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Micellar electrokinetic chromatography as a screening method for the analysis of vanilla flavourings and vanilla extracts

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Abstract

As a general example of the potential use of micellar elektrokinetic chromatography (MEKC) in food analysis, a rapid method for the determination of vanillin and related compounds and possible synthetic additives to vanilla flavourings by MEKC is described as a screening method for quality control. Under optimized conditions, baseline separation of nine vanilla constituents and three possible adulterants is possible within 9 min. The method was applied to additives to bakery products and flavoured beverages. The influence of organic solvents in the sample solution on peak shapes and migration times was investigated. Detection limits and precision of the method are given. It was shown that the use of an internal standard substantially increases the precision of the method. The linear calibration range covers two orders of magnitude with the use of an internal standard.

Keywords: Food analysis; Vanilla flavourings; Beverages; Vanillin and related compounds

1. Introduction

Vanilla extract is defined as a solution in aqueous ethanol of the sapid and odorous principals extractable from vanilla beans. Natural extract has been widely replaced as a flavouring agent by the cheaper vanillin (synthesized from lignin) or by synthetic ethylvanillin.

The high price of natural vanilla extracts has resulted in frequent attempts at adulteration. The need for quality control of flavourings claimed to be authentic vanilla extracts has given rise to many publications dealing with the analysis of vanilla flavourings. One approach for testing the authenticity of vanilla extracts is to determine the ratio of vanillin to related compounds present in vanilla beans and to verify the absence of those compounds which are frequently used for adulteration. Separation techniques including thin-layer chromatography (TLC) [1–3], gas chromatography [2,4,5] and high-performance liquid chromatography (HPLC) [6–10] have been used for this purpose. Recently, Poole and co-workers [11,12] reported the determination of vanillin and related compounds by TLC and automated multiple development, and further suggested [13] the determination of 5-(hydroxymethyl)-2-furfural (minor compound in vanilla extracts) by TLC as method for the routine control of vanilla extracts.

Natural vanillin is enriched in deuterium and carbon-13 compared with synthetic vanillin and

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the addition of synthetic vanillin to vanilla extracts can be detected by using isotope ratio measurements performed by mass spectrometry [14] and nuclear resonance [15] spectrometry. Fayet et al. [16] suggested a combination of the determination of the mass ratio of vanillin to minor compounds and the $^{13}C/^{12}C$ isotopic ratios in vanillin and 4-hydroxybenzaldehyde for the determination of the origin of vanilla beans.

Lamprecht et al. [17] demonstrated the versatility of the combination of two independent methods (stable isotope ratio analysis and chemical component analysis) for the estimation of the authenticity and the possible degree of adulteration in vanilla extracts.

We present in this paper a rapid method for the determination of vanillin and related compounds and possible synthetic additives to vanilla flavourings by micellar electrokinetic chromatography (MEKC) as a general example of the potential use of MEKC in food analysis. The proposed method can be used for the identification of components in ethanolic vanilla extracts.

MEKC, first introduced by Terabe and coworkers [18,19], renders possible the separation of neutral and charged solutes by distributing them between an aqueous mobile phase and a retarded micellar phase (pseudo-stationary phase). Since its introduction in 1985, MEKC has already found various applications, especially in the analysis of nitroaromatic compounds [20,21], in pharmaceutical analysis [22–25] and in the analysis of beverages [26].

Separations by MEKC are mostly performed within 10 min and do not require extensive sample clean-up, because irreversible sorptions of slow-migrating matrix components on a stationary phase are not possible with this technique, because the pseudo-stationary phase is replaced by rinsing procedures before each run. Irreversible sorption of matrix components in real samples on the inner surface of fused-silica capillaries, however, sometimes constitutes a severe problem, resulting in migration time shifts and deterioration of the separation [26]. If irreversible sorptions of matrix components in real samples on the inner surface of fused-silica capillaries do not occur or can be circumvented by rinsing procedures or the proper choice of the buffer composition [26], the features of MEKC, rapidity and low requirements for sample clean-up, permit the application of the MEKC separation method as a screening method in the routine quality control of food and beverages.

2. Experimental

2.1. Reference materials and reagents

4-Hydroxy-3-methoxybenzoic acid (vanillic acid), 3-ethoxy-4-hydroxybenzaldehyde (ethylvanillin), 3-methoxybenzoic acid, 3-methoxybenzaldehyde, 4-hydroxybenzyl alcohol and 3,4-dihydroxybenzaldehyde were purchased from Merck (Darmstadt, Germany), vanillin (analytical-reagent grade) from Janssen (Brüggen, Germany), 4-hydroxy-3-methoxybenzyl alcohol and 3,4-dihydroxybenzoic acid from Aldrich (Steinheim, Germany) and 4-hydroxybenzoic acid from Fluka (Neu-Ulm, Germany). Piperonal, coumarin and 4-hydroxybenzaldehyde were available at the Department of Chemistry, Marburg University.

Sodium tetraborate, boric acid and urea (Merck) and sodium dodecyl sulfate (SDS) (Roth, Karlsruhe, Germany), used for the preparation of the separation buffers, were of analytical-reagent grade. Water was doubly distilled.

2.2. Chromatographic measurements

All chromatographic measurements were carried out with a Beckman (Fullerton, CA, USA) P/ACE capillary electrophoresis system equipped with a UV absorbance detector. The temperature of the capillary was maintained at 25°C by liquid cooling. Samples were injected by application of pressure for 1.5 s. Detection was performed at 254 nm. All separations were carried out at a voltage of 25 kV. Data were recorded with Beckman System Gold software.

Fused silica-capillaries (75 μm I.D., 375 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 56.5 cm and the length to the

detector was 50 cm. The capillary rinsing procedures employed have been presented in detail elsewhere [27]. The elution time of the mobile phase, t_0, and the elution time of the micellar phase, t_M, were determined using formamide and quinine hydrochloride, respectively, as markers. Quinine hydrochloride was recommended by Terabe [28] as a suitable marker of the migration times of the micelles for anionic micellar systems. Peak identities were confirmed by spiking.

Buffer solutions were prepared in the following manner: to a solution of 10 mmol/l disodium borate and 100 mmol/l SDS, a solution of 10 mmol/l disodium borate, 100 mmol/l boric acid and 100 mmol/l SDS was added dropwise until the desired pH was obtained. The pH was measured with a Model 605 pH meter and an EA121 glass electrode (Metrohm, Herisau, Switzerland).

2.3. Preparations of standard solutions

Optimization studies and quantification were performed with solutions of the pure standards dissolved in a buffer solution containing 10 mmol/l disodium borate, 10 mmol/l boric acid and 10 mmol/l SDS. The concentration of the internal standard ethyl vanillin was 45.45 mg/l in the solutions used for determination of the linear calibration range.

2.4. Preparation of sample solutions

All samples were prepared in water. A weighed amount of the material to be investigated was added to water and then stirred and heated in a glass vessel. After reaching the boiling point, heat was removed after 30 s and the liquid was allowed to cool to room temperature. After adjustment to the desired volume, some of the samples (homogeneous solutions) were injected directly. After extraction, flavoured tea, flavoured coffee extract and dried pieces of vanilla beans were filtered through a paper filter (Binzer, Hatzfeld, Germany) prior to injection.

3. Results and discussion

3.1. Optimization of separation conditions

The principal polar aromatic flavour components in natural vanilla are vanillin, 4-hydroxybenzaldehyde, 4-hydroxy- 3-methoxybenzoic acid and 4-hydroxybenzoic acid, accompanied by smaller amounts of 4-hydroxybenzyl alcohol, 3,4-dihydroxybenzaldehyde, 3,5-dimethoxy-4-hydroxybenzoic acid and 4-hydroxy-3-methoxybenzyl alcohol [6–9]. In addition, extracts of *Vanilla tahitiensis* Moore contain significant amounts of 3-methoxybenzoic acid and 3-methoxybenzaldehyde [9]. Imitated vanilla extracts frequently contain coumarin, piperonal, added vanillin and ethylvanillin [6–14]. These compounds have to be separated from each other and from matrix components if the absence of adulterants and the ratio of vanillin to related compounds are to be examined.

The compounds that are to be separated are very different in polarity. At pH 7 some compounds are dissociated with a high degree of dissociation, α, whereas others are not dissociated. For HPLC separations, therefore, the mobile phase was acidified by adding glacial acetic acid [6–10] in order to suppress the dissociation. In MEKC, however, not only partitioning between the mobile phase and the retarded micellar phase works as a separation mechanism but also differences in effective electrophoretic mobilities. If the analytes have very different pK_s values, as in the present case, then those analytes which are mainly dissociated will be hardly included into the micellar phase. The mechanism by which they are separated is primarily capillary electrophoresis, even in the presence of micelles. For those analytes which are primarily not dissociated, distribution between the pseudo-stationary phase and the mobile phase will dominate the separation process. The formation of ion pairs does not have to be taken into account in this consideration, because in this case charged analytes and surfactant molecules exhibit the same charge.

In order to obtain sufficient separation between the analytes of interest, we chose the

following approach for the optimization of the buffer composition. The concentration of the anionic surfactant SDS was kept constant at 100 mmol/l while the pH of the buffer was altered by varying the concentration ratio of boric acid to disodium tetraborate. The concentration of disodium tetraborate was kept constant at 10 mmol/l. pH has a strong influence on the elution order via alteration of the degree of dissociation, α. It can be concluded that there is a pH-dependent switching of the separation mechanism from CZE to retardation by inclusion in the pseudo-stationary phase and vice versa.

In Fig. 1, the pseudo-retention factors of the solutes of interest (calculated neglecting the effective electrophoretic mobility of the solutes) are plotted against pH. The pseudo-retention factors of those compounds which do not change their degree of dissociation in the selected pH range mainly remain constant independent of the pH. The pseudo-retention factors for the benzoic acids, however, are strongly influenced by pH.

At a pH of 8.70 all solutes are sufficiently separated from each other. Therefore, this pH was chosen as the optimum. In Fig. 2 the separation of a test mixture under optimized conditions is presented. The electropherogram of the components of a test mixture dissolved in the separation buffer is given by the dotted line. No studies were performed with buffers of pH < 6.35.

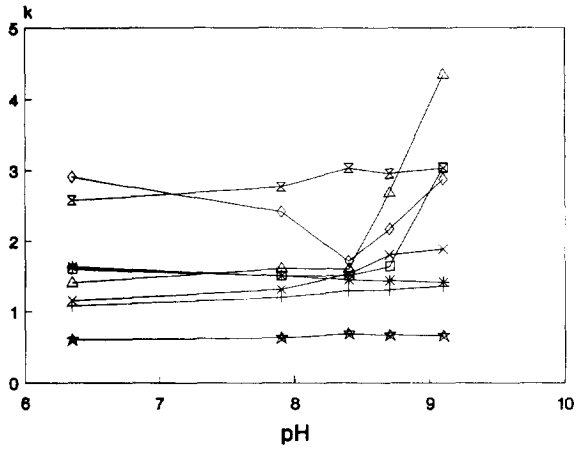

Fig. 1. Dependence of the pseudo-retention factors of selected constituents on the buffer pH. For measurement conditions, see Fig. 2. ☆ = 4-Hydroxy-3-methoxybenzyl alcohol; + = ethylvanillin; * = vanillin; □ = 3-methoxybenzoic acid; × = 4-hydroxybenzaldehyde; ◇ = vanillic acid; △ = 4-hydroxybenzoic acid; ⧖ = piperonal.

Generally, in MEKC a pH higher than 7 is favoured, because the electroosmotic flow, which determines the maximum migration velocity of uncharged solutes, decreases with a decrease in pH. In the case examined, the electroosmotic flow is reduced from 2.59 mm/s at pH 9.10 to 2.28 mm/s at pH 6.35. In addition, the problem of buffer depletion by electrolysis is less pronounced at pH > 8, because the dependence of the electroosmotic mobility on the pH at constant ionic strength is described by a curve of sigmoidal shape that approaches a constant value at pH ≥ 8 [29].

3.2. Sample preparation

It was reported by Ackermans et al. [30] and Crabtree et al. [31] that the addition of methanol [30] or acetonitrile [31] to the sample solution has a drastic effect on efficiency and elution time in MEKC. We studied the influence of various high contents of ethanol, methanol, acetone and acetonitrile in the sample solution on the separation of a text mixture containing vanillin, 4-hydroxybenzaldehyde, vanillic acid and 4-hydroxybenzoic acid (the major extractable polar flavouring components of vanilla beans). Plate numbers and migration times as a function of the content of organic solvent in the sample solution are presented in Table 1. The sample solution contained constant concentrations of the analytes, SDS and buffer components (see Experimental). The separation buffer was in all cases an aqueous solution containing 100 mmol/l SDS, 10 mmol/l boric acid and 10 mmol/l disodium borate.

The plate numbers, N, obtained for the test solutes and the migration times, t_M, for various compositions of the sample solution are listed. The plate numbers were calculated according to

$$N = 2\pi \cdot \frac{t_M^2 h^2}{a^2} \quad (1)$$

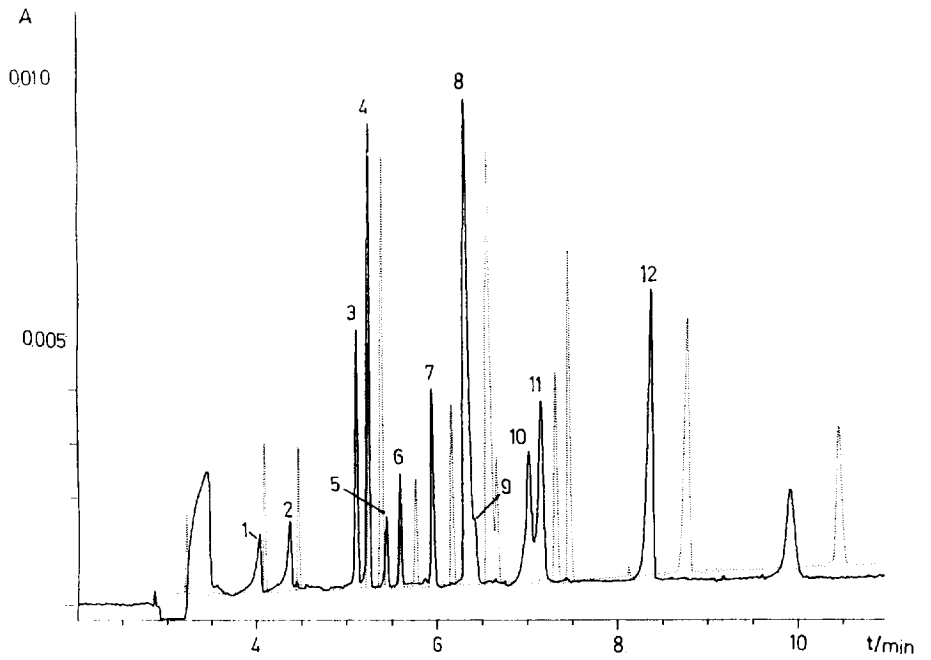

Fig. 2. Superimposed electropherograms of vanilla constituents and potential adulterants with various volume fractions of ethanol in the sample solution: dotted trace, φ(ethanol) = 0.0; solid trace, φ(ethanol) = 0.50. Buffer, SDS = 100 mmol/l, $Na_2B_4O_7$ = 10 mmol/l, H_3BO_3 = 10 mmol/l (pH 8.70); capillary, 565 (500) mm × 75 μm I.D.; voltage, 25 kV; temperature, 25°C; injection, pressure for 1.5 s; detection, UV at 254 nm. Peak identification: 1 = 4-hydroxybenzyl alcohol; 2 = 4-hydroxy-3-methoxybenzyl alcohol; 3 = 3-ethoxy-4-hydroxybenzaldehyde (ethylvanillin); 4 = vanillin; 5 = 3-methoxybenzoic acid; 6 = 4-hydroxybenzaldehyde; 7 = vanillic acid; 8 = hydroxybenzoic acid; 9 = piperonal; 10 = 3-methoxybenzaldehyde; 11 = coumarin; 12 = 3,4-dihydroxybenzoic acid.

where t_M = migration time, h = peak height and a = peak area.

Acetone in the sample solution has the strongest influence on the efficiency of the separation system. The addition of acetone to the sample solution lowers the efficiency for vanillin, even at a volume fraction of acetone of 0.39, from $N = 240\,000$ (sample solution in pure buffer) to 70 000.

The influences of ethanol, methanol and acetonitrile on the efficiency are very similar. Therefore, in Table 1 only plate numbers for ethanol in the sample solution are given. For all solutes investigated, a volume fraction of one of these organic solvents of up to 0.40 reduces the plate numbers measured for the test solutes to a negligible extent. At higher volume fractions of organic solvent, also with these solvents a decrease in the efficiency was measured.

There is a strong effect on the migration times even with volume fractions of organic solvent lower than 0.40. The migration times of all the solutes investigated decrease on addition of the organic solvents. For all organic solvents tested, the migration time decreases linearly with increasing volume fraction of organic solvent in the sample solution.

In Fig. 2, the separation of polar components in natural vanilla and potential adulterants with a separation buffer containing 100 mmol/l SDS, 10 mmol/l boric acid and 10 mmol/l disodium borate (pH 8.70) is shown. The electropherograms obtained with a sample solution containing only water as solvent and a sample solution containing ethanol ($\varphi = 0.50$) are superimposed. The addition of organic solvents to the sample solution produces disturbances of the baseline at the hold-up time. The migration times of the

Table 1
Plate numbers and migration times for vanilla principal components as a function of the volume fraction, φ, of organic solvents in the sampling solution, with measurement conditions as in Fig. 2

Compound	Ethanol								Acetone							
	$\varphi = 0.00$		$\varphi = 0.19$		$\varphi = 0.39$		$\varphi = 0.58$		$\varphi = 0.00$		$\varphi = 0.39$		$\varphi = 0.58$		$\varphi = 0.78$	
	N	t_M(min)	N	t_M(min)	N	t_M(min)	N	t_M(min)	N	t_M(min)	N	t_M(min)	N	t_M(min)	N	t_M(min)
Vanillin	242 000	5.83	234 000	5.72	241 000	5.57	203 000	5.47	242 000	5.83	66 000	5.67	65 000	5.53	62 000	5.41
4-Hydroxybenzaldehyde	230 000	6.28	238 000	6.15	207 000	5.97	201 000	5.85	230 000	6.28	158 000	6.09	134 000	5.91	102 000	5.77
Vanillic acid	224 000	6.74	240 000	6.57	236 000	6.33	206 000	6.19	224 000	6.74	176 000	6.55	138 000	6.33	101 000	6.16
4-Hydroxybenzoic acid	201 000	7.27	195 000	7.07	191 000	6.79	178 000	6.63	201 000	7.27	145 000	7.04	108 000	6.79	75 000	6.60

solutes and of the micelle marker are shortened on addition of ethanol to the sample solution. Some of the peaks show strong asymmetric deformation if the injection is performed with the sample solution containing ethanol, e.g., piperonal cannot be separated from 4-hydroxybenzoic acid if the sample solution contains ethanol ($\varphi = 0.50$).

In order to verify the hypothesis that predominantly the peak shapes of those solutes which strongly interact with the micelles are distorted, the migration times for the analytes, the separation of which is shown in Fig. 2, were determined with a buffer (pH 8.70) containing no surfactant. A comparison of the migration times listed in Table 2 with Fig. 2 shows that only the peak shapes of analytes eluted with the hold-up time marker employing a buffer containing no SDS are asymmetrically deformed by the addition of ethanol to the sample solution separated in an SDS-containing buffer. These analytes are primarily not dissociated under conditions of the measurement. Their retention in an SDS-containing buffer is due exclusively to interaction with the charged micelles.

These investigations show that matrix components can have a strong influence on the migration time and resolution of adjacent peaks that must be taken into account if peak identification is based on migration times. The addition of organic solvents to the sample solution must be restricted to volume fractions below 0.4. Ethanolic vanilla extracts must be diluted with water prior to analysis. If possible, samples should be prepared with the same solution as is employed as the separation buffer. If this is not possible, the standard solutions must contain the same matrix as the sample solution.

3.3. Analysis of vanilla flavourings

We investigated several flavourings that are commercially available as additives to bakery products. These flavourings are completely soluble in water. The resulting electropherograms were compared with an electropherogram obtained with an aqueous extract from a vanilla bean. Peak identification was performed by spiking.

In Fig. 3, a typical electropherogram obtained with imitated vanilla flavouring is shown. No components with the exception of vanillin can be identified. In Fig. 4 an electropherogram of a product imitating an extract of natural vanilla is shown. The product is dark brown and contains, according to the manufacturer's declaration, authentic vanilla extract. However, this vanilla extract is only a minor component of the product, because the mass ratio of vanillin, 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzoic acid and 4-hydroxybenzoic acid does not correspond to the mass ratio of these principal polar aromatic flavour components in natural vanilla extracts. Synthetic vanillin has been added to the diluted extract by the manufacturer.

Table 2
Migration times of vanilla constituents and potential adulterants with a separation buffer containing no anionic surfactant, with the same measurement conditions as in Fig. 2

Solute	t_M (min)	Solute	t_M (min)
Thiourea[a]	3.00	Vanillin	4.99
Coumarin	3.00	4-Hydroxybenzaldehyde	5.24
Piperonal	3.00	3-Methoxybenzoic acid	5.16
3-Methoxybenzaldehyde	3.00	Vanillic acid	5.59
4-Hydroxy-3-methoxybenzaldehyde	3.17	4-Hydroxybenzoic acid	5.89
4-Hydroxybenzyl alcohol	3.18	3,4-Dihydroxybenzoic acid	7.94
Ethylvanillin	4.69		

[a] Marker of hold-up time.

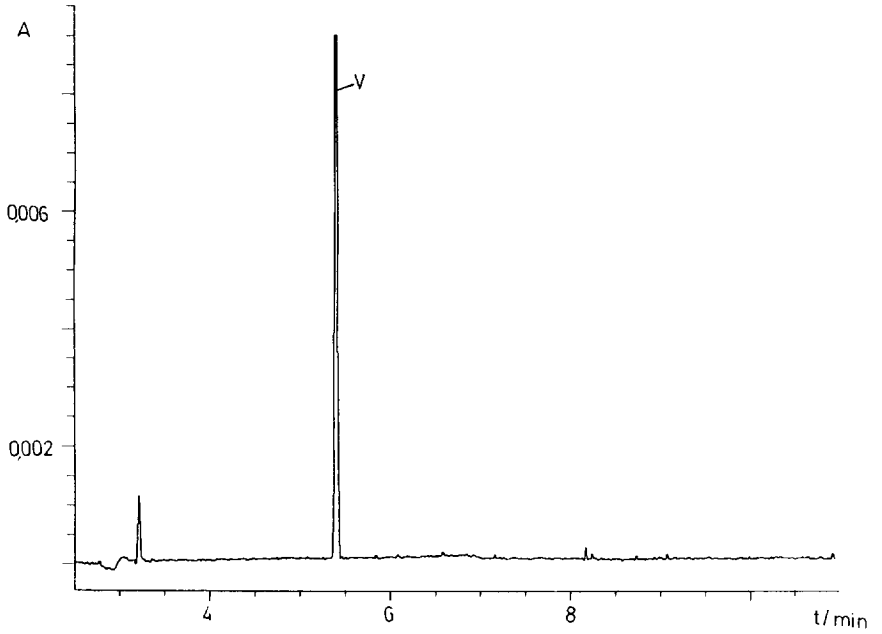

Fig. 3. Electropherogram of imitated vanilla flavouring. Peak V = vanillin. For measurement conditions, see Fig. 2.

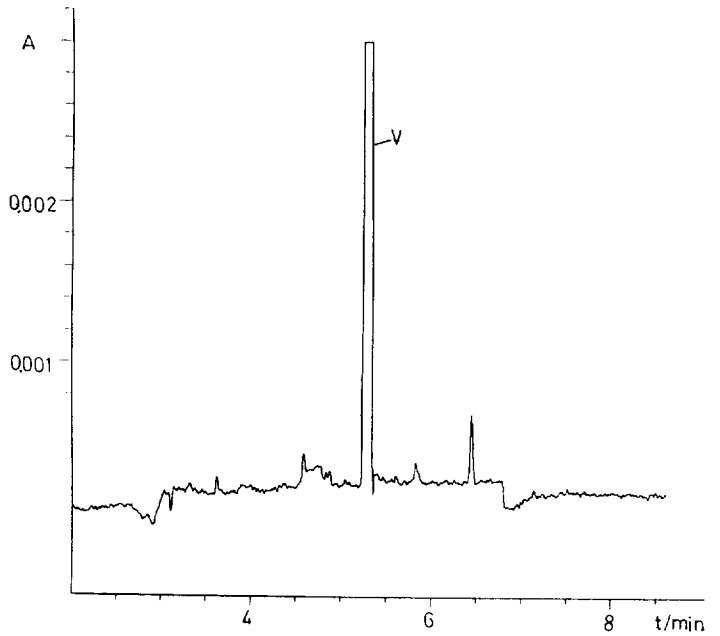

Fig. 4. Electropherogram of a product imitating an extract of natural vanilla. Peak V = vanillin. For measurement conditions, see Fig. 2.

In Fig. 5, the electropherogram of an aqueous solution of vanilla sugar is shown. This product does not contain any synthetic additives, according to manufacturer's declaration. The mass ratio of the principal polar aromatic flavour components vanillin, 4-hydroxybenzaldehyde, vanillic acid and 4-hydroxybenzoic acid and the minor components 4-hydroxybenzyl alcohol and 4-hydroxy-3-methoxybenzyl alcohol corresponds to the mass ratio of these constituents that we found in an aqueous extract of a vanilla bean. The electropherogram of the laboratory-made aqueous vanilla extract is shown in Fig. 6.

3.4. Analysis of beverages

In Figs. 7 and 8, the electropherograms obtained with vanilla-flavoured tea and vanilla-flavoured coffee prepared from coffee extract are compared. The beverages were injected directly after filtration through a paper filter. In Fig. 7 peaks can be attributed to compounds related to vanillin. The tea is flavoured with dried pieces of vanilla beans.

In Fig. 8, peaks due to compounds related to vanillin are absent. The mass ratio of vanillin, 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzoic acid and 4-hydroxybenzoic acid does not correspond to the mass ratio of these principal polar aromatic flavour components in natural vanilla extracts. The coffee extract is exclusively flavoured with vanillin. In addition to the aroma components, caffeine can also be quantified by direct injection of the beverages.

3.5. Quantitative analysis

In none of the vanilla flavourings investigated was ethylvanillin used as a flavouring agent. Therefore, it was selected by us as an internal standard. In Table 3 the quadratic correlation coefficient, the slope, the relative standard deviation (R.S.D.) of the slope of the calibration lines and the detection limits (signal-to-noise ratio =

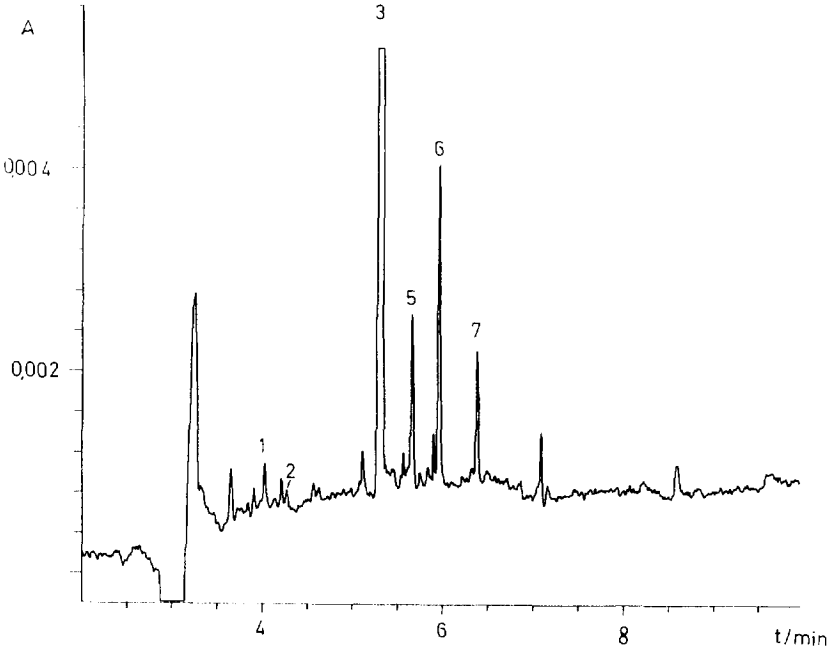

Fig. 5. Electropherogram of an aqueous solution of vanilla sugar. Peak identification: 1 = 4-hydroxybenzyl alcohol; 2 = 4-hydroxymethoxybenzyl alcohol; 3 = vanillin; 4 = 3-methoxybenzoic acid; 5 = 4-hydroxybenzaldehyde; 6 = vanillic acid; 7 = 4-hydroxybenzoic acid. For measurement conditions, see Fig. 2.

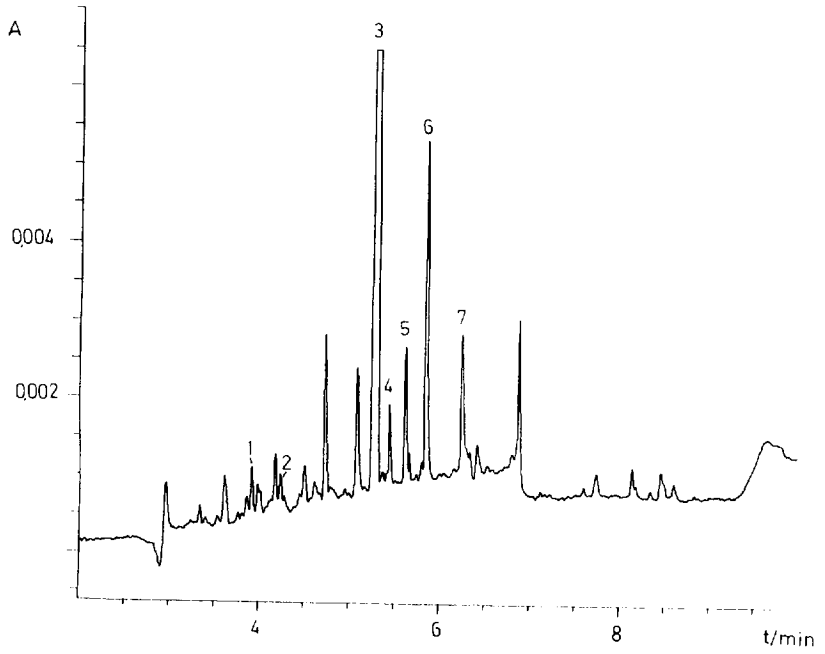

Fig. 6. Electropherogram of an aqueous extract from a vanilla bean. Peak identification as in Fig. 5. For measurement conditions, see Fig. 2.

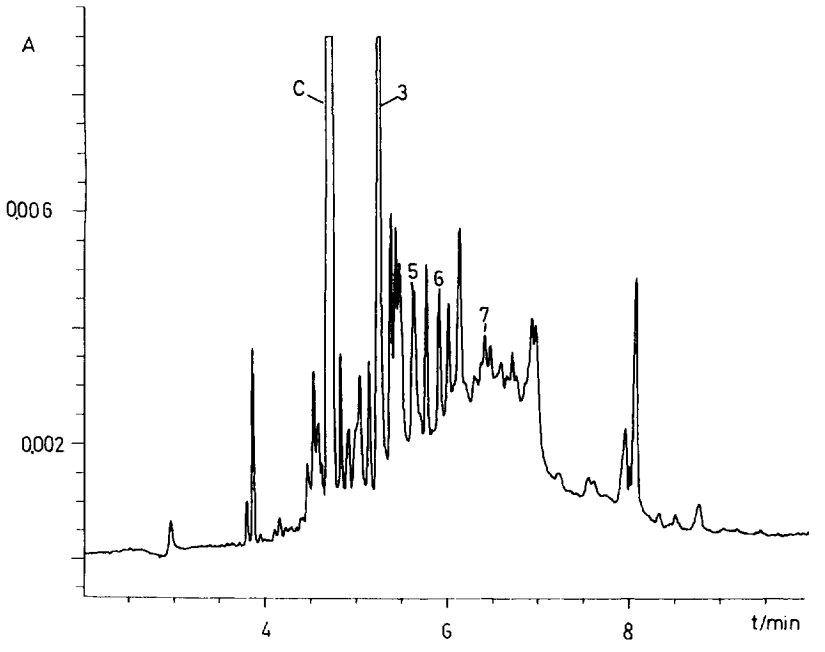

Fig. 7. Electropherogram of vanilla-flavoured tea. Peak identification as in Fig. 5; C = caffeine. For measurement conditions, see Fig. 2.

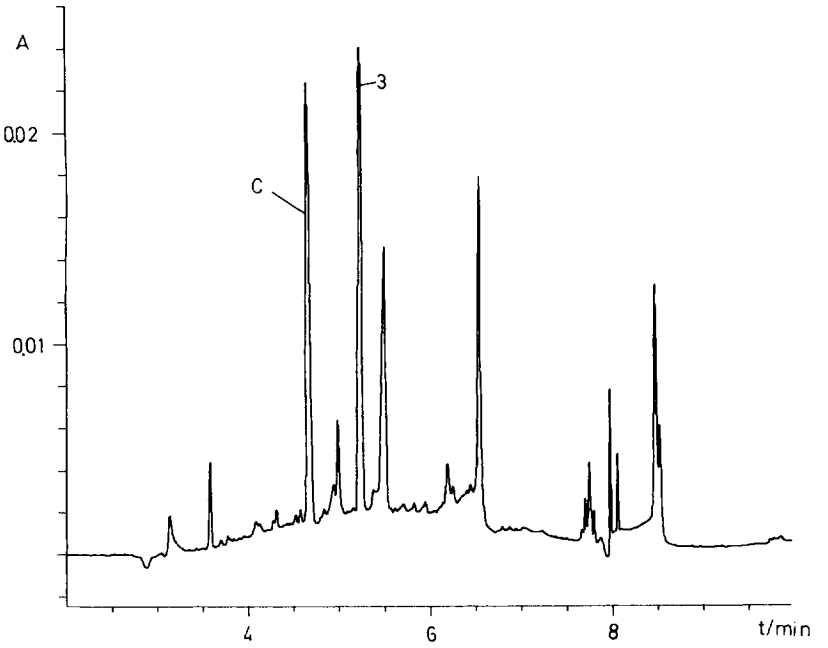

Fig. 8. Electropherogram of vanilla-flavoured coffee extract. Peak identification as in Fig. 5; C = caffeine. For measurement conditions, see Fig. 2.

3) for the four analytes are given. The linear calibration range covers two orders of magnitude (from 1 to 100 mg/l).

In Table 4 the R.S.D. of the peak area for four analytes calculated from ten consecutive runs is compared with the R.S.D. of the normalized peak areas (peak area of analyte/peak area of inner standard) obtained in the same runs. The R.S.D. is greatly reduced by the normalization, mostly owing to the elimination of variations in the injected volume. An injection time of 1.5 s was selected, because with longer injection times band broadening is observed. The R.S.D. of the migration times is below 1% in all cases.

4. Conclusions

MEKC can be used as a rapid screening method for the analysis of vanilla flavourings and vanilla extracts. The method permits the direct injection of vanilla extracts and beverages. The

Table 3
Quantification of principal polar compounds in natural vanilla extracts: slope, relative standard deviation of the slope and the quadratic correlation coefficient, r^2, of the regression lines used for calibration and detection limits (LOD)

Analyte	Slope ($mg^{-1}\,l^{-1}$)	R.S.D. (slope) (%)	r^2	LOD (mg/l)
4-Hydroxybenzaldehyde	0.0129	0.43	0.99991	0.9
Vanillic acid	0.0229	0.26	0.99997	0.5
Vanillin	0.0233	0.16	0.99999	0.5
4-Hydroxybenzoic acid	0.039	0.41	0.99992	0.3

Normalized peak areas (ethylvanillin as internal standard, β(ethyl vanillin) = 45.45 mg/l) are the database for the regression line. The data are the means of three consecutive runs. For measurement conditions, see Fig. 2.

Table 4
Comparison of relative standard deviations of the peak area [R.S.D.(A)] [β(analyte) = 92–95 mg/l] with those of the normalized peak areas [R.S.D.(A_N)] [ethylvanillin as internal standard, β(ethyl vanillin) = 45.45 mg/l] obtained for ten consecutive runs

Analyte	R.S.D.(A) (%)	R.S.D.(A_N) (%)
4-Hydroxybenzaldehyde	3.43	0.81
Vanillic acid	3.44	0.69
Vanillin	3.39	0.47
4-Hydroxybenzoic acid	3.38	0.60

For measurement conditions, see Fig. 2.

developed method can be part of a system of quality control for vanilla extracts in addition to stable isotope ratio measurements as a second independent method, quantifying the mean components and possible adulterants.

Analysis by MEKC is very sensitive to matrix constituents influencing migration times and peak shapes. Large amounts of an organic solvent in the sample solution either have to be avoided or must be taken into consideration for the preparation of the standard solutions. The use of an appropriate internal standard is indispensable with the experimental set-up employed in order to obtain high precision in quantitative analysis.

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