

In another attempt, the HPLC-fractions were extracted with diethylether, silica was added to the organic extract and the organic-solvent was evaporated. No losses of Vanillin were found and recoveries were around 100% and reproducible. The contribution of carbon from the silica used was below 1 µg for a single stable isotope determination.

Finally we applied the following procedure described in detail in experimental: After extraction of the aqueous fractions with diethylether, silica was added to the organic extract and the organic-solvent was evaporated. No losses of Vanillin were found and recoveries were around 100% and reproducible. The contribution of carbon from the silica used was below 1 µg for a single stable isotope determination.

3.4. Application

The analysed vanilla milk products and ice creams were purchased in local food stores in Vienna. Statements on the package about kind and amount of the added vanillin were different, ranging from detailed declaration up to no declaration. Parts of the vanilla plant were pictured on all packages, so that the consumer might expect, that the products contain authentic vanillin.

The method was verified by spiking vanillin-free ice cream with authentic vanilla extract. An aliquot of authentic vanilla extract was added to vanillin-free ice cream and worked up as described in experimental. In addition, vanillin of the authentic extract was isolated by preparative liquid chromatography as described in experimental. The isolated fractions of both samples were analysed by IRMS. For vanillin isolated from authentic extract and fortified ice cream, a δ^{12/13}C of 18.9 (n = 3, SD = 0.25) and 20.2 (n = 3, SD = 0.56) was found, respectively. Results of the stable isotope ratio analysis of vanillin from different sources, reported in a previous work (Lamprecht, Pichlmayer, & Schmid, 1994), showed a range of δ^{12/13}C from −14 to −22 for authentic vanillin and from −26 to −33 for synthetic vanillin.

The results of the stable isotope ratio analysis of the analysed samples are shown in Table 2.

Twelve samples were analysed in the category yoghurt and related products. According to the declaration of the producer, all products should contain authentic vanillin. It was found, however, that 3 samples contained synthetic vanillin.

The situation was different for the ice cream samples. Three samples (no. 17–19) were expected to contain authentic vanillin. No statement about the kind of vanillin added was given for the

![Fig. 3. Recovery of vanillin after extraction and evaporation of the solvent in dependence on pH of the solution.](image-url)
other samples. Two ice cream samples (no. 18 and 19) contained macerated vanilla beans as the only vanillin source. Problems were encountered with sample no. 19, where approximately 0.25 g macerated vanilla beans were found in 1.0 kg ice cream. Although several attempts were performed to isolate a sufficient amount of vanillin for isotope analysis, all efforts failed. In view of the low content of vanillin, the declaration of that product as vanilla ice cream is questionable.

**References**


