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A new liquid chromatography method with charge aerosol detector (CAD) for the determination of phospholipid classes. Application to milk phospholipids

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ABSTRACT

A new rapid method for the quantitative analysis of five classes of phospholipids (PLs) (phosphatidylcholine—PC, lysophosphatidylcholine—LPC, phosphatidylethanolamine—PE and phosphatidylserine—PS and phosphatidylinositol—PI) using liquid chromatography with charge aerosol detector (CAD) is described. The separation of the compounds of interest was achieved on a diol stationary phase with a mobile phase consisting of 13% HCOOH, hexane and 2-propanol in 19 min elution program, including 10 min equilibration of the column. The method was applied to characterize the phospholipid fractions of cow milk. PLs present in cow milk were separated by solid-phase extraction (SPE) procedure with Si cartridges before LC analysis with recovery ranging from 95.3% to 104.4%. The use of CAD detection of the eluted compounds was precise, linear and sensitive.

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1. Introduction

Phospholipids (PLs) are the major constituents of all biological membranes and plays a crucial role in the biochemistry of cells. Taking into consideration the chemical structure of phospholipids we can differentiate them between glycerophospholipids (GP) and sphingophospholipids (SP). GP and SP are amphiphilic molecules consisting of one or two hydrophobic acyl chains and a hydrophilic head. Glycerophospholipids are composed of glycerol backbone with fatty acids esterified at the *sn*-1 and *sn*-2 positions. The *sn*-3 position has a phosphate group attached to a polar head group like choline, serine, ethanolamine or inositol to give phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). In the case of SP, the hydrophobic part consists of a ceramide moiety and the hydrophilic part consists of organophosphate group (e.g., phosphocholine in the case of sphingomyelin, SM) [1,2].

The commercial sources of PLs are vegetable oils such as soybeans, rapeseed or sunflower [3]. In the animals world egg yolk is the main source [4], although marine raw materials like krill or some fish [5] processing by-products also have to be mentioned. The phospholipid and sphingolipid composition of milk is also of considerable interest regarding their nutritional and functional properties. These complex biological lipids are quantitatively minor constituents of milk fat (about 1% of the lipid fraction) [6] but are of great interest because they define the structural properties of the milk fat globule membrane (MFGM) surrounding the triacylglycerol core of fat globules [7]. Therefore they influence the physical functionality of dairy ingredients and food products [8–10]. Furthermore many health benefits have been attributed to bovine milk PLs as a group, as well as individual compounds [11]. In particular, sphingomyelin, which is referred to as a tumor suppressor molecule, is a highly biological active compound that is associated with cell regulation [12].

Detailed investigation of phospholipids content in milk require accurate methods for the separation and quantification of PLs classes.

The analysis of milk PLs involves three main steps: total milk lipids extraction, purification of PLs from neutral lipids and separation particular polar lipid classes.

The most popular method of lipids extraction was developed by Folch et al. [13]. This technique uses chloroform: methanol mixture (2:1, v/v) as a solvent. Later variations of this were introduced by Bligh and Dryer [14]. The obtained lipids fraction can be separated into polar and neutral lipids by various techniques like thin layer chromatography (TLC) [15], column chromatography [16,17] or solid-phase extraction (SPE) [18]. After extraction and purification steps, analysis for the detection and quantification of specific phospholipid classes is performed.

Nowadays, the most commonly used chromatographic method for the determination of phospholipids is the liquid chromatography (LC). The most powerful means to influence the separation

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in liquid chromatography is by the selectivity properties of the phase system. As the stationary phase the most common in PLs LC analysis silica columns were used [18–21]. However, diol [22], amino [23] and cyano [24] phases have also been applied.

Many of the mobile phases have been used with silica columns. Almost all methods use gradient elution with either a binary or ternary solvent system. One of the most popular linear binary system (A—chloroform:methanol:ammonium hydroxide, 80:19.5:0.5 (v/v) and B—chloroform:methanol:water:ammonium hydroxide, 60:34:5.5:0.5 (v/v)) was proposed by Becart et al. [25] and have been applied by other authors [18,26]. Later variation of this method was introduced by Boselli et al. [27] and Narváez-Rivas et al. [28]. Unfortunately, alkaline pH is causing the dissolution of silica phases and considerably reduces the column life. Therefore using of acidic buffer instead of ammonium hydroxide was proposed by Rombaut et al. [20] and more than 1500 runs were performed without loss of column performance. Several other LC methods were described but more complex gradient elution procedures were used [19,29–31].

A highly important aspect of PLs analysis is their detection. There are various detectors available for use on conjunction with LC. Most commonly, natural PLs are monitored by low-wavelength UV detectors [32] and evaporative light scattering detectors (ELSD) [28]. However refractive index [33] and fluorescent detectors [23] as well as ^{31}P NMR spectroscopy [34] have been also used for characterization of phospholipids. The main drawback of the latter method is that NMR methodology precludes its routine use in analyses of PLs and therefore LC methods are most suitable.

However most of these detection techniques have some limitations. UV detection of PLs requires use of low wavelengths (190–210 nm), which limits solvent selection (e.g., chloroform and dichloromethane) and increases the possibility of matrix interference. Fluorescent detectors have high sensitivity for PLs detection; however phospholipids samples required derivatization as they do not naturally contain fluorophores for detection [35]. The refractive index detector (RID) is the least sensitive of all the commonly used detectors. It is very sensitive to changes in ambient temperature, pressure changes and flow-rate changes; furthermore, it requires isocratic elution, which is not capable of fully resolving all of phospholipid classes [35,36]. Relatively recently, a new alternative detection method based upon aerosol charging (charge aerosol detector—CAD) has been introduced [37]. It has been proven that CAD can provide greater sensitivity and better precision than ELSD [31,38]. Moreover, CAD is quite user-friendly since it does not require any optimization of operating parameters; therefore it can be operated by any chromatographer without significant additional training [39]. Its principle of operation is based on charging of aerosol particles by corona discharge and subsequent measurement of the charged particles using electrometer.

The aim of the presented study was to develop and validate a new chromatographic method for LC-CAD, enabling the determination of phospholipid classes in a short run time using a simple gradient elution. To evaluate the applicability of the method, the cow milk samples were analyzed.

2. Experimental

2.1. Chemicals

All solvents for liquid chromatography were freshly opened bottles of Merck LiChrosolv® Reag. obtained from Merck. All other solvents were of analytical grade and were purchased from POCh (Poland, Gliwice). Thermo Betasil DIOL (5 μm, 150 × 4.6 mm) and Waters Spherisorb®Silica (5 μm, 150 × 4.6 mm) columns were used for HPLC analysis. All standards, L-α-phosphatidylcholine (PC) (purity ≥99%), L-α-lysophosphatidylcholine (LPC) (purity ≥99%), L-α-phosphatidylethanolamine (PE) (purity ≥97%) and L-α-phosphatidyl-L-serine (PS) (purity ≥97%) were purchased from Sigma-Aldrich Chemical Co. L-α-phosphatidylinositol (PI) (purity ≥97%) was purchased from Larodan. The bovine milk samples were from high yielding dairy cows in full lactation fed balanced diets using total mixed ration (TMR) system. The experiment was carried out in 2011 by the Department of Animal Nutrition and Feed Management of University of Agriculture in Krakow. Representative samples of milk from 4 cows were taken during morning milking, then pooled proportionally and kept frozen (−20 °C) for further analyses.

2.2. Lipids extraction

Prior to all extractions, milk samples were warmed to 35 °C. Milk lipids were isolated as a crude lipid fraction by extraction according to Folch et al. [13]. Milk sample (5 mL) was dissolved in 100 mL chloroform:methanol (2:1, v/v) and 10 mL of saline solution (0.05 M of NaCl), shaken vigorously for 15 min on a rotary shaker (200 rpm) and centrifuged at 5000 rpm for 5 min. After centrifuged the lower chloroform layer was released and the process was repeated adding 70 mL of chloroform to the upper (methanol–water) phase. The two chloroform phases were pooled and evaporated in vacuo. Separated lipids were exposed to a stream of N_2 and frozen at −20 °C until further analysis. Each extraction was repeated 3 times.

2.3. Solid-phase extraction (SPE)

The crude milk lipids extract (100 mg) was dissolved in 1.0 mL of chloroform:methanol (95:5, v/v) mixture and applied to SPE cartridge. A silica gel column (Discovery® DSC-Si SPE 500 mg) was used. After conditioning with 10 mL of chloroform:methanol (95:5, v/v), the neutral lipids were eluted with 20 mL of chloroform:methanol (95:5, v/v). The recovery of PLs was performed by using of 10 mL of methanol and then 10 mL of chloroform:methanol:water (5:3:2, v/v/v). The PLs fraction was evaporated using a rotary vacuum evaporator at 45 °C and was re-dissolved in 1 mL of chloroform:methanol (2:1, v/v) for LC analysis.

2.4. Chromatographic system and conditions

The LC was performed on an Ultimate 3000 from DIONEX chromatograph equipped with DGP-3600A dual-pump fluid control module, a TCC-3200 thermostated column compartment and an WPS-3000 autosampler. The system was controlled and data acquisition was carried out using Chromeleon 6.80 software (Dionex Corporation). The Corona™ Charged Aerosol Detector (CAD) was from ESA Biosciences. The following parameters were used: acquisition range 100 pA, digital filter set to none, N_2 pressure 35 psi. Data acquisition for CAD was carried out using the Chromeleon 6.80 software.

2.4.1. LC conditions for the analysis of the phospholipid fractions

LC analysis was carried out using a Betasil DIOL 5 μm (150 × 4.6 mm) column. The gradient elution was used for the separation of phospholipids (Table 1). The column temperature was maintained at 30 °C. The phospholipids components were identified by comparison of their retention times with those of commercial standards.

2.5. Repeatability and recovery assays

Repeatability and recovery were determined for all the analytes. The recovery was calculated by comparing the peak area of PLs added to a blank milk fat matrix with the results obtained by LC direct injection of PLs standards. All the samples were analyzed consecutively in the same day, for the same analyst to study repeatability. In each case six replicates were determined.

3. Results and discussion

3.1. Method development

The LC method presented in this paper enables the separation of six different phospholipid classes (PS, PE, PI, PC, SM and LPC) in 19 min elution program, including 10 min equilibration of the column. Phospholipids were eluted as well-defined peaks and separated based on the head group polarity, in order from non-polar to polar. A chromatograms of a standard mixture and milk PLs are depicted in Fig. 1.

Very important aspect of the method development was finding an appropriate mobile phase to achieve adequate resolution among all analytes. Furthermore, because the CAD process involves nebulization to remove the mobile phase, volatile mobile phases and its additives must be used. The CAD system does not pose a problem at low additives concentration, but at higher concentrations the background level can increase to an unacceptably high levels, masking the peaks of interest [40,41]. Taking all these factors into account, hexane and 2-propanol were chosen as the mobile phase organic components. Formic acid (13% v/v) was the third solvent. The use of appropriate concentration of formic acid as a volatile additive was necessary to increase the resolution since PC, PS and PI co-eluted when using the mobile phase without the pH modifier. Although a good resolution between PC, PS and PI can be obtained at the lower than 13% acid concentration, but in these conditions PE and PS fused together. With increasing the formic acid concentration in the mobile phase the separation between molecular species of LPC was also observed (Fig. 1).

Two columns (diol and bare silica) were evaluated. All phospholipids can be well resolved, with the same elution order, using both columns. However, longer retention and slightly higher resolution between particular PLs at the same analysis conditions were observed for silica column (data not shown). Differences were probably due to the fact that polar interactions with the diol layer are not as strong as with the silanols on a bare silica surface. However higher base-line signal was observed in the case of bare silica column. The lack of stability of the stationary phase with regard to the eluent can disturb the detection by an increase in base-line irregularities such as drift, noise and erratic signal. This has been noted when gradient elution at increasing water content was flushed through the bare silica column. When the water content of the mobile phase reaches 10%, the baseline drift and the background noise increases considerably. This was probably caused by the so-called column bleed effect and refers to the dissolution or washing out of the LC column packing material [42]. When this occurs, the particles from the column enter the CAD detector and result in increased baseline noise.

The effect of temperature on base line signal was also pronounced, and with the higher column temperature the higher signal was observed (Fig. 2).

In contrast to unmodified silica column, diol-bonded silica show far less column bleed with the same gradient elution program and was also highly stable at elevated temperatures. Therefore diol column was finally applied as the stationary phase.

To speed up the separation, 1.5 ml min^{-1} flow-rate was used. However, higher flow-rate resulted in high column back pressure (> 170 bars at 20 °C). To reduce this negative effect 30 °C column temperature was applied. With an increase in temperature, column back pressure decreases because of a decrease in viscosity of the mobile phase.

3.2. Response model

Calibration curves for each compound were calculated from the area values obtained by injecting 10 μL of chloroform–methanol

Table 1
Solvent gradient elution system required for the elution of phospholipids.

	Percent solvent			Flow (mL/min)
Time (min)	A[a] (%)	B[b] (%)	C[c] (%)	
0.0	3	40	57	1.5
4.0	10	40	50	1.5
9.0	10	40	50	1.5
9.1	3	40	57	1.5
19.0	3	40	57	1.5

[a] 13% Formic acid.
[b] Hexane.

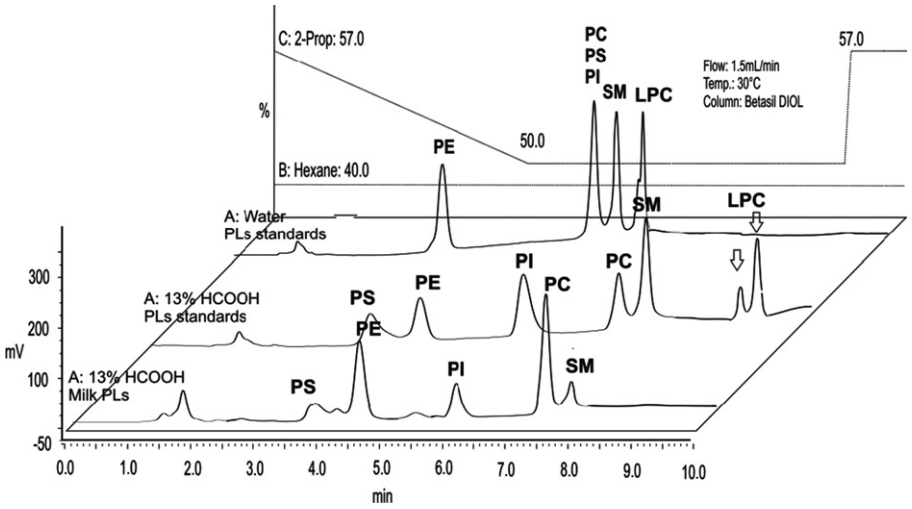

Fig. 1. LC-CAD chromatograms of phospholipid standards and milk phospholipids after purification by SPE and effect of formic acid on the separation selectivity.

[c] 2-Propanol.

(2:1, v/v) solutions of PS (0.02–1.66 μg), PE (0.01–1.57 μg), PI (0.03–2.00 μg), PC (0.03–2.16 μg), SM (0.03–1.99 μg) and LPC (0.05–1.44 μg). Calibration curve of lysophosphatidylcholine (LPC) was calculated based on the higher-peak area. At least six standard concentrations of every compound were prepared. Each standard mixture dissolved in chloroform–methanol (2:1, v/v) was injected five times. The resulting peak areas were plotted as a function of the individual phospholipid class amount and fitted using linear model ($y=a+bx$). Results (Table 2) showed high coefficient of determination, $R^2=0.9948–0.9996$ for a linear fit. This study demonstrates that the CAD response, in the two orders of magnitude, was linear. The linearity of CAD detector in a specific concentration range was also described by other authors [38].

3.3. Limit of detection (LOD) and limit of quantitation (LOQ)

Table 2 shows the limit of detection (LOD) and limit of quantitation (LOQ) found for each one of the phospholipid class. Due to the fact that CAD was linear in the indicated concentration range of all analytes, the limit of detection (LOD) was calculated based on the standard deviation (SD) of the response and on the slope (S) of the calibration curve, which is in fact the sensitivity. The SD of the response was calculated as the SD of y-intercept of regression line. As can be seen in Table 2, the lowest values of LOD and LOQ corresponded to PE while the SM presented the highest values. The method described here, therefore, exhibits the necessary sensitivity to cover PL concentrations present in dairy products and also in biological systems.

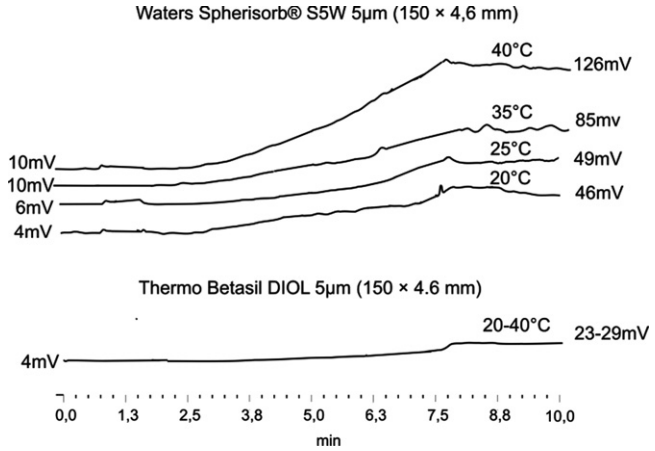

Fig. 2. Dependence of the CAD response on temperature and mobile phase composition for two different LC columns. Flow rate of mobile phase: 1.5 ml min^{-1}, with solvent A—water, B—hexane, C—2-propanol. Gradient timetable (%A/%B/%C, v/v/v)—at 0 min 3/40/57, at 4 min 10/40/50, at 9 min 10/40/50, at 9.1 min 3/40/57 and at 19 min 3/40/57.

3.4. Precision

To test the precision, or more specifically the instrument precision and injection repeatability, 10 injections of the same sample solution were performed in the conditions described above. Similar amount (0.4–0.5 μg) of all standards was used. As evaluation data for the repeatability of the method, both the retention time and the area under peaks, were evaluated (Table 3).

Relative standard deviation (RSD) was used to express the precision. The RSD values for different phospholipid classes indicate that this method is sufficiently stable. For the mean retention time all values of RSD were lower than 0.77%. Considering the peak area, the RSD was lower than 0.80%.

3.5. SPE recovery

The recovery of the PLs standards was determined by comparing the average peak area from six replicate analysis of phospholipids added on a blank milk fat with the average peak area obtained after direct LC analysis of phospholipid standards. Excellent SPE recoveries (95.3–104.4%) for all of the analytes with good repeatability (%RSD—1.30–11.46%) were obtained. The results are summarized in Table 4.

3.6. Application of the LC–CAD method for the quantification of milk phospholipids

Finally, a practical evaluation was done on milk samples from 4 dairy cows in full lactation. The method repeatability and the phospholipid classes content of milk samples are reported in Table 5 and the example of the obtained chromatogram is presented in Fig. 1.

Results demonstrated a good precision for the analysis of individual PL samples. The RSD values ranged from 0.70% to 11.82% for all analysis. Total amount of PLs in the milk samples ranged from 22.7 to 31.3 mg/100 mL of milk. Phosphatidylcholine (PC) was by far the most predominant PL (43.2–46.4%) followed by PE (29.9–34.2%), PS (8.1–8.6%), PI (7.7–11.5%) and SM (4.0–5.1%).

4. Conclusions

The analytical procedures presented herein allow rapid analysis and quantification of five classes of phospholipids presented in milk. SPE methodology permitted the separation of the phospholipids present in this matrix from other lipids with a high recovery.

Table 2
Calibration curves, limit of detection (LOD) and limit of quantification (LOQ) for standard compounds with CAD detection.

Compound	Range (μg)	Linear model ($y=a+bx$)		LOD (ng) [a] 3.3 *x* SD/S	LOQ (ng) [a] 10 *x* SD/S
		Equation	R^2		
PS	0.02–1.66	$40.459(\pm0.27)x-1.133(\pm0.24)$	0.9992	19.6	59.3
PE	0.02–1.57	$34.476(\pm0.15)x-0.160(\pm0.13)$	0.9996	12.4	37.7
PI	0.03–2.00	$40.538(\pm0.44)x+1.262(\pm0.47)$	0.9977	38.3	115.9
PC	0.03–2.16	$25.549(\pm0.18)x+0.422(\pm0.21)$	0.9990	27.1	22.2
SM	0.03–1.99	$44.567(\pm0.73)x+1.715(\pm0.78)$	0.9948	57.7	175.0
LPC	0.05–1.44	$27.760(\pm0.23)x+0.388(\pm0.16)$	0.9989	19.0	57.6

[a] SD—standard deviation of the response (intercept); S—sensitivity (slope); *x*—concentration of the injected amount.

Table 3
Repeatability of CAD response and retention times of standard compounds.

Compound	Amount (μg)	Peak area (mV/min)			Retention time (min)		
		Mean	SD	RSD%	Mean	SD	RSD%
PS	0.4	14.809	0.10	0.69	4.635	0.03	0.77
PE	0.4	13.314	1.03	0.31	5.276	0.01	0.40
PI	0.5	22.611	0.08	0.37	6.625	0.01	0.14
PC	0.5	14.836	0.12	0.80	7.880	0.01	0.14
SM	0.5	26.171	0.27	1.05	8.309	0.01	0.13
LPC	0.5	13.971	0.33	2.34	9.656	0.07	0.69

Table 4
Recovery of PLs determined according to peak area by direct analysis and by SPE isolation from milk fat ($n=6$).

		PS	PE	PI	PC	SM	LPC
Direct analysis	Mean	10.81	68.21	7.98	50.56	28.25	18.70[a]
	SD	0.50	0.91	0.42	0.46	1.12	2.18
	%RSD	4.60	1.34	5.23	0.91	3.98	11.67
SPE purification	Mean	10.30	66.37	8.19	50.83	28.19	19.53[a]
	SD	0.34	0.87	0.30	1.07	0.77	2.24
	%RSD	3.26	1.30	3.71	2.10	2.74	11.46
	Recovery	95.3%	97.3%	102.6%	100.5%	99.8%	104.4%

[a] The sum of the two peak areas.

Table 5
The phospholipid classes content of milk samples from dairy cows determined by LC-CAD.

Sample number	1			2			3			4		
	Mean	SD	%RSD	Mean	SD	%RSD	Mean	SD	%RSD	Mean	SD	%RSD
PLs (mg/100 mL milk)	24.35	1.00	4.11	22.74	0.67	2.95	25.04	1.97	7.87	31.34	0.94	2.97
PS (%)[a]	8.60	0.20	2.32	8.19	0.30	3.66	8.46	0.73	8.63	8.06	0.61	7.87
PE (%)[a]	34.19	0.37	1.08	29.93	1.89	6.31	32.99	0.93	2.82	33.41	0.61	1.82
PI (%)[a]	7.69	0.35	4.55	11.49	0.52	4.52	7.91	0.07	0.88	10.28	0.21	2.04
PC (%)[a]	45.46	0.32	0.70	46.36	1.51	3.26	46.17	1.15	2.49	43.19	0.64	1.48
SM (%)[a]	4.06	0.48	11.82	4.02	0.28	6.96	4.46	0.05	1.12	5.06	0.13	2.57

[a] % of total phospholipids.

It was shown that this new method is sensitive and reproducible for the most important evaluation parameters such as linearity and precision.

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