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Analysis of phospholipids in lecithins Comparison between micellar electrokinetic chromatography and high-performance liquid chromatography

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Abstract

The determination of phospholipids in lecithins by HPLC and MEKC has been compared. MEKC conditions have been optimized to provide a robust analytical method. MEKC offers the advantage of a much higher peak capacity which results in an improved resolution, especially for phosphatidylserine. Although the repeatability of the MEKC data is somewhat lower than for HPLC, good quantitative results are obtained.

Keywords: Lecithins; Phospholipids

1. Introduction

Lecithins obtained from crude oil source are complex mixtures consisting mainly of the phospholipids [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA)] in combination with various amounts of other substances such as triglycerides, fatty acids, partially hydrolyzed phospholipids and carbohydrates. The content of individual phospholipids may vary, depending on the nature of the source, and may become a criterion for the applicability of lecithins in the food, pharmaceutical and cosmetic industries.

Normal-phase separation by thin-layer chromatography (TLC) [1,2] and especially HPLC [3–7], are nowadays methods of choice for the characterisation of phospholipids in different matrices, ranging from animal [3,4] and plant [1] tissues, to seed oil extracts [2]. The full separation of individual molecular species of phospholipids by HPLC depends strongly on gradient elution [3–6]. Peak and baseline disturbances are often characteristic for this type of separation. Also by the presence of minor components, the resolution is often not sufficient for a reproducible quantitative analysis. Separation of the major phospholipids was also reported employing light scattering detection [8] and β-cyclodextrin bonded silica columns [9].

In micellar electrokinetic chromatography (MEKC) solutes are separated based on their distribution between a mobile (usually aqueous) and a pseudostationary (micellar) phase. In comparison

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with classical HPLC, MEKC offers a considerably higher separation efficiency due to the 'plug like' velocity profile of the electroosmotic flow and the dynamic nature of a micellar stationary phase. MEKC is suitable for the separation of neutral, charged, hydrophillic as well as hydrophobic compounds [10–13]. Recently a method for the analysis of phospholipids by MEKC was published [14].

In this contribution we present a modified and more robust MEKC method for the analysis of phospholipids in lecithins from plant sources. The results obtained have been compared with results obtained by a standard IUPAC HPLC method [7]

2. Experimental

2.1. Equipment

2.1.1. HPLC

HPLC was performed using a Gilson pump (Model 305, Gilson, France) equipped with: Gilson sample injector Model 231 (Gilson, France), a manometric module 805S (Gilson), 100×4 mm I.D. column packed with 10 μm Nucleosil 50-5 (Machery Nagel, Germany). The volume of the injection loop was 5 μl. All separations were performed at ambient temperature with UV detection system (UV-975, Jasco, Japan) operating at 206 nm. Isocratic elution system was used for all experiments with a flow-rate of 1 ml/min.

2.1.2. MEKC

The MEKC experiments reported were performed on a Beckmann P/ACE 5000 capillary electrophoresis system (Beckmann Instruments, USA). Fused-silica capillaries were 57 cm (52 cm to detector)× 375 μm O.D.×50 μm I.D. Samples were hydrodynamically introduced by applying pressure during 3 s, analyzed with an voltage of 25 kV (positive at injection side) and UV detected at 200 nm. If not stated otherwise, the capillary was thermostated at 50°C. Between analyses, the column was rinsed with approximately 10 capillary volumes of 50 m*M* borax and 10 capillary volumes of separation buffer. A PC workstation with System Gold software (Version 8, Beckmann Instruments) was used for instrument control. Data were collected and analyzed using Turbochrom software (Version 4, Perkin-Elmer Nelson Division, USA). The MEKC separations could be perfectly reproduced on a Hewlett-Packard HP^{3D} CE system.

2.2. Chemicals

Phospholipid standards, used for peak identification, were obtained from Sigma and used without further purification. All buffer solutions were prepared with deionized water (Milli-Q, Millipore, USA). All chemicals were of analytical- or HPLC-grade purity. The separation buffer for MEKC experiments consisted of 6 m*M* borax, 10 m*M* phosphate (pH 8.5), 35 m*M* deoxycholic acid (sodium salt) and 30% (v/v) *n*-propanol. The mobile phase for HPLC analysis consisted of *n*-hexane–2-propanol–acetate buffer pH 4.2 (8:8:1, v/v). Samples of commercially available lecithins were obtained from Unimills (Zwijndrecht, Netherlands).

2.3. Sample preparation

2.3.1. HPLC

Samples of commercially available lecithins were dissolved in the mobile phase in concentrations of approximately 0.1 mg/ml and filtered through a 0.45-μm filter (Millex-HV, Millipore) before injection.

2.3.2. MEKC

Lecithin samples were purified prior to the MEKC analysis from triglycerides, fatty acids and carbohydrates according to the official USP monograph for lecithin, determination of the content of acetone insoluble matter in lecithins [15]. The acetone precipitate, containing mainly phospholipids, was then redissolved in the separation buffer at a concentration of approximately 0.2 mg/ml and filtered through a 0.45-μm filter (Millex-HV, Millipore) prior to analysis.

3. Results and discussion

3.1. HPLC analysis

For the determination of lecithin phospholipids by HPLC the method according to IUPAC specifications was applied, except that a shorter column (see Section 2 for details) as advised by IUPAC was used, e.g., 100×4 mm I.D. instead of 200×4.6 mm I.D. The flow-rate was therefore adjusted to 1 ml/min. From a comparison of chromatograms reported in Ref. [7] and obtained by us, this did not have a noticeable effect neither on efficiency nor on separation.

Fig. 1 shows a typical chromatogram of a phospholipid profile of soybean lecithin. PC, PE, PI and PA are baseline separated within a relatively short time using isocratic elution. The shoulder on the PE peak corresponds, according to IUPAC specifications, to a *N*-acyl-PE. Due to the lack of this compound in pure form, we could not confirm its presence in a MEKC chromatogram and therefore it was excluded from further evaluation. The IUPAC method completely ignores the presence of PS, most probably because it is indeed only a minor component in soybean lecithins [16]. By spiking a sample of lecithin with a standard of PS, we found that this compound elutes between PI and PA and cannot be separated from them under the conditions proposed in the IUPAC method (Fig. 1b).

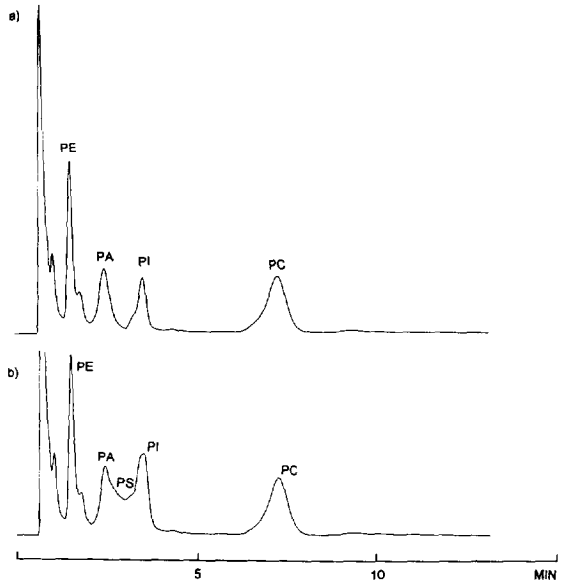

Fig. 1. HPLC separation of phospholipids in lecithin. (a) Commercially available soybean lecithin; (b) same as (a) but spiked with PS. See Section 2 for conditions.

3.2. MEKC analysis

From the theory of resolution in MEKC it follows that hydrophobic compounds will elute with poor resolution, as the retention factor is increased along with micellar solubilization [17]. Various methods have been proposed to reduce the retention factors. A published MEKC method for analysis of phospholipids [14] employs cholic acid as pseudo-stationary phase and 1-propanol as modifier. Bile salts are used for their lower solubilizing power compared to sodium dodecyl sulfate (SDS), thus allowing the analysis of hydrophobic compounds [10]. Short size *n*-alkyl alcohols, e.g., methanol, ethanol and 1-propanol, affect the mobile phase. They increase the solubility of more hydrophobic compounds in the aqueous phase thus decreasing retention factors towards more favourable values [18].

By modification of the MEKC method [14] faster analysis with smaller baseline disturbances was obtained. The data obtained in the two laboratories (on three CE systems) were very similar ilustrating the robustness of the method. Sodium cholate was replaced with sodium deoxycholate and a lower concentration (35 m*M* NaDCA vs. 75 m*M* NaCA) was applied. Fig. 2a shows the analysis of phospholipids in a commercially available soybean lecithin using the modified conditions. It can be seen that the peak corresponding to PS is negligible in this sample in comparison with the other phospholipids. PS can, however, be well separated from all major peaks as shown by a spiked sample (Fig. 2b).

Advantage of MEKC over the HPLC method for the analysis of phospholipids lies in the higher peak capacity due to its unique separation efficiency. From the comparison of Fig. 1 and Fig. 2 it is obvious that more peaks, seemingly minor unknown components, are detected in the MEKC chromatogram. It was also noticed that by decreasing the

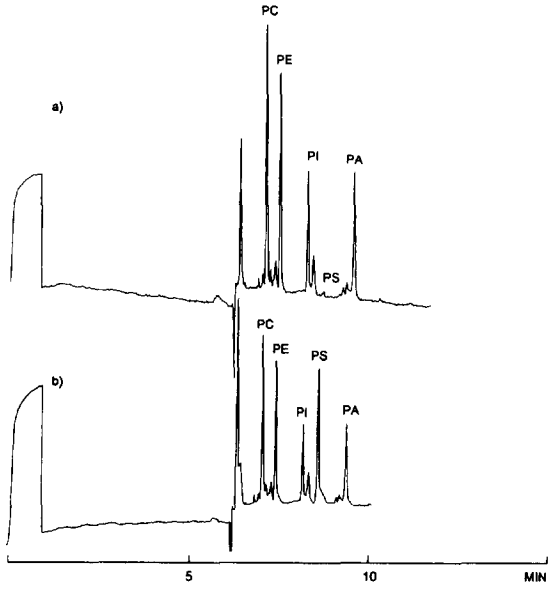

Fig. 2. MEKC separation of phospholipids in lecithin. (a) Commercially available soybean lecithin; (b) same as (a) but spiked with PS. See Section 2 for conditions.

temperature of cooling liquid from 50 to 15°C, peaks of PC, PE and PI started to split into 2–3 particular peaks (Fig. 3). We speculate that these peaks could correspond to a carbon number distribution of individual phospholipids. The identification of these components will be discussed in a separate paper.

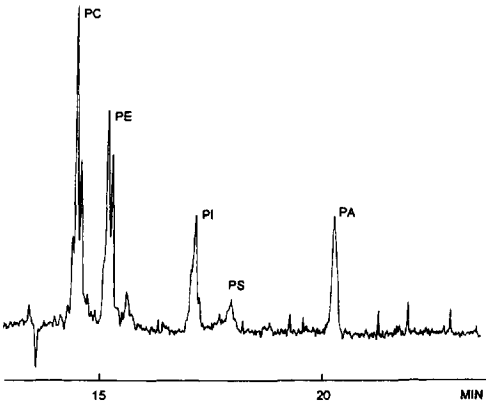

Fig. 3. MEKC separation of phospholipids in lecithin. All conditions as in Fig. 2, except that the temperature was kept at 15°C.

3.3. MEKC versus HPLC

During a repeatability study, we analyzed 11 commercially available samples of soybean lecithins each 5 times by both techniques. The resulting relative standard deviation (R.S.D.) of absolute peak areas was in the range 1.5–3% and 2.5–5% for HPLC and MEKC, respectively.

The comparison of peak areas in MEKC is more complicated than in HPLC, because all compounds are moving with different velocities. Therefore the integrated signal for an apparently slower moving

Table 1
Relative composition of lecithins found by HPLC and MEKC

No.	MEKC[a]				HPLC			
	PC	PE	PI	PA	PC	PE	PI	PA
1	39.38 (1.58)	28.78 (0.57)	15.97 (0.48)	15.87 (0.25)	44.03 (0.11)	24.84 (0.07)	15.55 (0.04)	15.58 (0.12)
2	41.98 (0.82)	33.43 (0.54)	15.79 (0.29)	8.80 (0.31)	45.06 (0.09)	30.25 (0.10)	15.66 (0.05)	9.03 (0.05)
3	44.33 (0.22)	29.64 (0.71)	17.07 (0.41)	8.96 (0.36)	47.08 (0.05)	27.50 (0.08)	16.02 (0.04)	9.41 (0.02)
4	42.16 (0.40)	29.33 (0.66)	17.17 (0.74)	11.34 (0.19)	44.70 (0.21)	27.22 (0.17)	15.92 (0.04)	12.17 (0.07)
5	47.10 (0.46)	27.14 (0.32)	17.75 (0.15)	8.01 (0.47)	48.63 (0.09)	27.72 (0.05)	15.92 (0.03)	12.17 (0.06)
6	37.21 (0.43)	31.44 (0.49)	15.12 (0.79)	16.23 (0.31)	38.23 (0.09)	28.13 (0.07)	14.53 (0.08)	19.10 (0.09)
7	37.27 (0.72)	31.16 (0.48)	15.14 (0.32)	16.42 (0.40)	39.28 (0.05)	28.20 (0.10)	14.84 (0.05)	17.68 (0.03)
8	35.14 (1.56)	30.95 (0.38)	15.01 (0.61)	18.90 (1.38)	37.46 (0.05)	26.98 (0.07)	14.10 (0.06)	21.45 (0.05)
9	37.48 (1.15)	32.99 (0.38)	16.08 (0.41)	13.45 (0.26)	41.95 (0.04)	29.88 (0.08)	15.06 (0.04)	13.11 (0.04)
10	38.55 (0.71)	34.08 (0.42)	15.65 (0.17)	11.72 (0.25)	43.43 (0.04)	31.60 (0.13)	14.17 (0.05)	10.80 (0.06)
11	42.63 (0.33)	29.93 (0.21)	15.89 (0.10)	11.42 (0.27)	44.90 (0.23)	28.56 (0.38)	15.28 (0.13)	11.25 (0.04)

Values are area% with S.D. values in parentheses.
[a]Correction for migration time.

Table 2
Differences in relative composition found by HPLC and MEKC

	$\bar{d}$ [a]	S.D.[b]	t[c]	[d]
PC	−2.87	1.3	7.4	S
PE	2.54	1.3	6.5	S
PI	0.87	0.5	5.5	S
PA	−0.97	1.6	2.0	S

[a] $\bar{d} = \frac{\sum_{i=1}^{n} (\text{Area\%}_{\text{CZE},i} - \text{Area\%}_{\text{HPLC},i})}{n}$; $n = 11$.

[b] Standard deviation ($n = 11$).

[c] $t = \frac{|\bar{d}|\sqrt{n}}{\text{S.D.}} t_{n-1}^{\alpha} = t_{10}^{0.05} = 1.812$.

[d] S: significant, NS: not significant.

analyte will be higher than that of the faster moving analyte assuming equal concentration and response factor. Therefore we compensated for this effect by dividing, for all MEKC experiments, the observed area by the elution time.

Table 1 shows the calculated relative phospholipid composition based on relative peak area, together with standard deviations calculated from 5 repeated injections. This relative composition does not reflect the real composition because it does not include the effects of the different response factors of individual phospholipids. The R.S.D for HPLC and MEKC was in this case considerably lower than for absolute peak areas, 0.5–1.0% and 1.0–2.5%, respectively. The small deviation in the relative phospholipid composition found by HPLC and MEKC, although statistically significant (see Table 2), can be attributed to systematic errors in the determination of peak areas and a response variation caused by differences in solvent composition and detector wavelength. By proper calibration with external standards, those deviations should be eliminated.

4. Conclusions

The presented MEKC separation of phospholipids is preferable over the IUPAC HPLC method as it offers a number of improvements because of its far better peak capacity. As a result, PS can be baseline separated from other phospholipids. Furthermore the high resolution power of MEKC will possibly allow the separation of phospholipids based on fatty acid chain differences. Although the repeatability of the MEKC method is somewhat lower than that for HPLC, good quantitative results were obtained. Small differences in relative peak areas for both methods should be eliminated by appropriate external calibration.

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References

[1] G. Lendrath, A. Nasner and L. Kraus, J. Chromatogr., 502 (1990) 385.
[2] B. Rivnay, J. Chromatogr., 294 (1984) 303.
[3] S.F. Chen and P.H. Chan, J. Chromatogr., 344 (1985) 297.
[4] L.A. Dethloff, L.B. Gilmore and G.E.R. Hook, J. Chromatogr., 382 (1986) 79.
[5] M. Seewald and H.M. Eichinger, J. Chromatogr., 469 (1989) 271.
[6] A.G. Andrews, J. Chromatogr., 336 (1984) 139.
[7] IUPAC Standard Methods for the Analysis of Oils Fats and Derivatives. Blackwell Scientific Publications, 7th Edition, 1987, IUPAC Method 5.302.
[8] l. Breton, B. Serkiz, J. Volland and J. Lepagnol, J. Chromatogr., 497 (1989) 243.
[9] S.L. Abidi, T.L. Mounts and K.A. Rennick, J. Liq. Chromatogr., 17 (1994) 3750.
[10] H. Nishi, T. Fukuyama, M. Matsuto and S. Terabe, J.Chromatogr., 513 (1990) 279.
[11] S. Terabe, Y. Miyashita, O. Shibata, E.R. Barnhart, L.R. Alexander, D.G. Patterson, B.L. Karger, K. Hosoya and N. Tanaka, J. Chromatogr., 516 (1990) 23.
[12] S. Terabe, Y. Ischihama, H. Nishi, T. Fukuyama and K. Otsuka, J. Chromatogr., 545 (1991) 359.
[13] J. Vindevogel and P. Sandra, Anal. Chem., 63 (1993) 1530.
[14] L. Ingvardsen, S. Michaelsen and H. Sørensen, J. Am. Oil Chem. Soc., 71 (1994) 183.
[15] United States Pharmacopeia 23 - NF 18: the United States Pharmacopeia – the National Formulary. Official from January 1, 1995.
[16] B.F. Szuhaj, Lecithins: Sources, Manufacture and Uses, The American Oil Chemists Society, Chicago, IL, 1989.
[17] J. Vindevogel and P. Sandra, Introduction to Micellar Electrokinetic Chromatography, Hüthig, Heidelberg, 1992.
[18] E. Van Hove, R. Szücs and P. Sandra, J. High. Resolut. Chromatogr., submitted for publication.