# Determination of individual phospholipids by micellar electrokinetic chromatography using bile salt

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## INTRODUCTION

Phospholipids are for various reasons getting increased research attention, which include their dietary link to health. These compounds constitute thus an important group of naturally occuring lipids, also in rapeseed [1]. Due to their high content of polyunsaturated fatty acids, phospholipids are found interesting in connection to quality and nutritional value of rapeseed and rapeseed meal products, in which they are accumulated after oil extraction. In addition, various interests in phospholipids are related to these compounds owing to their biochemical and functional activities both in vivo and in vitro. This call for attention to structures of the individual compounds in relation to the risk for rancidity problems, the nutritional aspects, and use of phospholipids for non-food purposes as pharmaceutical or pharmacological agents..

Phospholipids quantitatively dominating in lecithins or gum fractions from the oil mills are phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE), but lecithins including rapeseed phospholipids contain several other constituents in minor amounts [1]. Commercially important lecithins are those from soybean, rapeseed, egg, cottonseed and other oilseed plants. Sosada *et. al* [2] found that especially rapeseed lecithins from 00-varieties low in erucic acid and glucosinolates offers the chance to increase its application in food industry, cosmetics and pharmacy.

Accurate analysis of lecithins and phospholipids are increasingly important both for producers and for users. Recently, HPLC has been developed as a method of analysis for separation and quantitation of both intact phospholipids, and as anthroyl-derivatized compounds [3-7]. However, these methods are experimentally rather complicated, relatively expensive and time consuming, and proper identification of individual phospholipids can be difficult. In this respect, we have developed a cheap, fast, simple and efficient method of analysis by which phospholipids can be determined, as described in details elsewhere [8].

## MATERIALS AND METHODS

HPCE was performed using an ABI Model 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA), with a 760 mm x 0.05 mm I.D. fused-silica

capillary tube. Detection was carried out at a position 530 mm from the injection end of the capillary by on-column measurements of UV absorbtion at 200 nm. For data processing, a Shimadzu (Kyoto, Japan) Chromatopac C-R3A was used. The separations were performed at 50°C and 30 kV.

The separation buffer was a solution of 75 mM sodium cholate (NaCh), 10 mM di-sodium hydrogenphosphate, 6 mM di-sodium tetraborate and 30% 1-propanol adjusted to pH 8.5 with 1 M HCl. Prior to use, the buffer was filtrated through a 0.20 μm membrane filter.

The names and structures of the phospholipids in the stock solution are presented in Fig. 1.

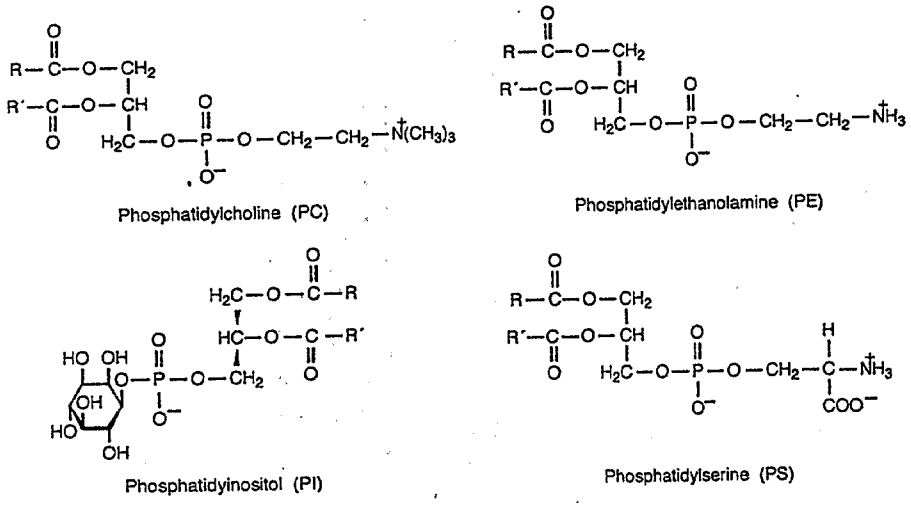


*Figure 1. Structures, names and abbreviations of phospholipids used in HPCE analyses.*

All samples were dissolved in chloroform (about 10 mg/ml) and diluted to appropriate concentrations with 5% (116 mM) NaCh in 50% 1-propanol and introduced from the positive end of the capillary by vacuum for 3 seconds. When running several successive samples or changing buffer composition, the capillary was washed with 1.0 M NaOH for 4 minutes followed by buffer for 10 minutes between each analysis.

## RESULTS AND DISCUSSION

HPCE-MECC was found to be attractive to overcome the analytical difficulties encountered in determination of individual phospholipids [8]. The influence of various solvents was studied, as phospholipids, according to their provenience from biological membranes, have an expected tendency to renewed membrane/bilayer/micellar formation in the aqueous environment required for MECC. Promising results were obtained, when NaCh was used as the micellar phase [9]. The explanation for this is probably related to the structure of the micelles, as NaCh at concentrations higher than the critical micelle concentration (CMC; 5-10 mM), has been suggested to form rodlike or cylindrical micelles with the hydrophobic part situated on

the surface and the hydrophilic part turned inward [10-12]. These inverse micelles favours hydrophobic interaction with the amphipathic phospholipids, which are either neutral or negatively charged. Separation can thus occur as a consequence of different partitioning coefficients for the analytes between the micellar phase and the aqueous phase.

Separation of the phospholipids in a standard mixture of PC, PE, PI and PS was efficiently achieved as illustrated in Figure 2. Identification of the peaks corresponding to the individual compounds was based on use of authentic compounds and defined by RMT values. Calculation of RMT was performed with chloroform as the reference compound, which also acts as a marker for the electroendoosmotic flow (EOF) [8].

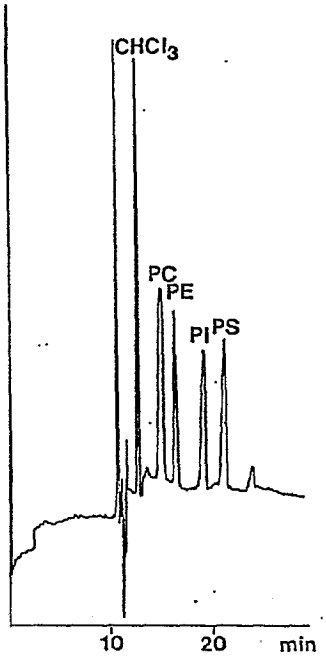


*Figure 2. Electropherogram of a mixture of phospholipids separated by the MECC method. Abbreviations as in Fig. 1.*

Optimization of the separation parameters has been done by use of this mixture, and a brief summary of the effect of changes for the chosen separation parameters is stated below.

pH in the buffer had a great influence on the migration times of the individual compounds and especially for higher values, a shift in elution order was observed. To obtain a separation of the amphipathic analytes, it was necessary to use a buffer including micelles acting as a pseudostationary phase. NaCh, at concentrations higher than CMC, was found suitable due to the formation of "inverse" micelles. 1-propanol was included to stabilize the micelles, and to affect the interaction of analytes and micelles. Use of less than 20% 1-propanol gave insufficient separation of PE and PC, and a high concentration was found to positively affect the separation. The electrolyte concentration did not affect the separation notably, neither did voltage and temperature except for reducing the migration times.

Under the chosen separation conditions the number of theoretical plates per meter of capillary were 28.500, 16.400, and 25.300 for PE, PI, and PS, respectively. Resolution values for PC-PE, PE-PI, and PI-PS were calculated to 1.98, 4.04, and 2.74, respectively [8].

For the standard solution of phospholipids containing PC, PE, PI and PS repeatabilities of migration times, relative migration times (using chloroform as reference compound), normalized peak areas, and relative normalized peak areas (using either PS or PE as reference compound) were evaluated and the results are shown in Table 1. The experiments were done by changing the buffer at the inlet side between each analysis. Uncertainty caused by evaporation from sample vials during the test [13] was minimized using anti-evaporation septa on the vials. Under the conditions mentioned, the instrument performed very well with respect to repeatabilities. When RMT, NA and RNA values were used compared to MT and uncorrected peak areas, the repeatabilities expressed as relative standard deviations were reduced considerably. For quantitation of the analytes, the results indicate the necessity of an internal standard, which ideally should have the same properties as the phospholipids.

*Table 1. Relative standard deviation of migration times (MT), relative migration times (RMT), normalized peak areas (NA) and relative normalized peak areas (RNA) for phospholipids. Separation conditions as in MATERIALS. Phospholipid abbreviations as in Fig. 1. For all calculations n = 7.*

| Phospholipid | Relative standard deviation (%) | | | | |
|---|---|---|---|---|---|
| | MT | RMT | NA | RNA[a] | RNA[b] |
| PC | 1.46 | 0.26 | 6.49 | 2.86 | 1.74 |
| PE | 1.12 | 0.31 | 5.64 | 1.74 | - |
| PI | 1.66 | 0.47 | 5.31 | 2.53 | 2.35 |
| PS | 1.56 | 0.48 | 4.36 | - | 1.75 |

[a]Relative to phosphatidylserine
[b]Relative to phosphatidylethanolamine

Linearity was determined as the correlation between decreasing concentrations of PC, PE, PI and PS and their corresponding values of normalized peak areas. Correlation coefficients were between 0.9984 and 0.9998, and would probably have increased if the test were made with an internal standard [8,14]. The linear increase in normalized peak areas with increasing concentrations of phospholipids injected shows, that the method now developed [8] may be used to quantitate phospholipids.

Phospholipids in commercial rapeseed lecithin were separated by anion-exchange chromatography. The fractions obtained corresponding to the effluent ($A^*$) and the eluate ($B^*$) were then analyzed by the HPCE method developed (Figure 3).

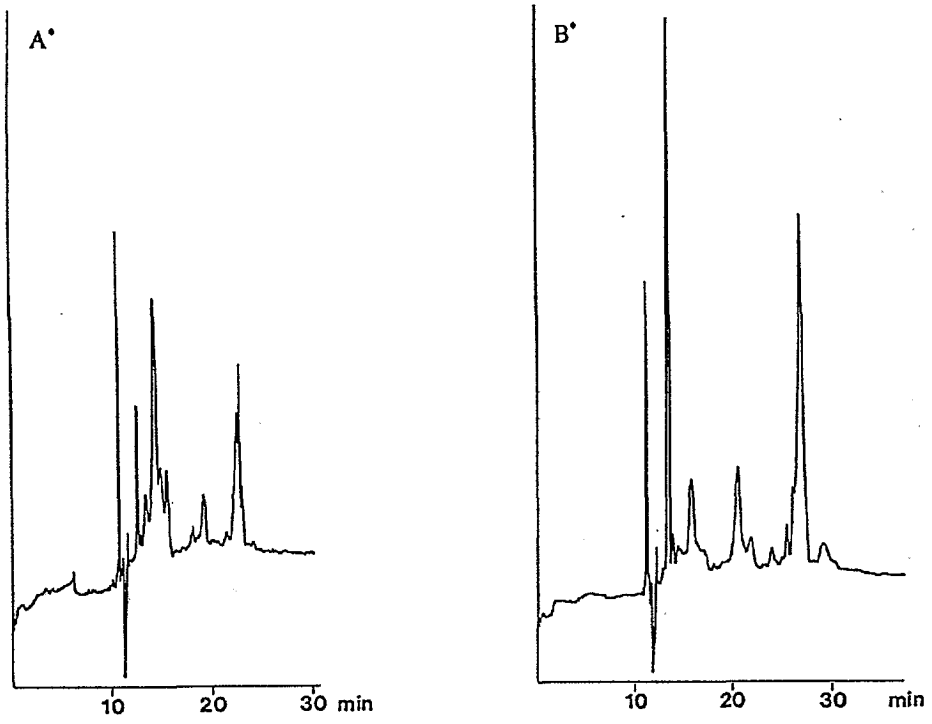


*Figure 3. Electropherograms of phospholipids isolated from rapeseed lecithin. A* is the effluent and B* is the eluate from anion-exchange chromatography. HPCE separation conditions as in MATERIALS, and the first peak after the solvent peak in both electropherograms represent chloroform.*

## CONCLUSIONS

The HPCE-method using bile salt as pseudostationary phase is found suitable for analysis of phospholipids. Due to the high separation efficiency of the apparatus and the various possibilities of changing the separation conditions, the HPCE technique is recommended for studies of intact, individual phospholipids in respect to variation in fatty acid composition. Identification and quantitation shall be done by use of an internal standard and, relative migration times and normalized peak area, respectively. By following the conditions decribed, a quick, easy and reproducible analysis of phospholipids can be done.

## ACKNOWLEDGEMENTS

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