Journal of Chromatography A, 1218 (2011) 6476–6482

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of phospholipids in milk samples by means of hydrophilic interaction liquid chromatography coupled to evaporative light scattering and mass spectrometry detection

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ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 8 July 2011
Accepted 12 July 2011
Available online 23 July 2011

Keywords:
Phospholipids
Cow's milk
Donkey's milk
HILIC
ELSD
MS-IT-TOF

ABSTRACT

The combined use of the state-of-the-art hybrid mass spectrometers together with high efficient liquid chromatography could surely be a useful tool for such a challenging task, as phospholipids (PLs) analysis. In this research, we used hydrophilic interaction liquid chromatography (150 mm × 2.1 mm I.D., 2.7 μm d.p. partially porous column) to achieve the separation of major PLs classes in cow's and donkey's milk samples. Solid-phase extraction (SPE) was performed in order to pre-concentrate minor PLs from non polar lipids (triacylglycerols) and the recovery for the extraction method was assayed on a milk sample, fortified with 5 μg/mL of SM pure standard, and analyzed in triplicate. A value of 89.99% was calculated, with a coefficient of variation (CV%) of 1.93. A 70-min long stepwise gradient of water/acetonitrile afforded baseline separation of PLs classes, at 50 μL/min flow rate. Accurate detection by an ion trap-time of flight (IT-TOF) mass spectrometer (in both positive and negative ionization mode) allowed to fully characterize the distinctive phospholipid profile and fatty acid composition of cow's and donkey's milk, the latter being analyzed for the first time. Evaporative light scattering detection was further employed to attain the quantitative evaluation of major PLs classes identified, by the external calibration method using reference material solutions in the 5–200 μg/mL concentration range. Major difference between the two analyzed samples consisted in the total PLs amount, which in cow's milk was determined as over 20-fold higher than the donkey's.

1. Introduction

Polar lipids are the main constituents of biological membranes, and are also found in food matrices, in different amounts. Among them, phospholipids (PLs) comprise an important kind of amphiphilic molecules, with lipophilic acyl chains and a hydrophilic head. Glycerolphospholipids consist of a glycerol backbone esterified with two fatty acids (FAs) at *sn*-1 and *sn*-2 positions, while the *sn*-3 position is occupied by a phosphate group attached to a polar head of different nature. On the other hand, sphingolipids are a class of lipids derived from the aliphatic amino alcohol sphingosine. The latter is the most prevalent sphingoid base in mammalian sphingolipids, containing 18 carbon atoms, two hydroxyl groups and one double bond [1]. A ceramide is formed when the amino group of this sphingoid base is linked with a saturated fatty acid. The bonding to an organophosphate group or to a saccharide brings to sphingophospholipids or glycosylceramides, respectively.

These compounds possess important physiological functions, as well as positive nutritional properties. Recent studies have in fact given considerable evidence that PLs can exert beneficial effects on human health, such as anti-inflammatory activity and reduction of the risk of cardiovascular disease [2–4]. PLs are also used as emulsifiers or emulsion stabilisers in the food industry, in the form of complexes with proteins [5–8].

Recently, a number of biological activities have been attributed to lysophospholipids (LPs) as well, which differ for the presence of only one acyl group at the glycerol residue and include well-studied members lysophosphatidic acid (LPA) and sphingosine 1-phosphate. Documented cellular effects of these molecules

Abbreviations: CDL, curved-desolvation line; CID, collision-induced dissociation; HILIC, hydrophilic interaction liquid chromatography.

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doi:10.1016/j.chroma.2011.07.036

encompass growth-factor-like influences on cells, including but not limited to survival, migration, adhesion differentiation, as well as pathophysiological actions associated with cancer. Consequently, LPs receptors have gained considerable attention as molecular targets for novel anti-tumoral therapies [9].

For the above mentioned reasons, the characterization of phospholipids in natural matrices and foodstuffs, such as milk and dairy products, is a very interesting task in food-related research and lipidomics. Five major classes of phospholipids are found in milk fat, and they are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS) [10–13].

Phospholipids are located on the milk fat globule membrane (MFGM), where they contribute significantly to the emulsification role of the membrane by virtue of both lipophilic and hydrophilic properties [14,15].

The challenge in phospholipids analysis arises from their low abundance with respect to the nonpolar triglycerides, and the simultaneous occurrence of a number of positional and structural isomers. Taking into account the high variety of fatty acids that can be found bound to the particular structure of a single PLs group, the number of different compounds that should be identified is extremely great. As a result, PLs analysis typically involves different steps, consisting in fat extraction from milk, isolation of PL fraction from the other lipid classes, separation and detection of the different phospholipid classes and or species.

As far as the isolation of PL fraction from the other lipid classes is concerned, thin layer chromatography [16,17], column chromatography [18] and solid phase extraction (SPE) have been applied [19–21]. High performance liquid chromatography with UV or evaporative light scattering detector (ELSD) has been used for the analysis of the different PLs [22–24]. More recently, HPLC/ELSD methods applying on line pre-concentration were developed [25].

Rodríguez-Alcalá and Fontecha [26] developed an HPLC–ELSD method for the analysis of the lipid classes of buttermilk and milk from different species, focused on the phospholipids fraction without a prior fractionation step and in a single run. Separation of lipid classes was accomplished on a 5 μm d.p. Rx-Sil column; the identification and quantification of the different compounds were achieved using calibration curves made with individual PLs standards (LOQ values were determined in the 0.3–0.8 μg range).

HPLC with ELS detection was also employed to quantify major PLs classes in donkey's serum [27], duck meat after purification by SPE on aminopropyl-silica column [28], dairy products after extraction with chloroform/methanol [29].

Avalli and Contarini [30] evaluated the performance of different methods for both milk lipid extraction [31,32] and separation of phospholipids (different SPE cartridges and solvent programs); the quantification of PC, PE, PI, PS and SM was then performed by HPLC/ELSD on a 5 μm d.p. Rx-Sil column.

Separation of the different phospholipid classes is generally achieved by means of NP LC, whereby distinctive retention occurs according to their polar head group (PE, PC, etc.). Different molecular species arising from peculiar fatty acid substitution within a particular PL class can be afterward separated in a secondary chromatographic step, consisting or RP LC, which differentiates PLs according to their FA chain lengths and degree of saturation [33–35].

Hydrophilic interaction liquid chromatography (HILIC) is a relatively new LC technique that uses a hydrophilic stationary phase, in most cases, with organic-dominant mobile phase. Its mechanism can be described as liquid–liquid partition chromatography, or a version of normal phase liquid chromatography which can be performed with partially aqueous mobile phases. HILIC separates compounds by passing a hydrophobic or mostly organic mobile phase across a neutral hydrophilic stationary phase, causing solutes to elute in order of increasing hydrophilicity; the elution order of substances in HILIC mode is therefore roughly the reverse of that in reversed-phase mode. In LC–MS of amphiphilic molecules like phospholipids, HILIC may be useful to avoid extremely water-rich, or ion-pair containing mobile phases used under reversed-phase mode. The use of mobile phases of higher-organic contents is advantageous in providing larger diffusion constants of analytes during their migration through the column, and also better ionization efficiency in electrospray ionization (ESI). By this means, HILIC is an ESI-MS compatible separation tool for PL classes separation. RP LC is also ESI-MS compatible, however it would be difficult to separate classes of PLs containing more than one sub-component [36].

As far as detection is concerned, electrospray ionization (ESI) mass spectrometry (MS) is one of the preferred detection techniques combined with HPLC, as it records intact molecule related ions and offers structural information via MS/MS experiments. Particularly, hybrid IT-TOF instruments offer the advantages of high mass accuracy, enabling exact measurement of intact molecules, and MS^n capabilities for selective fragmentation of target precursor ions which helps in identification of unknown structures and sometimes also in distinguishing between isomers [37].

In this work, a new LC separation methodology based on the use of a partially porous (Fused-core, 2.7 μm d.p.) HILIC column, coupled to an hybrid ion trap-time of flight (IT-TOF) mass analyzer was developed for the characterization of the phospholipid fraction in cow's and donkey's milk, the latter being analyzed for the first time. In addition, an ELS detector equipped with a miniaturised interface was employed for the quantification of several classes of phospholipids, sphingolipids and lysophospholipids in the milk samples. Furthermore, molecular species within the different PL classes were partially separated, and the relative fatty acid composition characterized in the milk samples.

2. Experimental

2.1. Chemicals and materials

Chloroform, methanol, hexane and diethyl-ether employed for the extraction procedure were obtained from VWR (Milan, Italy). SPE cartridges were Supelclean LC-SI (6 mL volume, 1 g sorbents) from Sigma–Aldrich/Supelco (Bellefonte, PA).

For LC analyses, acetonitrile and water, both LC–MS grade, were purchased from Riedel-de Haën (Germany). The standard phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) were obtained from Sigma–Aldrich/Supelco (Bellefonte, PA).

Two milk samples were analyzed: a whole pasteurized cow's milk purchased at a local market and a donkey's milk kindly donated by a local producer.

2.2. Extraction of the lipid fraction of the samples

Extraction of the lipid fraction was carried out from 10 mL of the milk samples, according to the Folch method to ensure the exhaustive extraction of the whole lipid content [32]. The total extract was evaporated under vacuum, and the final dry residue (400 and 150 mg for cow's and donkey's milk, respectively) was re-dissolved in chloroform/methanol 2:1 (*v/v*) and stored at −18 °C until use.

2.2.1. SPE (solid phase extraction)

PLs extraction was obtained by SPE, carried out in order to partially remove non-polar lipids in the fat samples, thus concentrating the PLs fraction. Lipid extract (100 mg) was afterward dissolved in 1 mL mixture of chloroform/methanol (2:1, *v/v*). After the cartridge has been conditioned with hexane, the non-polar lipids were

eluted with 3 mL of hexane/diethyl-ether (8:2, *v/v*) and 3 mL of hexane/diethyl-ether (1:1, *v/v*). Recovery of PLs from the cartridge was obtained by two-step elution, using 4 mL of methanol as first extraction solvent, and subsequently 2 mL of methanol followed by 2 mL of chloroform/methanol/water (3:5:2, *v/v/v*). The recovered fraction was dried under a gentle stream of nitrogen, yielding 3.8 and 49.7 mg dry residue, for donkey's and cow's milk, respectively. The residue was finally re-dissolved in chloroform/methanol (2:1, *v/v*) [30].

Recovery for the extraction method was assayed on a milk sample, fortified with a known amount of SM pure standard (5 μg/mL), applied to SPE cartridge after chloroform/methanol extraction, and analyzed in triplicate.

2.3. Instrumentation and software

The LC analyses were carried out using a Shimadzu Prominence LC-20A system (Shimadzu, Milan, Italy), including a CBM-20A controller, two LC-20 AD dual-plunger parallel-flow pumps, a DGU-20A5 on-line degasser, a CTO-20A column oven and a SIL 20A autosampler. The instrument was hyphenated online either to an LCMS-IT-TOF mass spectrometer (Shimadzu, Milan, Italy) through an ESI interface, or to an evaporative light scattering detector equipped with a miniaturised interface (ELSD-LT, Shimadzu, Milan, Italy).

LCsolution Version 1.23 A and LCMSsolution Ver. 3.50.346 software (Shimadzu, Milan, Italy) were used for data acquisition and processing.

2.4. LC conditions

The analytical column used was an Ascentis Express HILIC, 150 mm × 2.1 mm I.D. with partially porous (Fused-core) particles of 2.7 μm (Sigma–Aldrich/Supelco, Bellefonte, PA). Mobile phases consisted of (A) acetonitrile and (B) acetonitrile–water (2:1, *v/v*). The chromatographic separation was carried out using the following stepwise binary gradient: 0–10 min 0% B, 15 min 20% B, 35 min 45% B, 50 min 80% B, 70 min 100% B (hold for 30 min). A flow-rate of 50 or 100 μL/min was used for LC–ELSD and LC–MS analyses, respectively. 5 μL of the standard or milk samples was injected, in triplicate. Pure phospholipid standards were chromatographed individually to confirm retention times and purity.

2.5. MS and MS/MS conditions

The MS acquisition was performed using an ESI interface simultaneously operated in both positive and negative ionization mode, under the following conditions: CDL temperature, 200 °C; block heater temperature, 200 °C; nebulizing gas flow (N_2), 1.5 L/min. MS data were acquired in the 500–1000 *m/z* range, using 10 ms ion accumulation time (repeat = 2). MS/MS data were acquired in the 200–1000 *m/z* range, using 10 ms ion accumulation time (repeat = 2, tolerance 0.05 *m/z*) and automatic precursor ion selection (width: 3 Da, CID parameters: energy 50%, collision gas (argon) 50%, execution trigger (BPC) intensity at 95% stop level).

Resolution, sensitivity, and mass number calibration of the ion trap and the TOF analyzer were adjusted using a standard sample solution of trifluoroacetic acid (TFA, approx. 0.25 mL/L) and sodium hydrate (approx. 0.1 g/L). After the calibrant had flowed, cleaning operation of the tube and ESI probe was made by flowing acetonitrile (0.2 mL/min, 20 min).

2.6. ELSD conditions

A nebulizing gas (N_2) flow of 2 mL/min (180 kPa) was used for micro-ELSD, and a drift tube temperature of 50 °C. ELSD detection was employed to attain the quantitative evaluation of major PL classes identified, by the external calibration method. Five-point calibration curves for PLs were obtained from the area values obtained by injecting 5 μL of chloroform–methanol (2:1, *v/v*) independent solutions of the standards, i.e. PI (5–100 μg/mL), PS (5–100 μg/mL), PE (5–200 μg/mL), PC (2–100 μg/mL) and SM (2–100 μg/mL). Each solution was prepared and injected in triplicate, and the average data were subjected to linear regression analysis ($y = ax + b$). The limit of detection (LOD) and the limit of quantification (LOQ) were estimated, as the concentration level giving three-fold and ten-fold the noise signal, respectively. By interpolation of the calibration curves, major classes of PLs identified the SPE-extracted samples were quantified.

3. Results and discussion

The aim of this study was to attain the characterization of PLs content of extracted milk samples, through LC–HILIC analysis followed by identification and quantification by IT-TOF and ELS detection, respectively. After preliminary extraction of the whole lipid content of the samples (as described in Section 2.2) by chloroform/methanol mixture, selective concentration of the PL fraction was attained on SPE cartridge, in order to partially remove non-polar lipids (as described in Section 2.2.1).

3.1. Identification of PLs in cow's and donkey's milk samples

Separation of the PLs in the extracted samples was achieved under gradient conditions on a 15 cm-long, narrow bore silica HILIC column. Such a choice lies on a number of considerations. Although HILIC is typically used for polar compounds, baseline separation can be also achieved for amphiphilic molecules like phospholipids [36]. With respect to normal-phase (NP) chromatography, which is usually used for PL classes separation, HILIC has the double advantage of using water-miscible solvents, which are compatible with ESI detection, making on-line hyphenation to MS detection straightforward. The high organic content in mobile phases (typically >80%) promotes enhanced ESI-MS response, thus increasing the sensitivity of detection. This chromatographic mode offers complementary selectivity to reversed-phase chromatography, and was therefore taken into consideration as first step toward the implementation of a multidimensional separation system. Furthermore, it allowed to shorten the sample separation procedure, by directly inject the final SPE extract, with no need for prior evaporation and reconstitution steps.

The column used hereby was packed with 2.7 μm d.p. partially porous particles (Fused-core technology), which offer superior efficiency than totally porous particles of the same dimensions, still at moderate backpressures. Such stationary phase performed successfully either in one-dimensional [38–40], or in multidimensional comprehensive setups; in the latter case aiming to attain higher resolution power in D1 (on long column lengths), or shorter retention times in D2 (fast repetitive gradients with brief reconditioning time) [41–46].

In the HILIC–ELSD chromatogram of the whole lipid fraction extracted from the milk samples, a huge amount of non-polar lipids (triglycerides) eluted at the beginning of the gradient run (chromatogram not shown). Triglycerides were almost completely removed upon PLs concentration on SPE cartridge, as can be noticed from the chromatograms in Figs. 1 and 2. Lipid classes are defined by the polar head-group nature, while different molecular species may occur within any single class, according to the nature (length and degree of saturation) of the fatty acids. Baseline separation of five PL classes was achieved, under gradient conditions, within a runtime of 55 min, eluting according to decreasing polarity, i.e.:

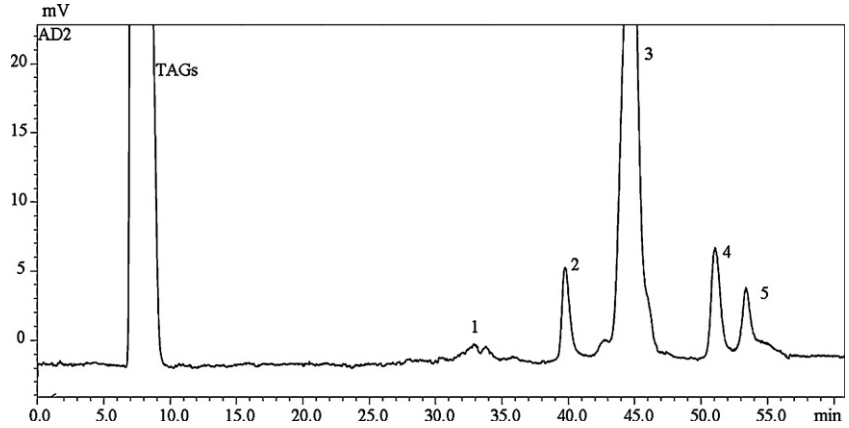

Fig. 1. HILIC-ELSD chromatogram of SPE-extracted PLs from a cow's milk sample: 1. Phosphatidylinositol; 2. Phosphatidylserine; 3. Phosphatidylethanolamine; 4. Phosphatidylcholine; 5. Sphingomyelin. Chromatographic conditions in the text.

PI > PS > PE > PC > SM. Identification was achieved first by comparison of the retention times with those of the reference materials. One more species can be observed in the cow's milk chromatogram, eluting after SM at the end of the gradient, for which no matching standard material was available.

The hyphenation of LC to an IT-TOF mass spectrometer allowed to further discriminate the separated compounds, all detected as intense $[M+H]^+$ or $[M+Na]^+$ ions, the only exception being PI, which was therefore recorded in the negative ionization mode. Besides the individual classes, a partial separation of the different PL molecular species within each subclass was also observed, with elution order depending upon the chain length and degree of saturation of the acyl substituents at the glycerol backbone. The individual components were identified on the basis of the observed *m/z* values and the calculated ECN (effective carbon number); compounds with higher ECN were eluted earlier under such chromatographic conditions, having higher lipophilicity. ECN was calculated as the number of carbon atoms (NC) of the FA chains, minus twice the number of double bonds (DB). Table 1 reports, for each individual component, theoretical and observed monoisotopic masses of the pseudomolecular ion, and the calculated mass accuracy, expressed as error in ppm units. Besides, molecular formulas and fatty acid composition of the species eluting at different retention times were compared with literature data [25,26,28,30,36,47]. Tandem MS data were used to infer the exact fatty acid composition, in those cases when more than one molecular species could be derived from a molecular formula. An example is shown in Fig. 3, where the MS spectrum (negative ESI mode) of different PI molecular species detected in the milk sample is reported, as $[M-H]^-$ ions, or $[M+Na]^+$ adducts. Different *m/z* values displayed correspond to distinct PI species, arising from a different FA substitution at the glycerol backbone (different acyl length and degree of unsaturation). In detail, *m/z* 833.5161 was measured for the isomer containing palmitic and linoleic acid, i.e. (16:0/18:2)-PI; *m/z* 835.5353 for the isomer containing palmitic and oleic acid, i.e. (16:0/18:1)-PI, *m/z* 861.5491 for the isomer containing stearic and linoleic acid (18:0/18:2)-PI, *m/z* 863.5654 for the isomer containing stearic and oleic acid (18:0/18:1)-PI. In hybrid mass spectrometer configurations based on ion-trap, the possibility to manually select desired precursor ions for collision-induced dissociation (CID) further helps in identification of analyzed molecules. By selecting different *m/z* values for CID experiments, distinctive fragmentation is in fact obtained, as can be seen from the two lower spectra. In the example shown here, fragmentation of both the parent ions gave signals at different *m/z* values, for ions corresponding to the loss of the fatty acids, one of the fatty acid and the sugar moiety, and the fatty acids, themselves.

In both cow's and donkey's milk samples, phosphatidylethanolamine (PE) gave the most intense signal, with symmetrical peak shape. In contrast, phosphatidylcholine (PC) eluted as a broader peak, as a result of a wider variety in fatty acid composition, and the partial separation of the different molecular species. Fagan and Wijesundera [25] observed for normal-phase chromatography of PC standards a larger range of retention times, compared to the PE standards; this is in agreement with a variation of the capacity factors of diacyl PC, which was found

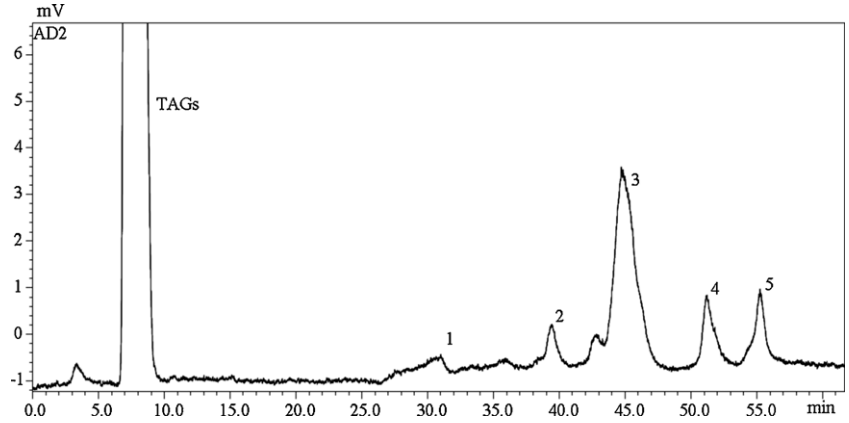

Fig. 2. HILIC-ELSD chromatogram of SPE-extracted PLs from a donkey's milk sample: 1. Phosphatidylinositol; 2. Phosphatidylserine; 3. Phosphatidylethanolamine; 4. Phosphatidylcholine; 5. Sphingomyelin. Chromatographic conditions in the text.

Table 1
Molecular species of PLs identified in cow's and donkey's milk (MS-ESI-IT-TOF).

PL class	Molecular formula	Molecular species	$[M+H]^+$ observed	$[M+H]^+$ calculated	Error (ppm)
PI[a]	$C_{43}H_{77}PO_{13}$	C16:0/C18:2	833.5161	833.5174	−1.56
	$C_{43}H_{79}PO_{13}$	C16:0/C18:1	835.5353	835.5331	+2.63
	$C_{45}H_{83}PO_{13}$	C18:0/C18:2	861.5491	861.5498	−0.81
	$C_{45}H_{85}PO_{13}$	C18:0/C18:1	863.5654	863.5655	−0.11
PS	$C_{38}H_{72}NPO_{10}$	C16:0/C16:0	734.4937	734.4966	−3.94
	$C_{40}H_{74}NPO_{10}$	C16:0/C18:1	760.5134	760.5123	+1.44
	$C_{40}H_{76}NPO_{10}$	C16:0/C18:0	762.5261	762.5279	−2.36
PE	$C_{39}H_{74}NPO_8$	C16:0/C18:2	716.5522	716.5224	+4.15
	$C_{39}H_{76}NPO_8$	C16:0/C18:1	718.5388	718.5381	+0.97
	$C_{41}H_{78}NPO_6$	C18:0/C18:2	744.5536	744.5537	−0.13
PC	$C_{40}H_{78}NPO_8$	C16:0/C16:1	732.5558	732.5537	−2.86
	$C_{40}H_{80}NPO_8$	C16:0/C16:0	734.5679	734.5694	−2.88
	$C_{42}H_{78}NPO_8$	C16:1/C18:2	756.5535	756.5537	−1.78
	$C_{42}H_{80}NPO_8$	C16:0/C18:2	758.5680	758.5694	−1.84
	$C_{42}H_{82}NPO_8$	C16:0/C18:1	760.5854	760.5850	+0.52
	$C_{44}H_{82}NPO_8$	C18:1/C18:2	784.5880	784.5850	+ 3.82
	$C_{44}H_{84}NPO_8$	C18:0/C18:2	786.6009	786.6007	−1.65
	$C_{44}H_{86}NPO_8$	C18:0/C18:1	788.6154	788.6163	−1.41
SM	$C_{39}H_{79}N_2PO_6$	C16:0	703.5749	703.5748	+0.28
	$C_{41}H_{77}N_2PO_6$	C18:1	725.5574	725.5591	−2.34
LPC[b]	$C_{26}H_{48}NPO_7$	C18:3	518.3243	518.3241	+0.38
	$C_{26}H_{52}NPO_7$	C18:1	522.3549	522.3554	−0.95

[a]Detected as $[M-H]^-$ ion.
[b]Observed in cow's milk only.

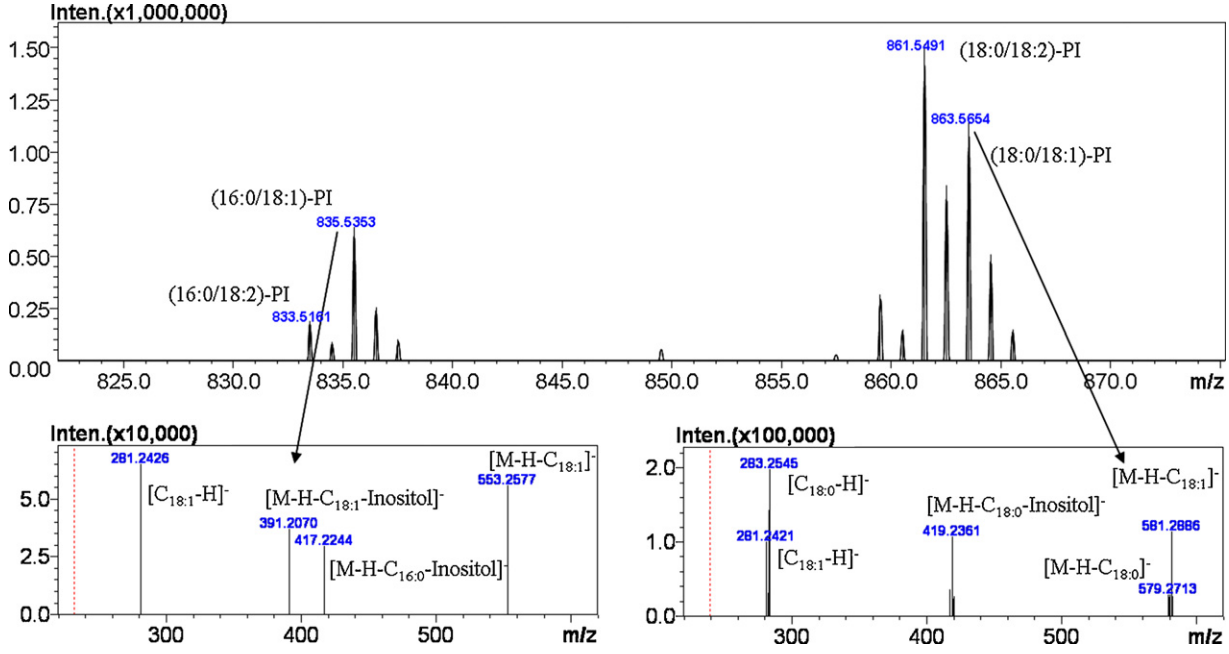

Fig. 3. Top: MS spectra (negative ESI mode) of different PI molecular species detected in the milk sample. Bottom: MS/MS data obtained upon CID fragmentation of selected precursor ions (indicated by arrows).

Table 2
Calibration equation coefficients, correlation coefficients, limits of detection and quantification, and concentration ranges for standard phospholipids in the assayed LC–ELSD method. Linear regression equation: $y = ax + b$ (x = μg injected; y = peak area in mV).

PL	a	b	R^2	LOD (μg/mL)	LOQ (μg/mL)	Range (μg/mL)
PI	6797	−22,771	0.998	4.04	4.05	5–100
PS	31,567	+51,756	0.999	1.68	1.70	5–100
PE	15,697	−52,589	0.998	3.38	3.39	5–200
PC	18,873	−47,948	0.999	1.83	1.85	5–100
SM	11,174	+19,940	0.999	2.57	2.58	5–100

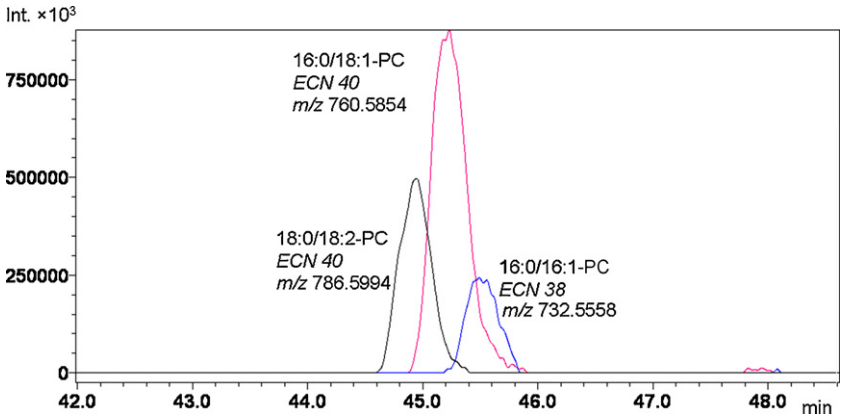

Fig. 4. Individual chromatogram traces (MS-ESI-IT-TOF) of the $[M+H]^+$ ions of three phosphatidylcholine (PC) species detected in cow's milk at different retention times; the elution order within a PC class increased with increasing hydrophilicity (lower ECN) and decreased with increasing lipophilicity (higher ECN). Experimental conditions in the text.

being 3–10 times higher than the equivalent diacyl PE [48]. Fig. 4 shows as an illustrative example the individual chromatogram traces of the $[M+H]^+$ ions of three phosphatidylcholine species recorded at different retention times; the elution order within each PC class increased with increasing hydrophilicity (lower ECN) and decreased with increasing lipophilicity (higher ECN). Stearyl-linoleylphosphatidylcholine (18:0/18:2), with ECN equal to 40, eluted first at a retention time (RT) of 44.94 min, followed by palmitoyl-oleylphosphatidylcholine (16:0/18:1), which was more retained, even if having same ECN. This species containing a shorter acyl chain eluted with RT of 45.22 min, reflecting its greater polarity. The species containing palmitoyl and palmitoleyl acid moieties, i.e. 16:0/16:1 eluted with RT of 45.50 min, consistently with lower lipophilicity (ECN 38). Higher degree of interaction with the stationary phase is also due to higher degree of saturation. It must stressed, however, that the higher intensity of *m/z* signal of a certain species, with respect to another one, does not necessarily means higher abundance, as it is well known that different PLs show different MS ionization efficiencies, depending on the type of acyl substitution at the glycerol backbone.

A total of 10 different molecular species were detected within PC class, as reported in Table 1, not including the $[M+Na]^+$ ions. Phosphatidylserine (PS), phosphatidylethanolamine (PE), and sphingomyelin (SM) were also detected in the positive ionization mode (as $[M+H]^+$ or $[M+Na]^+$ ions), at retention times of 29, 35 and 48.5 min, respectively; the elution order observed was in agreement with the literature [25,28,33]. On the other hand, phosphatidylinositol (PI), which eluted earlier with RT of 27.5, was better detected as $[M-H]^-$ ion. A lower number of molecular species were observed for PI, PS, PE, and SM, as can be seen in Table 1.

The higher sensitivity of MS (ESI-IT-TOF) detector, with respect to ELSD, gave evidence of the presence of one more PL component, eluting in cow's milk after SM. On the basis of its MS spectrum, it was identified as lysophosphatidylcholine (LPC), one member of the lysophospholipids which differ from PLs for the presence of only one acyl group at the glycerol residue. Due to the increased hydrophilicity, resulting from the presence of an hydroxy group instead of an acyl chain at position 2 of the glycerol backbone, it was most retained (RT > 50 min) under the chromatographic conditions employed for the analysis. Molecular species of LPC class were also partially separated, and detected as $[M+H]^+$ ions at *m/z* 522.3549 (oleyl-LPC, 18:1) and 518.3243 (linolneyl-LPC, 18:3). This species was not observed in the donkey's milk, representing a qualitative difference between the two samples.

3.2. Quantification of PLs cow's and donkey's milk samples

In this study, ELS detector was used to quantify phospholipid classes in donkey's and cow's milk, by external standardization. Total lipids were first extracted from the samples by homogenisation with chloroform–methanol (2:1); the fat solution was afterward applied to SPE cartridge, for selective PLs extraction. Recovery for the extraction method was assayed on a milk sample, fortified with 5 μg/mL of SM pure standard, and analyzed in triplicate. A value of 89.99% was calculated, with a coefficient of variation (CV%) of 1.93, using the following formula for the calculation:

$$\text{Recovery}\,\% = \frac{\text{conc. fortified sample} - \text{conc. unfortified sample}}{\text{fortification}} \times 100$$

The use of ELSD can give a precise relative content of PL classes and/or molecular species, while the quantification with an ion trap may be affected by the different ionization potentials of the analytes. Also, elution of all molecular species of a defined class in a single peak is desirable for the most accurate results, when aiming at quantifying PL class distribution in a sample.

Calibration curves for detector response *vs.* mass of PL injected were obtained by applying the linear model. Five independent concentrations of pure standards were injected in triplicate, at levels similar to those contained in the sample analyzed; Table 2 shows the results obtained by linear curve fitting. The response of ELS detector is non-linear, but within a limited range it can be described by a linear model. We are aware of the fact that, under certain circumstances, quadratic dependence gives better results than the linear, this usually applies for very low concentration ranges [49]. We applied both regression equations to our experimental results, i.e. the linear ($y = a + bx$) and the power ($y = axb$) but the results we obtained were nearly identical, with regression coefficients varying very slightly between PLs, in turn higher or lower. For this reason, we presented only the results obtained in one way. The method showed linearity for each PL class over the range stated; correlation coefficients (R^2) obtained from the plot of experimental values as a function of theoretical values were always above 0.9998 for the range of linearity. Besides, LOD and LOQ values were estimated, for the standard PLs in the assayed method, as the concentration level giving three-fold and ten-fold the noise signal, respectively, according to the EURACHEM Guidelines (at 95% confidence level, from ten replicate injections) [50]. By interpolation of the calibration curves, major classes of PLs identified the SPE-extracted samples were quantified, and the values are reported in Table 3, together with the standard deviation calculated from the replicate analyses.

According to these results, the total PLs content in cow's milk was much higher than the donkey's milk, with values of 46.21 and

Table 3
Values in μg/mL ± SD of major PLs classes in cow's and donkey's milk samples.

No.	PL class	Cow's milk	Donkey's milk
1	Phosphatidylinositol	0.64 ± 0.018	0.07 ± 0.003
2	Phosphatidylserine	5.34 ± 0.475	0.33 ± 0.003
3	Phosphatidylethanolamine	33.37 ± 2.102	1.77 ± 0.156
4	Phosphatidylcholine	3.69 ± 0.066	0.51 ± 0.010
5	Sphingomyelin	3.23 ± 0.188	0.26 ± 0.003
TOT		46.21 ± 1.827	2.94 ± 0.215

2.94 μg/mL, respectively. In both samples, PE was by far the most abundant PL class, i.e. 9–50 times fold the amount of the other classes; in contrast PI was detected at very low amounts, i.e. 0.64 and 0.07 μg/mL, in cow's and donkey's milk, respectively. PS, PC and SM were detected at intermediate levels, and accounted for the rest. The other major difference in quantitative PLs profile of the two samples was represented by the second abundant compound, i.e. PS in cow's milk, accounting for the 11% of the total PLs, and PC in donkey's milk, accounting for 17.34%.

4. Conclusions

In this work, hydrophilic interaction liquid chromatography on a partially porous column was employed to achieve the baseline separation of five phospholipid classes after SPE extraction from milk samples. Accurate detection by an ion trap-time of flight LCMS-IT-TOF mass spectrometer allowed to fully characterize the distinctive phospholipid profile and fatty acid composition of cow's and donkey's milk, the latter being analyzed for the first time. Furthermore, molecular species within the different PL classes were partially separated and the relative fatty acid composition characterized for the first time in milk samples. Evaporative light scattering detection was further employed to attain the quantitative evaluation of major PLs classes identified. Further effort is currently put in investigating the linear dynamic range of the hybrid mass spectrometer, to attain quantitative profiling of both PL classes, and molecular species. Besides, the possibility of coupling a second reversed-phase column in a multidimensional heart-cutting or comprehensive LC system is under evaluation, capable to deliver orthogonal selectivity; such a system would be afterward employed to attain detailed fingerprint of the PLs and FAs composition of milk samples from different origins.

Acknowledgments

The authots gratefully acknowledge Shimadzu and Sigma–Aldrich/Supelco Corporations for their continuous support.

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