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Determination of phenolic carboxylic acids by micellar electrokinetic capillary chromatography and evaluation of factors affecting the method

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ABSTRACT

Micellar electrokinetic capillary chromatography (MECC) based on cetyltrimethylammonium bromide (CTAB) was developed for separation of individual cinnamic and benzoic acid derivatives. This method was adapted to the separation of the phenolic carboxylic acids from other anions, phenolics and glucosinolates in samples prepared from plant materials. The influence of temperature, voltage, pH, electrolyte and detergent concentrations in the buffer on migration times for the compounds considered, peak areas, resolution and number of theoretical plates were investigated. It is shown that rapid and efficient separations (> 270 000 plates/m) are possible, even for structural closely related phenolics.

INTRODUCTION

Higher plants contain a large number of different cinnamic and benzoic acid derivatives, including several compounds with appreciable physiological effects [1,2]. This group of naturally occurring phenolics occur in plants, mainly as esters with various types of alcohol residues depending on the plant genera and plant parts examined [3]. Phenolic choline esters dominate in cruciferous seed material, whereas the phenolic acids are present as esters of malate and carbohydrates in vegetative plant parts [2,4]. Furthermore, recent investigations on dietary fibres (DF) isolated from peas, rapeseeds and cereals (rye and wheat) have revealed a considerable amount of phenolic acids, bound as esters or associated in other forms to the DF fraction [5].

Interest in DF has risen as a result of the large number of reports on various physiological effects connected with the intake of DF [6]. Evaluation of the casual relationship between DF and these effects demands thorough characterization of the chemical composition, including the type and level of the associated phenolics. Current methods for determination of the free phenolic carboxylic acids comprise gas chromatography (GC) and high-performance liquid chromatography (HPLC) [2,7], both of which are relatively expensive and time-consuming techniques. However, a promising alternative has been found in free zone high-performance capillary electrophoresis (HPCE), as demonstrated for various types of phenolic carboxylic acids [8]. HPCE provides opportunities for rapid, simple and cheap analyses. Moreover, the use of detergents in the buffer system gives promise of even better results, as shown for several aliphatic low-molecular-weight carboxylic acids and other groups of compounds [9–12].

Micellar electrokinetic capillary chromatography (MECC) was introduced by Terabe *et al.* in 1984 [9] as a technique for separating neutral molecules, which otherwise migrate as a group with the electroosmotic flow. MECC can also be used to enhance the separation of charged species [13]. In this

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paper, we present a technique for determination of individual cinnamic and benzoic acid derivatives using MECC with cetyltrimethylammonium bromide (CTAB). The method was systematically evaluated by studying the effects of various factors on the migration time, peak area, number of theoretical plates and resolution. Application of the developed conditions, including temperature, voltage, pH, electrolyte and detergent concentrations in the buffer, have been shown to provide an inexpensive and efficient method for the separation of individual phenolic carboxylic acids in solutions, including samples prepared directly from DF and other plant materials.

EXPERIMENTAL

Apparatus

The apparatus used was an ABI Model 270A capillary electrophoresis system (Applied Biosystems, Ramsey, NJ, USA), with a 657 mm × 0.05 mm I.D. fused-silica capillary tube. Detection was performed by on-column measurements of UV absorption at a position 445 mm from the injection end of the capillary. For data processing, a Shimadzu (Kyoto, Japan) Chromatopac C-R3A was used.

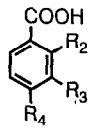
Samples and reagents

Cinnamic and benzoic acid derivatives (Fig. 1) and flavonoids were plant products isolated and characterized by traditional methods [2,4] and/or obtained from Sigma (St. Louis, MO, USA).

Total dissolution of the compounds tested required the use of ethanol, which may alter the separation conditions in MECC. Evaporation of the ethanol prior to the final addition of water, for obtaining suitable peak heights, was therefore performed. The concentrations of the individual phenolic carboxylic acids in standard solutions ranged from $5 \cdot 10^{-4}$ to $1 \cdot 10^{-2}$ *M*.

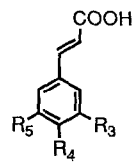
DF samples obtained from Polish-grown double low rapeseed (cv. Bronowski), rapeseed hulls, wheat (cv. Java) and rye (cv. Danikowskie) consisted of insoluble (IDF) and soluble dietary fibres (SDF), isolated by an enzymatic gravimetric method described elsewhere [14]. Phenolic carboxylic acids were isolated either directly from DF or from DF solutions obtained by water extraction (25 ml/g, 2 h, 70°C) of DF as described [5]. The procedure consisted of hydrolysis under alkaline conditions (70 mg of DF + 5 ml of 1.0 *M* ammonia solution or 5 ml of water extract + 0.41 ml of 25% ammonia solution, 2 h, 100°C under reflux), centrifugation and evaporation of the supernatant to dryness, addition of 1 ml of 1.0 *M* HCl, extraction with chloroform (3 × 2 ml) and evaporation to dryness. The residue was dissolved in 0.5 ml of 1.0 *M* ammonia solution, evaporated to dryness, redissolved in 4 × 50 μl of water and used for MECC after centrifugation at 3000 *g* for 10 min.

Sodium tetraborate and sodium phosphate were obtained from Sigma and CTAB from BDH (Poole, UK). All chemicals were of analytical-reagent grade.



Benzoic acid derivatives

No.	Name	R_2	R_3	R_4
7	Salicylic acid	- OH	- H	- H
1	4-Hydroxybenzoic acid	- H	- H	- OH
2	Isovanillic acid	- H	- OH	- OCH_3



Cinnamic acid derivatives (trans (E) isomers)

No.	Name	R_3	R_4	R_5
5	Coumaric acid	- H	- OH	- H
4	Ferulic acid	- OCH_3	- OH	- H
6	Isoferulic acid	- OH	- OCH_3	- H
3	Sinapic acid	- OCH_3	- OH	- OCH_3

Fig. 1. Structures of cinnamic and benzoic acid derivatives used in MECC analyses. Numbers indicated are used in connection with the other figures.

Procedure

Buffer solutions were prepared from stock solutions of sodium tetraborate (100 m*M*), sodium phosphate (150 m*M*) and CTAB (100 m*M*). The

different run buffers were mixed from these stock solutions, water was added to the desired concentration (borate plus phosphate, 16–48 m*M*; CTAB, 20–50 m*M*), adjusted to the required pH (6.0–8.0) and then filtered through a 0.45-μm membrane filter prior to use. Buffers were changed manually. The samples were introduced from the negative end of the capillary by 1-s vacuum injection, resulting in injection volumes of few nanolitres. Separations were performed at 30–60°C and 17–23 kV. On-column UV detection at 280 nm was applied.

Standard conditions, while varying individual running parameters, were a temperature of 40°C, a voltage of 20 kV and electrolyte and detergent concentrations of 18 m*M* (borate), 30 m*M* (phosphate) and 50 m*M* (CTAB). Calculations of relative migration times (*RMT*) and normalized peak areas (*NA*) were performed according to the equations

$$RMT = MT_1/MT_2 \quad (1)$$

where MT_1 is the migration time of the actual phenolic carboxylic acid and MT_2 that of the phenolic carboxylic acid in the mixture given the value 1, and

$$NA = A/MT \quad (2)$$

where A is the measured peak area.

The number of theoretical plates (N) and resolution (R_s) were calculated as described elsewhere [15,16]. Linearity of the method was based on least-squares estimates.

Washing with buffer was performed between each analysis for a minimum of 5 min. After various numbers of analyses, the capillary was washed for 2–4 min with 1.0 *M* NaOH and 0.1 *M* NaOH solutions.

RESULTS AND DISCUSSION

The applied MECC method is based on the electrophoretic mobility of the analytes, electroosmotic flow of the solvent and electrophoretic mobility of CTAB micelles, as described previously [17]. The pH of the applied buffer creates negatively charged silica groups on the capillary wall, giving the possibility of CTAB forming a double layer, associating one positive end to the capillary wall and the other to negative ions from the run buffer. Thereby, the direction of the electroosmotic flow is shifted towards the anode, compared with free zone capillary electrophoresis. Micelles formed by CTAB, as the concentration in solution exceeds critical micelle concentration (CMC), move toward the cathode, opposite to the electroosmotic flow. With injection of the negatively charged analytes at the negative end of the capillary, it means, that the electroosmotic flow increases the analyte speed, whereas CTAB micelles has a retarding effect. The selective retention obtained in this MECC technique is therefore due to differential partitioning of the phenolics between the aqueous buffer and the micellar phases. A comprehensive evaluation of the migration behaviour of cationic analytes in an anionic micelle sys-

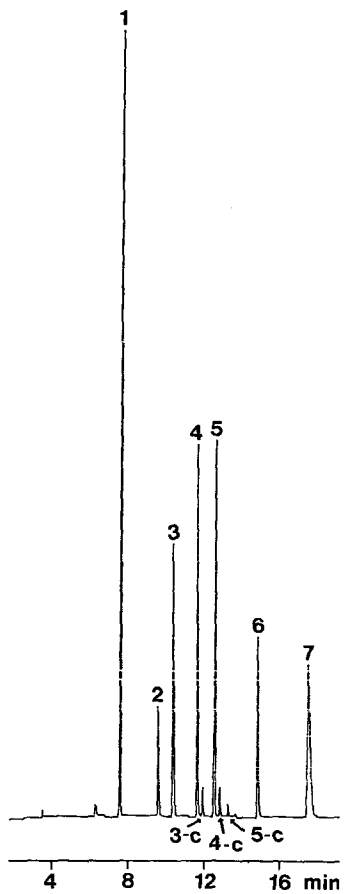


Fig. 2. Elution order of cinnamic and benzoic acid derivatives separated by the MECC technique. Numbers as in Fig. 1. Arrows show the occurrence of *cis* forms (c) of sinapic acid, ferulic acid and coumaric acid. Separation conditions: temperature, 40°C; voltage, 20 kV; buffer composition, 18 m*M* borate–30 m*M* phosphate–50 m*M* CTAB adjusted to pH 7.0; UV detection at 280 nm.

tem has recently been described by Strasters and Khaledi [18]. Further, the complex mechanisms of the interactions of ions and micelles have been evaluated by Wallingford and Ewing [19].

The elution order of the structurally closely related phenolic carboxylic acids investigated is presented in Fig. 2.

The ability of MECC to separate structural isomers was demonstrated by the separation of ferulic and isoferulic acid and of 4-hydroxybenzoic and salicylic acid, respectively. Moreover, the *cis* forms of the cinnamic acid derivatives sinapic, ferulic and coumaric acid, indicated by arrows in Fig. 2, were efficiently distinguished from the dominating *trans* forms.

Modifications of the MECC conditions implied changes of temperature, voltage, pH and composition of buffer, including concentrations of borate plus phosphate and detergent.

Temperature

Shorter migration times were seen with increasing temperatures, in agreement with the simultaneous decrease in viscosity of the solvent in the capillary. The lowering of viscosity was also reflected in an increased current intensity. *RMT* values, which in all instances were calculated relative to ferulic acid, were unchanged over the applied temperature range.

NA values increased with increase in temperature, the increment occurs for all phenolics, as illustrated in Fig. 3.

Corrections of peak areas (*NA*) were performed to eliminate the influences of alterations in migration time. Under the present circumstances, the changes in migration times were induced by the change in temperature, but may also be seen after series of analyses. In this case, the phenomenon may be limited by regular changes of the buffer and washing with NaOH for regeneration of the capillary. Studies of repeatability, subjected to the above and also to the problems of evaporation, showed constant normalized peak areas, even after numerous analyses [13]. However, under the present conditions, corrections for changes in migration times were insufficient to keep the *NA* values constant. As the magnitude of these changes differed markedly among the analytes tested (Fig. 3), it was assumed that evaporation could not explain the observed al-

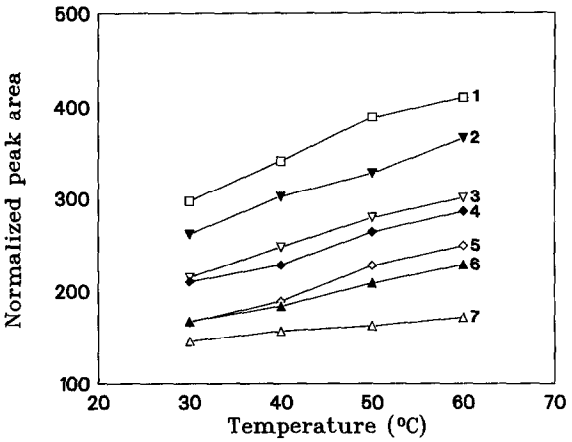


Fig. 3. Relationship between temperature and normalized peak areas of cinnamic and benzoic acid derivatives. Numbers as in Fig. 1. Other separation conditions as in Fig. 2.

terations. However, investigations on glucosinolates have shown that changes in electrolyte concentration in the run buffer, due to evaporation, had different effects on the *NA* values, depending on the migration times of the compounds involved (unpublished results). Another explanation of varying *NA* values may be the occurrence of a temperature-related alteration of solvent viscosity, injected sample volume as well as molar absorptivities with associated changes in UV absorption, as also demonstrated for different proteins [20], and related to changed interactions of phenolics with micelles (see below). Moreover, salicylic acid has the ability to form internal hydrogen bonds between the carboxy and hydroxy groups in the molecule, changing the chromophore. However, increasing voltage, electrolyte and CTAB concentrations, which may also result in higher capillary temperatures, did not confirm this hypothesis of a temperature-related effect (see below). Whatever the reason, the determination of phenolics in series of samples implies constancy of the parameters affecting the *NA* values.

The effect of temperature on separation efficiency was minor, although a tendency for decreases in N and R_s values were seen for some of the compounds tested. This observation may be explained by changes in the interaction of phenolics with micelles, leading to minor reductions in peak width compared with reductions in migration time. Hence, increased temperature may change the

CMC to a higher value, affecting the micelle concentration, aggregation number and size and shape of the micelles in the solution [10,21]. The changes in N and R_s values were restricted to the cinnamic acid derivatives, indicating that the structure of the compounds may be of importance.

In MECC, in contrast to reversed-phase HPLC [2], the micellar phase (semi-stationary) will pass through the detector together with the analytes to be detected. It is then obvious that changes in the micellar interaction of phenolics may affect the molar absorptivities and thereby the *NA* values (see above).

Voltage

An increase in applied voltage resulted in reductions in migration times, as illustrated in Fig. 4. The reduction was highest for compounds having long migration times, meaning that the overall time of analysis was reduced considerably at high voltage. Both *RMT* and *NA* values remained constant.

According to the HPCE theory, an increased field strength leads to improved separation efficiency, which again should be reflected in N and R_s values [15,22,23]. However, this could not be confirmed by the results obtained, as the N and R_s values were not greatly affected. Despite air-controlled temperature regulation in the apparatus, limited dissipation of the developed heat may result in band broadening and less sharp peak profiles [15,23]. This means that the reduced separation window, owing to a shorter total time of the analysis and with a limited simultaneous reduction in peak width, will affect the R_s values in a negative direction.

pH

Changing the pH from 6.0 to 8.0 had no marked effect on migration times, either actual or relative. The *NA* values were not affected, except for 4-hydroxybenzoic acid, which showed a considerable increment as demonstrated in Fig 5. Moreover, at high pH, the *NA* value for salicylic acid also had a tendency to increase.

Again, an effect on the molar absorptivities, here induced by the change in $[H^+]$, may be a suitable explanation. The observed results are probably due to ionization of the hydroxyl groups under the weakly alkaline conditions, leading to alterations in chromophore properties.

No clear effects on N and R_s values were observed with increase in pH, although a tendency for increasing separation efficiency was seen for some of the compounds tested. The reason for this is uncertain, as no change in migration time occurred and narrower peaks were not observed.

Electrolyte concentration

Changes in the concentrations of borate and phosphate ions in the run buffer had a marked effect on the migration times of phenolics, as illustrated in Fig. 6.

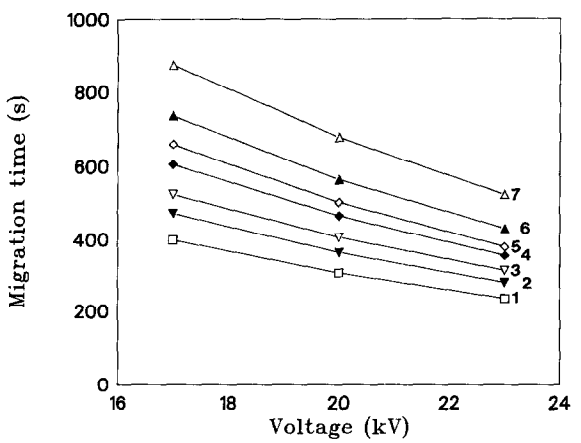


Fig. 4. Relationship between voltage and migration time of cinnamic and benzoic acid derivatives. Numbers as in Fig. 1. Other separation conditions as in Fig. 2.

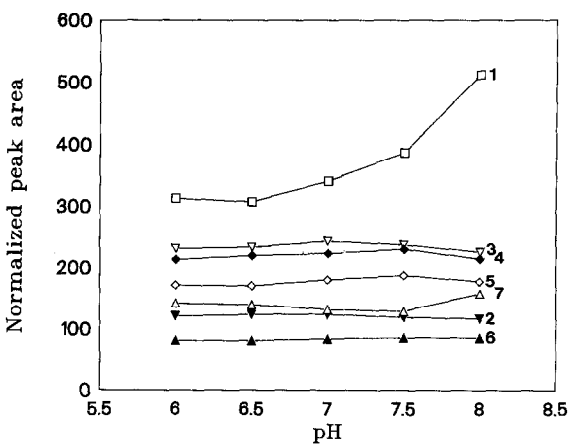


Fig. 5. Relationship between pH and normalized peak areas of cinnamic and benzoic acid derivatives. Numbers as in Fig. 1. Other separation conditions as in Fig. 2.

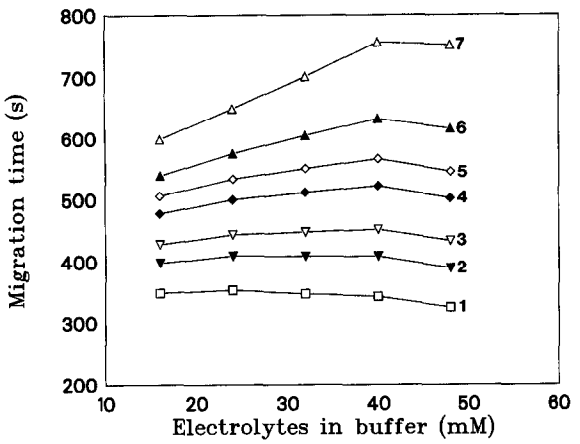


Fig. 6. Relationship between concentration of borate and phosphate ions in the buffer and migration time of cinnamic and benzoic acid derivatives. Numbers as in Fig. 1. Other separation conditions as in Fig. 2.

The increment in migration time was greatest for compounds late in the electropherogram. These compounds are associated with micelles to a greater extent than compounds with short migration times, indicating an effect on the micellar phase. In aqueous solution, the presence of electrolytes causes a decrease in CMC, owing to decreased electrical repulsions between the ionic ends of the surfactants in micelles [21]. This may result in higher micelle concentrations and thereby the observed delay in migration times, similarly to the effects found at increased concentrations of CTAB (see below). However, as the CMC of CTAB is less than 1 m*M* [21], the change in CMC will be small compared with the concentration of CTAB (50 m*M*).

A probably more important factor is the electroosmotic flow, which is considered to be changed as a result of an effect on the zeta potential. The difference in concentration of anions between the middle of the capillary and the capillary wall will decrease as the electrolyte concentration increases, leading to a reduction in electroosmotic flow [24]. Moreover, competition between CTAB and cations from the buffer may reduce the extent of the double layer on the capillary wall, leading to the same effect.

In the system described here, borate, phosphate and bromide ions are present as counter ions to CTAB micelles. An increase in borate and phosphate concentrations in the buffer may affect the ratio of the various counter ions on the surface of micelles, and thereby change the interaction of analytes with the micellar phase. The reason for the sudden decrease in migration time, when the concentration of borate plus phosphate exceeds 40 m*M* is uncertain, but a possible explanation may be an increase in temperature, when the electrolyte concentration and thereby the current in the solution are increased to a certain level.

As the concentration of electrolytes in the run buffer increased, higher *RMT* values were obtained for analytes with long migration times, whereas the *RMT* values decreased for the first eluted phenolics. This is also in accordance with the changes in migration time discussed above. Changes in *RMT* values confirm that identification of peaks should be performed with a knowledge of *RMT* values under the actual running parameters. However, the *RMT* values may vary even under standardized conditions, as demonstrated by Michaelsen *et al.* [13].

The *NA* values remained relatively constant. The *N* values were affected in a non-systematic way, whereas the R_s values, especially for the most hydrophobic compounds, increased markedly as the electrolyte concentration increased. This may be explained by the changes in CMC, as described above.

CTAB concentration

A positive linear relationship was found between migration time and increasing concentration of CTAB in the run buffer. This is in accordance with the theory, as a higher micellar concentration leads to changes in the partitioning of compounds in the direction of the micellar phase. Moreover, alterations in the electroosmotic flow may contribute to the effect observed. Only minor changes in *RMT* values were seen.

NA values were affected in a non-systematic way, the explanation for this being uncertain. However, changes in the interaction of analytes with micelles as the micelle concentration increased could be expected to influence the chromophoric systems, as previously mentioned for temperature–*NA* value interactions.

There was a tendency for improved *N* and R_s values as the CTAB concentration in the buffer increased. Several factors may contribute to the observed effect, including changes in the shape, size and aggregation number of micelles.

The conflict existing between the demand for rapid analysis and high separation efficiency is reflected in the standard conditions chosen. Hence the short migration times obtained with application of high temperatures are obtained partly at the expense of N and R_s values. As a compromise, 40°C was chosen. Voltages exceeding 20 kV were avoided owing to the risk of considerable heat development. An electrolyte concentration at 48 mM was adapted in order to take advantage of the positive effect on R_s values together with the improvements in migration times at concentrations higher than 40 mM. The buffer pH was kept neutral, as the NA values seemed to remain relatively constant until this point. Finally, 50 mM CTAB provided the maximum separation efficiency.

Applications

The method described was applied to the identification of cinnamic and benzoic acid derivatives associated with DF. Fig. 7 shows electropherograms of phenolics in IDF isolated from whole rapeseed and rye.

The phenolic carboxylic acids, occurring as esters in IDF from rye, were quantitatively dominated by *trans*-ferulic acid, minor amounts of the *cis* isomer and with only trace amounts of sinapic and coumaric acid. IDF from oilseed rape had a much more complicated composition of ester-bound phenolic carboxylic acids, dominated by *trans*-sinapic acid, minor amounts of *cis*-sinapic acid, 4-hydroxybenzoic acid, vanillic acid, coumaric acid, ferulic acid and some additional unidentified compounds [3], appearing in the area of the electropherogram corresponding to flavonoids.

Flavonoids are easy to separate with the MECC technique; details will be presented elsewhere. These compounds appear mainly in areas of the electropherograms corresponding to higher migration times than the phenolic carboxylic acids, but changes in the electrophoretic conditions lead to reduced migration times, as shown in Fig. 8.

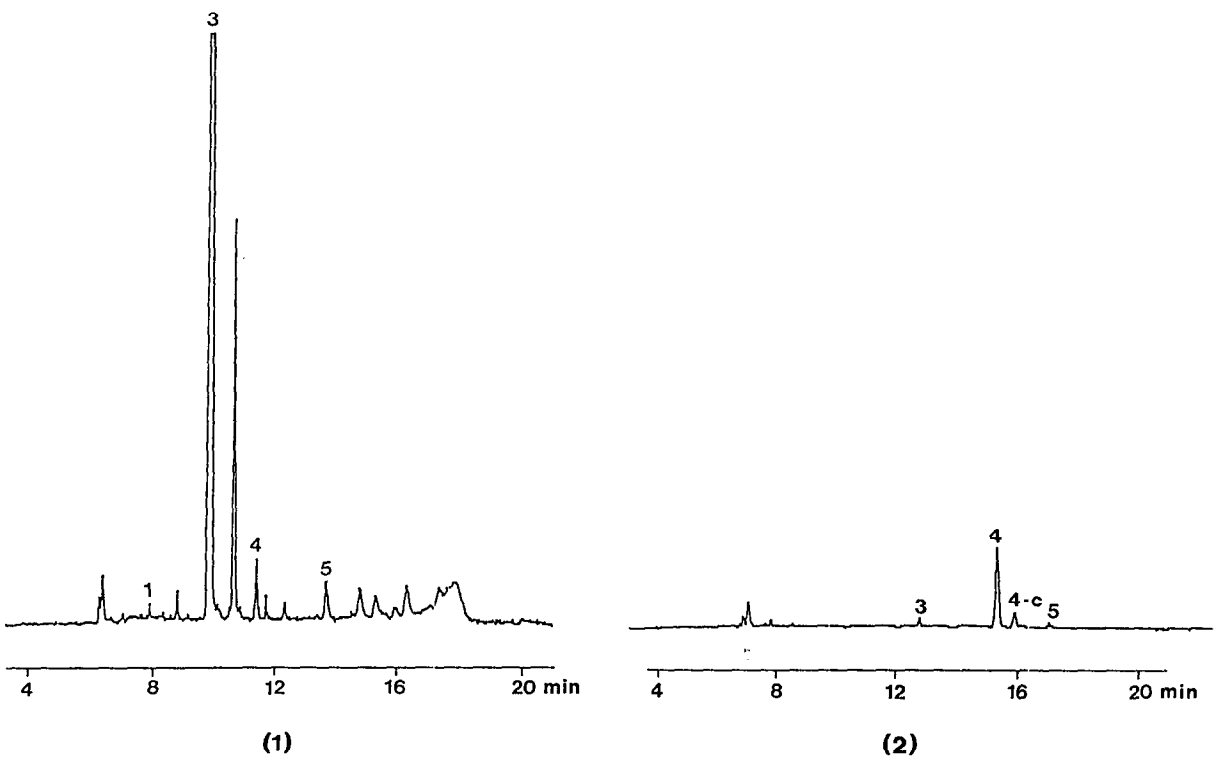


Fig. 7. Electropherograms of cinnamic and benzoic acid derivatives in IDF from (1) whole rapeseed and (2) rye (water extract). Numbers as in Fig. 1. Separation conditions as in Fig. 2.

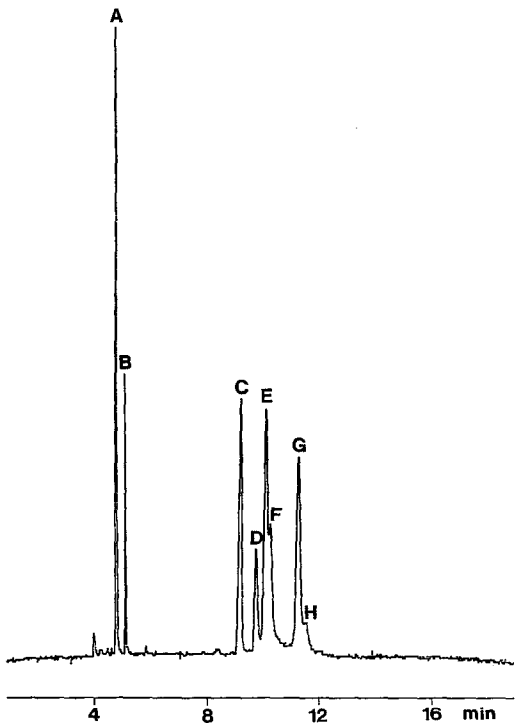


Fig. 8. Electropherogram of flavonoids. A = kaempferol-3-sophoroside-7-glucoside; B = kaempferol-3-sinapoylsophoroside-7-glucoside; C = rustoside; D = rutin; E = kaempferol-3-glucoside; F = quercetin-3-glucoside; G = kaempferol-3-(6″-carboxyglucoside); H = quercetin-3-(6″-carboxyglucoside). Separation conditions: temperature, 50°C; voltage, 26 kV; buffer composition, 12 m*M* borate–20 m*M* phosphate–60 m*M* CTAB adjusted to pH 7.0, 4% 1-propanol added; UV detection at 350 nm.

Phenolic carboxylic acids such as sinapic acid, isoferulic acid and benzoic acid derivatives occur in some plants as esters bound to glucosinolates [25]. These types of compounds appear in other areas of the electropherograms [13] (higher migration times than the free phenolic carboxylic acids), as for phenolic carboxylic acids bound as malate esters [2].

Identification of the compounds mentioned was based on traditional techniques described previously [2,13], including UV and NMR spectroscopy and with use of detection at various wavelengths in MECC.

Investigations on the changes in *NA* values with increasing concentrations of phenolics in standard mixtures showed good coherence. Correlation coefficients (r^2) ranged from 0.9946 to 0.9998, indicating a strong linear relationship. This means that the method described may be employed for quantitative analyses of phenolics, taking the different factors influencing *NA* values into account.

CONCLUSIONS

The MECC method described is highly effective, in comparison with HPLC and GC methods, for separating cinnamic and benzoic acid derivatives in standard solutions and in samples prepared from plant materials. The work has shown that migration times for free phenolic carboxylic acid derivatives are different from those of various types of other anions, glucosinolates, malate esters and flavonoids. This means that the separation of these compounds from the free acids is easy and possible with the MECC method. It is a rapid and inexpensive method compared with HPLC, and the separation capacity may be high, exceeding 270 000 plates/m. Evaluation of the method showed a considerable susceptibility towards different factors, making it relatively simple to choose the conditions that will separate just the compounds of interest. However, reliable identification requires a knowledge of the *RMT* values under the actual running parameters. Moreover, in order to obtain quantitative determinations, the variation in the molar absorptivities under different conditions is an interesting problem which has to be evaluated.

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