MECC Determination of aromatic choline esters accumulated in cruciferous seeds and associated to dietary fibres

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ABSTRACT

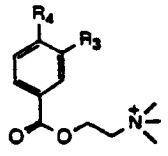
Micellar electrokinetic capillary chromatography (MECC) using different detergents as the micellar phase has been investigated for separation and quantification of individual aromatic choline esters. MECC based on sodium cholate (NaCh) in combination with taurine was found suitable and optimization of the method resulted in a total runtime of 10 minutes and up to 485.000 theoretical plates per meter of capillary. The relative standard deviations were low, ranging from 0.4-0.7% and 0.6-2.4% for the relative migration times and relative normalized areas, respectively, and this is a precondition for identification and quantitative determination. The high repeatability demanded certain precautions including regular shift of buffers and evaporation septa on the sample vials. The linearity of the method was good ($0.9950 < r^2 < 0.9984$) and the detection limits was found between 25 and 60 pg. Selected examples of analysis of aromatic choline esters accumulated in seeds from *Sinapis alba* and associated to dietary fibres from rapeseed meal (*Brassica napus*) are presented.

INTRODUCTION

Aromatic choline esters make up a well defined group of natural products occurring in plants and they are, due to their physiological effects, found interesting in connection with rapeseed quality (1,2). Sinapine is the best known and quantitatively dominating aromatic choline ester in rapeseed, where it co-occurs with several other aromatic choline esters (3). Available methods for determination of aromatic choline esters include high-performance liquid chromatography (HPLC), based on ion-pairing chromatography (4). However, HPLC methods suffer from some disadvantages compared to the potential possibilities with use of high-performance capillary electrophoresis (HPCE) (5,6). Separation and determination of cations have been achieved in capillary zone electrophoresis (7,8) and the technique of micellar electrokinetic capillary chromatography (MECC) (9) has also proved to be successful for charged analytes (10,11). We have developed an efficient MECC method for the analysis of aromatic choline esters using an anionic bile salt surfactant as the micellar phase supplemented with a zwitterionic compound to prevent adsorption of analytes to the capillary wall (12). With the combined technique of group separation, purification (3), and MECC (12), a rapid, simple and efficient method is obtained for the determination of aromatic choline esters accumulated in rapeseed and other cruciferous plants.

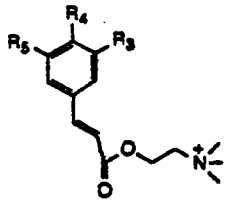
MATERIALS AND METHODS

The apparatus used was an ABI Model 270A capillary electrophoresis system (Applied Biosystems, USA), with a 760 mm x 0.05 mm I.D. fused-silica capillary tube. Detection was performed by on-column measurements of UV absorption (235 nm) at a position 530 mm from the injection end of the capillary. The names and structures of the benzoic- and cinnamic acid derivatives are presented in Fig. 1.



Benzoic acid derivatives

No.	R_3	R_4	Name
2	- H	- OH	4 - Hydroxybenzoylcholine
3	- OCH_3	- OH	Vanillylcholine
9	- OH	- OCH_3	Isovanillylcholine
10	- OCH_3	- OCH_3	Hesperaline



Cinnamic acid derivatives

No.	R_3	R_4	R_5	Name
4	- H	- OH	- H	p - coumaroylcholine
5	- OCH_3	- OH	- H	Feruloylcholine
6	- OCH_3	- OH	- OCH_3	Sinapine
7	- OCH_3	- OCH_3	- H	3,4 - dimethoxycinnamoylcholine
8	- OH	- OCH_3	- H	Isoferuloylcholine

Figure 1. Structures and names of benzoic and cinnamic acid derivatives used in MECC analyses.

Aromatic choline esters were isolated and purified by the procedure described by Clausen *et al.* (1985) (3). Disodium hydrogenphosphate, disodium tetraborate, taurine, and sodium cholate (NaCh), 2-propanol and other chemicals used were of analytical-reagent grade.

Buffers were filtrated through a 0.20 μm membrane filter. Washing of the capillary was performed with 1.0 M NaOH for 2 minutes and with buffer for 5 minutes before each analysis. When buffer composition was changed, the washing procedure was extended to 4 minutes with 1.0 M NaOH and 10 min with the new buffer. The sample was introduced at the anode by vacuum for 1 second. Unless otherwise stated, the separations were performed at 30°C and 20 kV.

RESULTS AND DISCUSSION

The preliminary work on a MECC-method for aromatic choline esters comprised investigations of different surfactants, including the positively charged tetradecyltrimethylammonium bromide and the negatively charged SDS. Unsatisfactory separation was obtained with both types of detergents, and for SDS, this was due to too strong ionic interaction, leading to unacceptable high migration times of the analytes in this system.

The negatively charged bile salt, NaCh is special in forming rodlike or cylindrical micelles with the hydrophobic part situated on the surface and the hydrophillic and charged portions turned inward (13,14). This is opposite to the structure of SDS-micelles, (9,15) and this inverse structure should prevent the strong ionic interaction, being the problem with SDS. 2-propanol was included in the buffer as an organic modifier in order to stabilize these special inverse structured micelles.

Under the separation conditions applied, micelles formed by NaCh move toward the anode whereas the direction of the electroosmotic flow (EOF) will be toward the cathode. Injection of the positively charged aromatic choline esters at the anode results in an increase of analyte speed due to EOF, whereas micelles act retarding. The selective retention obtained is thus a result of differential partitioning of the aromatic choline esters between the aqueous buffer and the hydrophobic micellar phase. Addition of taurine (zwitterion) are thought to reduce eventually interactions between the negatively charged capillary wall and the positively charged analytes

Fig. 2 shows the separation of 6 structural closely related aromatic choline esters included in the standard mixture. Compound 1 is an internal standard (trigonelline amide) used for calculation of relative migration times and relative normalized areas. The different separation parameters has been optimized with reference to this mixture, and notable facts about the effect of changes in these parameters is stated out below.

Variation in temperature and voltage affected the migration time of the compounds studied in accordance to theory but had less impact on separation efficiency than changes in the buffer composition. Micelle formation by NaCh was necessary for separation of aromatic choline esters, as coelution of analytes was found with concentrations of NaCh below the critical micellar concentration at 13 mM (16). 2-propanol had to be included in a certain concentration to stabilize the micelles. It was shown, that very small changes in combinations of NaCh and 2-propanol concentration affected separation efficiency markedly, emphasizing the interaction of 2-propanol with the micellar phase. It was found, that a high concentration of taurine ($>$ 400 mM) was necessary to prevent adsorption of the analytes to the capillary wall. A high concentration of taurine limited however the use of 2-propanol to below 12%, as higher amounts lead to precipitation of taurine. In the optimized buffer system with 2% 2-propanol, this was without importance. The concentration of phosphate had limited effect on separation capacity, but the concentration should not exceed 150 mM, as the corresponding high electrical current may cause heat damage. pH of the buffer was shown critical, as low

pH separation buffers were unstable and precipitated after few runs. The explanation for this has to be found in the pK_a-value at 6.4 for NaCh. As pH in the buffer approaches this pK_a, protonization of the carboxylic group of NaCh will occur. The surfactant hereby becomes uncharged, and hence more hydrophobic and this apparently leads to the precipitation observed. Choosing a pH at 7.3, as in the optimized system, overcomed this problem.

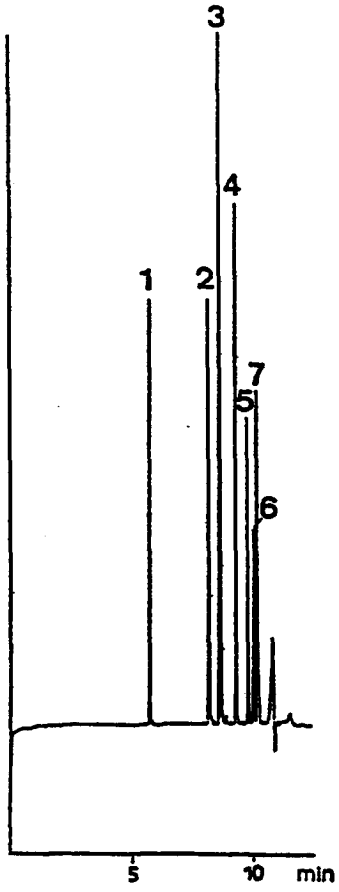


Figure 2. Electropherogram of a mixture of aromatic choline esters. Numbering as in Fig. 1. Buffer composition: 100 mM disodium hydrogenphosphate, 500 mM taurine, 35 mM NaCh and 2% 2-propanol; pH = 7.3, temperature 30 °C; voltage 20 kV.

The number of theoretical plates per meter of capillary under the chosen separation conditions were 287000 (2), 233000 (3), 403000 (4), 485000 (5), 388000 (6) and 281000 (7), respectively. R_s values for 2-3, 3-4, 4-5, 5-6 and 6-7 were calculated to 5.0, 8.0, 6.7, 3.3 and 1.5, respectively.

The results from determination of the repeatabilities of migration times, relative migration times, normalized peak areas and relative normalized peak areas are shown in Table 1. The repeatability experiments were done by changing the buffer at the inlet side between each analysis and at the outlet side after five analyses. Uncertainty caused by evaporation from sample vials during the test (6) was minimized using anti-evaporation septa on the vials.

Table 1. Relative standard deviation of migration times (MT), relative migration times (RMT), normalized peak areas (NA) and relative normalized peak areas (RNA) for aromatic choline esters. Separation conditions as in Fig. 2. Numbers in bold are aromatic choline ester numbers (see Fig. 1). For all calculations n = 8.

Aromatic choline esters	Relative standard deviation (%)			
	MT	RMT	NA	RNA[a]
2	1.44	0.38	1.68	1.02
3	1.51	0.45	1.73	0.64
4	1.59	0.54	1.67	0.66
5	1.67	0.63	1.31	1.11
6	1.71	0.66	1.60	2.43
7	1.73	0.68	1.55	1.03

[a] Relative to trigonelline amide (**1**)

The results from the linearity tests showed correlation coefficients ranging from 0.9950 to 0.9984. The linear increase in normalized peak areas with increasing injection time as well as increasing concentrations of aromatic choline esters injected shows, that the method now developed may be used to quantify aromatic choline esters. However, this provides use of an internal standard as trigonelline amide and response factors determined from the results obtained when testing the linearity.

Approximate detection limits were determined to 16-49 μM from a signal-to-noise ratio of 2:1 using various dilutions of the aromatic choline ester stock solution. According to Harbaugh *et al.* (1990) (17) and Vinther (1991) (18), the injected volume was calculated to be 4.28 nl, assuming a viscosity in the buffer and sample identical to water and with a capillary temperature of 30°C. This results in detection limits between 69 and 282 fmol for each of the aromatic choline esters, which again corresponds to 26-60 pg.

The isolation of aromatic choline esters from *Sinapis alba* and insoluble dietary fibres from dehulled extracted rapeseed meal was made by use of a vacuum system (Supelco) for the group separation (3). Thereby, the group separation and purification were reduced in time to few minutes. A sample of the eluate (500 μl) was evaporated to dryness and redissolved in water. This solution was used directly for the MECC, resulting in separations of individual aromatic choline esters as shown in Fig. 3.

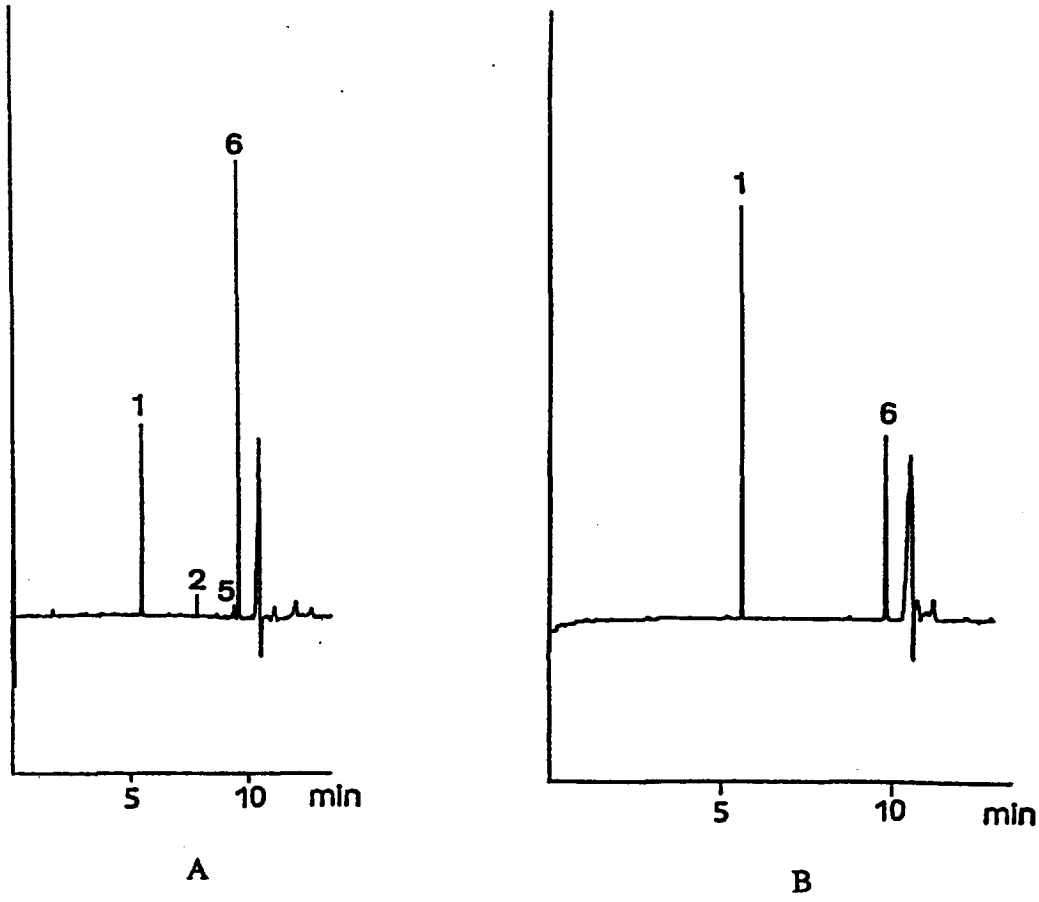


Figure 3. Electropherogram of the aromatic choline esters accumulated in seeds of (A) Sinapis alba L. and (B) insoluble dietary fibres from dehulled extracted rapeseed meal. Numbers as in Fig. 1. Other separation conditions as in Fig. 2.

CONCLUSIONS

The MECC technique performed with a bile salt as the surfactant and taurine as the zwitterion is recommended for the analysis of aromatic choline esters. The method can be used for qualitative as well as quantitative analysis. Relative migration times are to be known for identification, providing an internal standard to be present in the samples. For quantification, internal standard and normalized peak areas shall be used in combination with use of anti-evaporation septa on the samples and changing of the buffer at the inlet side preferable after each analysis and at the outlet side after 5 analyses. Taking these precautions, a very reproducible, effective (up to 485000 theoretical plates per meter) and quick (10 minutes) analysis is provided.

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