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Determination of Phospholipids in Milk by HPLC with Evaporative Light Scattering Detector: Optimization and Validation

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Abstract

Phospholipids are part of the milk fat globule membrane. Functional and technological properties make them interesting for the development of functional foods or as ingredients of technological interest. In this study an analytical procedure for determination of major phospholipids in milk (phosphatidyl Ethanolamine, phosphatidylinositol, phosphatidyl Choline, phosphatidyl Serine and sphingomyelin) by HPLC with evaporative light scattering detector was optimized and validated. The best calibrations were achieved when an exponential model was applied; the squared correlation coefficients show a satisfactory linearity ranging from 0.975 to 0.993. The limit of detection for the different phospholipids ranged from 0.17µg for phosphatidyl Serine to 0.61µg for phosphatidyl Ethanolamine; and the limit of quantification from 0.40µg for phosphatidyl Serine to 1.26µg for phosphatidyl Ethanolamine. The intra-day and inter-day precision of the method was below 10% for all the compounds except phosphatidylinositol in the intra-day assay with a value of 12.4%. However, the values of intra-day and inter-day repeatability show that the variations, due to the extraction procedure, do not depend whether the assays were performed after short intervals of time or not. The recovery ranged from 74% for phosphatidil Serine to 112% for phosphatidilcoline. The addition of formic acid to the mobile phase in order to achieve an acidic buffer extends the column life considerably.

Keywords: Phospholipids; Phosphatidyl ethanolamine; Phosphatidylinositol; Phosphatidyl Choline; Phosphatidyl serine; Sphingomyelin; Milk analysis

Abbreviations: ELSD: Evaporative Light Scattering Detector; PC: Phosphatidyl Choline; PE: Phosphatidyl Ethanolamine; PI: phosphatidyl Inositol; PS: phosphatidyl Serine; SM: Sphingo Myelin; IDF: International Dairy Federation; vol: volume; G: Gravitational field intensity; LOD: Limit Of Detection; LOQ: Limit of Quantification; SD: Standard Deviation; RSD%: Relative Standard Deviation

Introduction

Phospholipids are present in all living organisms and are the main structural and functional compounds of cellular membranes. They are amphipathic molecules with a hydrophobic moiety and a hydrophilic head group. The glycerophospholipids are characterized by a diglyceride that is covalently bonded to a phosphate group by an ester linkage, and different organic groups (choline, serine, ethanolamine and inositol) may be bound to the phosphate group. The sphingophospholipids consist of a sphingoid base on which a fatty acid is bound to form a ceramide and on this ceramide unit is linked an organophosphate group.

Phospholipids are recently been taken more into consideration because of their nutritional and technological characteristics [1]. Their inhibitory effect on some types of cancer [2-5], their ability to reduce blood cholesterol levels [5,6] and enhance brain functioning [5,7], their anti-bacterial and anti-inflammatory activity [5,8] and their protective effect on gastric mucosa [9] have been studied. Additionally, their emulsifying properties can be used in several applications in the food, pharmaceutical and cosmetic industry [10].

High-performance liquid chromatography (HPLC) coupled to an evaporative light scattering detector (ELSD) is a useful method for the determination of phospholipids in food matrices [11]. Most of the published HPLC-ELSD methods are normal-phase, with a silica gel or chemically modified silica gels (diol, cyanopropyl, aminopropyl phases) stationary phase and a gradient or isocratic elution, with different solvents; particularly, chloroform: methanol: ammonium hydroxide [12,13] and hexane: isopropanol: water/acids/bases [14]. Recently Pimentel et al. [15] reviewed the analysis procedures of phospholipids in dairy products including sample extraction and analysis by GC or HPLC using ELSD, CAD and MS detectors.

The aim of this work was to optimize a HPLC-ELSD method for qualitative and quantitative determination of the major phospholipids present in milk: phosphatidyl Ethanolamine (PE), phosphatidylinositol (PI), phosphatidyl Choline (PC), phosphatidyl Serine (PS) and Sphingo Myelin (SM).

Material and Methods

Chemicals

For sample extraction, all the reagents were of analytical grade, chloroform and methanol from Sigma-Aldrich (St. Louis, USA), HCl (25%) and NaCl from Panreac (Barcelona, Spain). For HPLC analysis, HPLC-grade chloroform, HPLC-grade methanol and ammonium hidroxide (max. 33%) were supplied by Sigma-Aldrich. Formic acid (98%) and HPLC-grade water were from Panreac.

Phospholipids standards PE and PC (from bovine brain) and PI (from bovine heart) were obtained from Larodan (Malmö, Sweden) and PS and SM (from bovine brain) from Sigma-Aldrich.

Samples for analysis

Raw cow milk samples were stored at -42°C prior to the extraction procedure.

Major components analysis

The fat content was determined by the Röse-Gottlieb method in accordance with IDF International Standard 1 [16]. All analyses were made in duplicate.

Extraction

The extraction procedure was a modification of the method described by Rombaut et al. [17]: 5 g of quark or 10 g of milk were mixed with 20mL of distilled water. The mixture was transferred into a separatory funnel and 80mL of chloroform: methanol (2:1vol/vol) was added. After shaking for 2 minutes, the mixture was separated into two layers, and the lower chloroform layer was released. Sometimes, it was necessary to centrifuge (318 x G, for 15-30 minutes at 10 °C to break the emulsion. Then, 40mL of chloroform: methanol (20:1vol/vol) was added to the upper phase. This step was repeated and the two chloroform phases were separated. In a fourth step, 40mL of chloroform: methanol: water with 1M HCl and 0.9% (w/v) NaCl (86:14:1 vol/vol/vol) was used and the lower phase was pooled with the other three, and then evaporated using a rotary vacuum evaporator at 35ºC. The lipid sample was redissolved in 10mL of chloroform: methanol (88:12vol/vol), filtered with a syringe filter (15mm×0.20µm), transferred into an amber vial and stored at -42°C until HPLC analyses. All samples were extracted and injected in duplicate.

Chromatographic analysis

Phospholipid separations were carried out using a Shimadzu HPLC system (Kyoto, Japan) composed of the following units: degasser, solvent delivery module, controller module, column oven, Rheodyne (Shimadzu) manual injector and an interface module. The detector was a Shimadzu ELSD-LTII. The optimal parameters stabilised for the detector were: pressure of gas nebulizer (N_2) 3.5 bar, temperature 50 °C and gain 3. A Prevail silica column (150×3mm) with 3 µm particle diameter (Grace Davison, Deerfield, IL, USA) and a precolumn with the same packing was used.

The gradient elution was a linear gradient of chloroform: methanol: buffer (0.5% formic acid with ammonium hydroxide until pH 6) 80:19.5:0.5 (vol/vol/vol) at t=0 minutes to 60:33:7 (vol/vol/vol) at t=17 minutes. The initial conditions were restored at t=20 minutes and the time required to reequilibrate the column was 15 minutes. The flow rate of the mobile phase was 0.5mL/minute, the temperature of the column oven was 35°C and the injection volume was 20µL.

The phospholipids were identified by comparing with the retention time of the pure standards.

Results and Discussion

Chromatography conditions

In the first stage of optimization of the chromatographic conditions, a mobile phase gradient of chloroform, methanol and 1M formic acid buffer, adjusted to pH 3 with triethylamine, as described by Rombaut et al. [17] was used. By this procedure the separation of the five major phospholipids (PE, PI, PS, PC and SM) was achieved. However, the resolution between PS and PI peaks was not acceptable. Therefore, several modifications were made with the aim of achieving a total separation.

Since the addition of organic ions to the mobile phase improves the resolution of acidic phospholipids such as PS and PI [18], pH and buffer concentration were changed. Buffers of different pH (3.5 and 4.0) and at different concentrations (1M and 2M formic acid) were tested, but without changing the gradient described in the method [17].

By the buffer modification the PS and PI separation was not improved. Therefore, it was decided to vary the gradient and maintain the initial buffer (1M formic acid, pH 3)

Finally, the conditions that achieved the separation of all compounds were:

Time 0 min: 87.5:12:0.5 (vol / vol / vol) chloroform: methanol: buffer

Time 20 min: 28:63:9 (vol / vol / vol) chloroform: methanol: buffer

Time 30 min: mobile phase returns to initial conditions.

Under these conditions an acceptable separation was achieved; but a prolonged use of triethylamine caused significant fluctuations in the baseline and ghost peaks appeared after minute 15, preventing the quantification of the phospholipids. According to other authors [19] another drawback of the use of modifiers like triethylamine and formic acid is the gradual deterioration in the PS peak shape.

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After that, tests were performed without the use of buffer (chloroform: methanol) and substituting the buffer by water (chloroform: methanol: water). In both cases, as expected, the separation of all peaks was not achieved, since triethylamine and formic acid increase the ELSD response [20].

Then it was decided to seek an alternative to triethylamine. Before testing mobile phase mixtures in gradient, a test in isocratic with isopropanol, hexane and water was made, but the results were not satisfactory.

Subsequently, different gradients of chloroform: methanol: ammonia, mobile phases used by other authors for the separation of phospholipids [13,21] were tested, and the separation of PE, PI, PS, PC and SM was acceptable. But the use of ammonia has a major drawback, since its basic pH dissolves silica and the column life diminishes [12]. To solve this problem, formic acid was added to the solution of ammonia in order to achieve an acid pH. Different mixtures of formic acid and ammonia, and different gradients were tested to optimize the separation conditions, for the identification and quantification of the five major phospholipids present in milk. The details of the final chromatographic method were described in section 2.5.

The resolution of the peaks was 4.5 for PE-PI, 4.7 for PI-PC, 2.7 for PC-PS and 1.2 for PS-SM. In all cases the resolution is higher than 1.5, except between the peaks of PS and SM. A resolution of 1.5 indicates the complete separation of the two components; whereas with a resolution of 1.0 the non-separated area is about 4% [22].

Each peak matches with a type of phospholipid, itself made up of molecular species with different fatty acids. This fact may explain the existence of peaks with shoulders and also double peaks. For the specific case of SM, when a standard of bovine origin was analyzed separately from the other phospholipids, 3 sub-peaks were detected, while in combination with the other phospholipids, it was observed that SM eluted as 2 subpeaks (Figure 1). Double or triple peak of the SM has been described in several studies [19, 23-25]; this has been attributed to the presence or absence of an extra hydroxyl group or due to the different fatty acids or sphingoid groups.

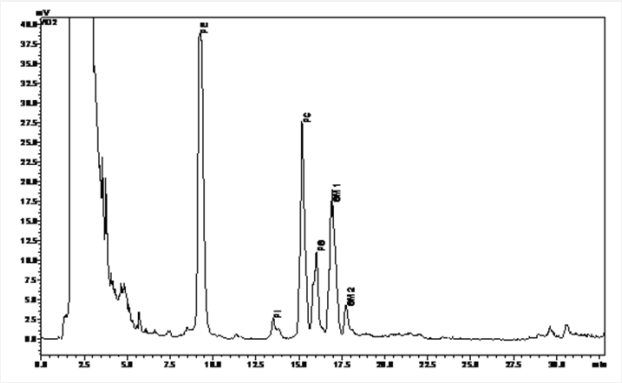

Figure 1: HPLC chromatogram of raw cow milk phospholipids.

In relation to the light scattering detector parameters, the gain can be modified from 1 to 12, and it is important to choose a gain that does not saturate the detector. The nitrogen pressure should be fixed at an optimum flow of gas to produce an adequate signal to noise ratio. The evaporating temperature is another important factor; at higher temperature, the baseline shows more noise; but the choice temperature has to be enough to evaporate the mobile phase, avoiding the analyte volatilization. Different temperatures (50 °C, 55 °C, 60 °C, 65 °C and 80 °C) were tested and it was noted that when temperature increases, the response of SM decreases. According to other authors [14] this could be due to the evaporation of some free fatty acids of low boiling point. These values were finally established as follows: gain 3, nitrogen pressure 3.5 bar and evaporation temperature 50 °C.

After the chromatographic conditions and the detection parameters were established, the method validation was carried out.

Calibration and detection limits

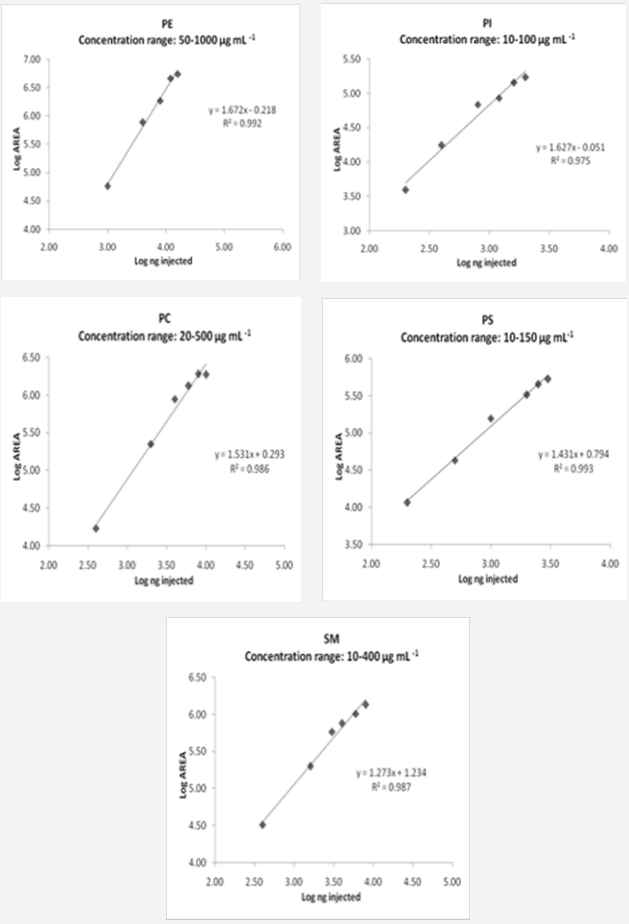

Figure 2: Equations, correlation coefficients and concentration range of phospholipids calibrations.

PE: Phosphatidyl Ethanolamine, PI: Phosphatidyl Inositol, PC: Phosphatidyl Choline, PS: Phosphatidyl Serine, SM: Sphingo Myelin.

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To evaluate linearity of the ELSD, two different equations were applied: linear and exponential. The response of the ELSD has been described as linear [13,17] and non-linear [26,27]. In the present study, the best results were obtained with the exponential model, which was plotted on a logarithmic scale to obtain a linear calibration. The calibration curves were calculated by applying logarithms to the area values and the mass of lipid standard injected on column. Six levels of concentration were used for the calibration of each compound. The calibration equations, correlation coefficients (R^2) and concentration range for PE, PI, PS, PC and SM are shown in Figure 2. The R^2 values show a satisfactory linearity, ranging from 0.993 to 0.975.

The limits of detection (LOD) and quantification (LOQ) were determined as the amount injected that provided a signal to noise ratio of 3 and 10 respectively. The LOD for the different phospholipids ranged from 0.17 µg for PS to 0.61 µg for PE and the LOQ from 0.40 µg for PS and 1.26 µg for PE (Table 1). Similar values were previously reported [19,28].

Table 1: Limit of detection (LOD) and limit of quantification (LDQ).

Phospholipid	LOD(µg)	LOQ (µg)
PE	0.61	1.26
PI	0.26	0.53
PC	0.2	0.47
PS	0.17	0.4
SM	0.22	0.59

PE: Phosphatidyl Ethanolamine, PI: Phosphatidy Linositol, PC: Phosphatidyl Choline, PS: Phosphatidy Serine, SM: Sphingo Myelin.

Intra-day and inter-day precision

For the determination of intra-day precision, the same sample (raw milk) was extracted 5 times, and each extraction was injected in duplicate on the same day. To evaluate the inter-day precision, 5 extractions were performed on 5 different days and each injected twice.

Table 2: Relative Standard Deviation (%RSD) of the intra-day and inter-day precision.

Phospholipid	Intra-day Precision %RSD	Inter-day Precision %RSD
PE	5.7	7.1
PI	12.4	7.7
PC	7	7
PS	8.2	8.7
SM	9.6	5.6

PE: Phosphatidyl Ethanolamine, PI: Phosphatidyl Inositol, PC: Phosphatidyl Choline, PS: Phosphatidyl Serine, SM: Sphingo Myelin.

For all the compounds, the RSD% was below 10%, except for PI in the intra-day assay with a RSD% of 12.4, and there is not a higher tendency for RSD% in inter-day assays (Table 2). These results show that the variations due to the extraction procedure are quite constant and they do not depend on the time factor. The RSD% obtained was in accordance with the results reported by other authors [13,14].

Recovery

To establish the efficiency of the extraction procedure, a mixture of the five phospholipids was added to the milk sample. The results range from 74% for PS to 112% for PC (Table 3). Taking into account the complexity of the extraction process, the results are acceptable.

Table 3: Recovery of the phospholipids added to the milk samples.

Compound	% Recovery	SD
PE	110	0.02
PI	109	0.1
PC	112	0.03
PS	74	0.12
SM	83	0.03

PE: Phosphatidyl Ethanolamine, PI: Phosphatidyl Inositol, PC: Phosphatidyl Choline, PS: Phosphatidy Serine, SM: Sphingo Myelin.

Conclusion

The HPLC-ELSD is an adequate technique for the analysis of the main phospholipids present in milk. The best calibrations were achieved when an exponential model was applied. The square correlation coefficients, limits of detection, limits of quantification, and intra-day, inter-day precision and recoveries were acceptable. However, the values of intra-day and inter-day repeatability show that the variations, due to the extraction procedure, do not depend whether the assays were performed after short intervals of time or not.

Since the basic pH of the ammonia used in the mobile phase dissolves silica, the addition of formic acid to the mobile phase in order to achieve an acidic buffer, extends the column life considerably.

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