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Development and validation of a UHPLC-ESI-MS/MS method for the simultaneous quantification of mammal lysophosphatidylcholines and lysophosphatidylethanolamines in serum

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ABSTRACT

Recent investigations based on non-targeted metabolomics have proposed lysophospholipids (Lyso-PLs) as biomarkers of different diseases. In particular, lysophosphatidylcholines (Lyso-PCs) and lysophosphatidylethanolamines (Lyso-PEs) have been associated with serious lipid pathologies. Methods to determine the different molecular species in a biological sample and to quantify even less abundant species are required for the evaluation of the Lyso-PL pattern as a novel comprehensive biomarker of dyslipidemia. This study describes the development and validation of an ultra-high-performance liquid chromatography coupled to tandem mass spectrometry assay for the determination of a large number of Lyso-PCs and Lyso-PEs in biological samples. The method was validated in rat serum using two simple methanol-based extractions with low sample volumes (5–50 μL) that covered the wide concentration range of these metabolites. In total, thirty-one Lyso-PLs were separated and quantified with low method limits of detection and quantification, reaching values of 0.2 and 0.8 nM, respectively. The method was subsequently applied in the identification of Lyso-PL-related changes produced by the chronic intake of a cafeteria diet. The results showed alterations in the majority of Lyso-PCs and Lyso-PEs in rat serum. Furthermore, multivariate analysis indicated that the comprehensive evaluation of serum Lyso-PLs could be an excellent indicator of the nutritional phenotype associated with an increased risk of lipid disorders.

1. Introduction

Glycerol-based lysophospholipids (Lyso-PLs) are structural components of cellular membranes that can also act as signaling molecules in a wide range of physiological and pathological events including inflammation, reproduction, nervous and vascular system development and carcinogenesis [1]. The molecular structure of Lyso-PLs is very simple and consists of a hydrophobic acyl chain attached to the *sn1*- or *sn2*-position of the glycerol backbone and a hydrophilic phosphate head group in the *sn3*-position whose nature determine the diverse classes of Lyso-PLs.

Among circulating lyso forms, those containing a choline group, namely lysophosphatidylcholines (Lyso-PCs), are the most abundant, with serum levels of several hundred micromolar [2,3]. Lyso-PCs have been mainly related to inflammatory diseases and atherosclerosis [4–7]. Conversely, lysophosphatidylethanolamines (Lyso-PEs) exhibit a smaller head group than Lyso-PCs and are present at lower circulating levels, reaching a maximum concentration of several hundred nanomolar [8,9]. Information regarding the biological significance of Lyso-PE in serum is scarce compared to Lyso-PCs, although both classes of Lyso-PLs have been postulated as biomarkers of the progression of different pathologies. Lipid profiling of patients with different development statuses of atherosclerotic plaques showed that certain modifications in the circulating levels of Lyso-PCs and Lyso-PEs were associated with disease severity [10]. Continuing with a holistic view, a recent study noted the importance of both Lyso-PLs as serum indicators of hepatocarcinogenesis progression in humans [11]. *In vivo* investigations demonstrated that dysregulation of serum and hepatic

Abbreviations: Lyso-PL, lysoglycerophospholipid; Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine; ST, standard chow; CAF, cafeteria diet; LSV and HSV, low and high sample volume extractions; PCA, principal component analysis; ROC, receiver operating characteristic

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levels of several Lyso-PCs and Lyso-PEs occurs in rodents fed high-calorie diets [12–14], while differential patterns of secreted Lyso-PLs have been observed in a cellular model of steatosis [9]. Therefore, the examination of circulating levels of Lyso-PLs holds remarkable potential in the diagnosis of lipid disorders and could be used to assess the therapeutic effects of drugs. Since the pathways leading to the synthesis of Lyso-PCs and Lyso-PEs are related [15], it would be of interest to quantify them in a high-throughput analysis with sufficient specificity to differentiate Lyso-PLs containing different side chains.

A variety of methodologies, including coupled techniques based on chromatography, have been developed for the evaluation of Lyso-PCs and Lyso-PEs in biological samples. One of the most common techniques involves separation by thin-layer chromatography (TLC) on silica gel plates [16]. However, once Lyso-PLs are isolated, they must be extracted for further analysis of the fatty acid composition, and thus this methodology is laborious and time-consuming [17,18]. Moreover, the quantification of molecular species present in low abundance using this approach is very difficult. In recent years, chromatographic techniques have become more robust and specific. The use of reverse phase high-performance liquid chromatography (HPLC) coupled to fluorescence [19], ultraviolet (UV) [20], radioactive [21], phosphorus [22], and evaporative light-scattering [23] detection permits enhanced separation of Lyso-PLs containing different acyl chains from complex mixtures with high resolution. However, these quantitative techniques have low sensitivity and poor selectivity for Lyso-PLs. By contrast, electrospray ionization mass spectrometry (ESI–MS) stands out as suitable technique due to the easily ionizable nature of the polar head group of Lyso-PLs [24]. The soft ionization achieved using ESI–MS offers a series of advantages that make it an ideal technique for the analysis of biological samples. ESI–MS detects intact molecules with very high sensitivity, and ESI-tandem MS (ESI–MS/MS) permits the determination of the structural composition of the relevant ions. Thus, HPLC-ESI–MS/MS can lead to real and simultaneous identification and quantification of the many compounds present in a biological sample. As a result, this approach is currently used in studies evaluating the Lyso-PL content of experimental rodent and human samples [2,25,26]. The recent emergence of ultra-HPLC (UHPLC) with sub-2-μm column particles has enabled much greater operating pressures compared with HPLC, thus reducing analysis times without sacrificing efficiency [27,28]. Such properties are essential for clinical research and the evaluation of biomarkers since these studies require the evaluation of a large number of samples. Because Lyso-PEs are the Lyso-PLs more recently postulated as biomarkers in mammals, most of the present quantitative methods which are focused on Lyso-PLs have considered few or none molecular species within the lipid subclass.

Therefore, the main objective of the present study was to develop a rapid, sensitive and reproducible methodology based on UHPLC-ESI–MS/MS for the exhaustive characterization of Lyso-PCs and Lyso-PEs as part of the evaluation of the comprehensive biomarker in serum. First, circulating members belonging to the two lipid subclasses were identified in a pooled serum sample using a UHPLC coupled to a quadrupole time-of-flight mass spectrometer (UHPLC-QTOF). Second, a quantitative method was applied using two simple methanol-based procedures (to cover the entire concentration range of the metabolites of interest) and coupling of the UHPLC to a triple quadrupole mass spectrometer (UHPLC-QqQ). To validate the developed method, several quality parameters were determined by spiking the rat serum with different Lyso-PL standards. The suitability of the quantitative methodology was demonstrated by comparison of the circulating levels of Lyso-PLs found in two groups of rats with different diets. Subsequent multivariate data analysis of all identified Lyso-PLs was also conducted in both animal groups to evaluate the efficiency of the biomarker in serum.

2. Material and methods

2.1. Chemicals and reagents

Methanol (Scharlab, Barcelona, Spain), acetonitrile (Millipore, Darmstadt, Germany), isopropanol, glacial acetic acid (Panreac Applichem, Barcelona, Spain), chloroform and 7.5 M ammonium acetate solution (Sigma-Aldrich, St. Louis, MO, USA) were of the highest grade commercially available. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Lyso-PL standards were all of > 99% purity and were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Lyso-PC standards included 1-tridecanoyl-*sn-glycero*-3-phosphocoline, Lyso-PC (13:0); 1-palmitoyl-*sn-glycero*-3-phosphocoline, Lyso-PC (16:0); 1-stearoyl-*sn-glycero*-3-phosphocoline, Lyso-PC (18:0); and 1-arachidoyl-*sn-glycero*-3-phosphocoline, Lyso-PC (20:0). Lyso-PE calibrators were 1-palmitoyl-*sn-glycero*-3-phosphoethanolamine, Lyso-PE (16:0); 1-stearoyl-*sn-glycero*-3-phosphoethanolamine, Lyso-PE (18:0); and 1-oleoyl-*sn-glycero*-3-phosphoethanolamine, Lyso-PE (18:1). The standards contained a small proportion of the corresponding *sn2*-Lyso-PL isomer. The standards were individually dissolved in methanol/chloroform/water (65:35:8 v/v/v) at 2 mg/mL and stored in dark-glass vials at −20 °C prior to use. On the day of LC–MS/MS analysis, a mixed standard solution with a concentration of 100 mg/L was prepared using methanol and diluted with water/isopropanol/acetonitrile (4:3:3 v/v/v) to the desired concentrations. Lyso-PC (13:0) was separately handled similarly to the standards for use as internal standard (IS). Butylated hydroxytoluene (BHT; Sigma-Aldrich, St. Louis, MO, USA) was used as an antioxidant.

2.2. Animal procedure

Serum samples were obtained from female Sprague-Dawley rats (3-month-old). The animals were maintained under standard conditions of temperature (22 ± 1 °C) and relative humidity (50 ± 10%) with a light/dark period of 12 h. The rats were randomly divided into two groups (n = 5 per group) with free access to food and water. For 8 weeks, the animals were fed *ad libitum* either standard chow (ST) or a highly palatable diet rich in fat and carbohydrates known as the cafeteria diet (CAF) [14]. The CAF was renewed daily and consisted of the followings components (quantity per rat/day): bacon (8–12 g), biscuits with pâté (12–15 g) or cream cheese (10–12 g), sweet roll (8–10 g), carrot (6–8 g), milk with sugar (220 g/L; 50 mL) and ST chow. After overnight fasting, ST- and CAF-fed rats were alternately sacrificed by beheading to avoid interference due to chemical drugs, and total blood was collected in falcon tubes. Serum samples were obtained by allowing blood to clot at room temperature for 30 min. The samples were then centrifuged at 2000 g and 4 °C for 15 min, aliquoted and stored at −80 °C until metabolite extraction and LC–MS/MS quantification. All procedures were performed in accordance with the European Communities Council Directive regarding the protection of experimental animals (86/609/EEC).

2.3. Identification of Lyso-PCs and Lyso-PEs in rat serum

Serum volumes of 10 μL from each animal were pooled (n = 10) for determination of the specific members of Lyso-PCs and Lyso-PEs in rat serum. Metabolites were extracted from serum using a hydroalcoholic solution. Briefly, 900 μL of methanol/water (8:1 v/v) was added to 100 μL of pooled sample, vortexed (30 s) and ultrasonicated (30 s). The mixture was incubated on ice for 10 min and centrifuged (20,000*g*, 10 min, 4 °C). The supernatants were dried under nitrogen flow and re-dissolved in 200 μL of water/methanol (1:1 v/v) prior to injection.

The exhaustive characterization of the two circulating subclasses of lipids was performed using a UHPLC 1290 coupled to a Q-TOF 6550 Series mass spectrometer equipped with a Dual ESI source using Agilent Jet Stream Technology (AJS ESI) (Agilent Technologies, Palo Alto, CA,

Table 1
Target lysophospholipids (Lyso-PLs) and abbreviations, molecular formula and weight, retention time, transitions and optimal collision energies used for UHPLC-QqQ analysis.

Compound	Lipid abbreviation	Molecular formula	Molecular weight	Retention time (min)	Parent ion (*m/z*)	Daughter ions (*m/z*)	Collision energies (V)
Lysophosphatidylcholines							
1-tridecanoyl-*sn-glycero*-3-phosphocholine [IS]	Lyso-PC (13:0)	$C_{21}H_{44}NO_7P$	453.55	8.9	454.3	184.0*, 104.1, 86.0	24, 28, 40
1-myristoyl-*sn-glycero*-3-phosphocholine [A]	Lyso-PC (14:0)	$C_{22}H_{46}NO_7P$	467.58	10.3	468.3	184.0*, 104.1, 86.0	24, 28, 40
1-pentadecanoyl-*sn-glycero*-3-phosphocholine [A]	Lyso-PC (15:0)	$C_{23}H_{48}NO_7P$	481.60	11.6	482.3	184.0*, 104.1, 86.0	24, 28, 40
1-palmitoyl-*sn-glycero*-3-phosphocholine [A]	Lyso-PC (16:0)	$C_{24}H_{50}NO_7P$	495.63	13.0	496.3	184.0*, 104.1, 86.0	24, 28, 40
1-palmitoleoyl-*sn-glycero*-3-phosphocholine [A]	Lyso-PC (16:1)	$C_{24}H_{48}NO_7P$	493.61	11.1	494.3	184.0*, 104.1, 86.0	24, 28, 40
1-heptadecanoyl-*sn-glycero*-3-phosphocholine [B]	Lyso-PC (17:0)	$C_{25}H_{52}NO_7P$	509.66	14.3	510.3	184.0*, 104.1, 86.0	32, 28, 40
1-heptadecenoyl-*sn-glycero*-3-phosphocholine [B]	Lyso-PC (17:1)	$C_{25}H_{50}NO_7P$	507.64	12.4	508.3	184.0*, 104.1, 86.0	32, 28, 40
1-stearoyl-*sn-glycero*-3-phosphocholine [B]	Lyso-PC (18:0)	$C_{26}H_{54}NO_7P$	523.68	15.5	524.3	184.0*, 104.1, 86.0	32, 28, 40
1-oleoyl-*sn-glycero*-3-phosphocholine [B]	Lyso-PC (18:1)	$C_{26}H_{52}NO_7P$	521.67	13.7	522.3	184.0*, 104.1, 86.0	32, 28, 40
1-linoleoyl-*sn-glycero*-3-phosphocholine [B]	Lyso-PC (18:2)	$C_{26}H_{50}NO_7P$	519.65	11.9	520.3	184.0*, 104.1, 86.0	32, 28, 40
1-linolenoyl-*sn-glycero*-3-phosphocholine [B]	Lyso-PC (18:3)	$C_{26}H_{48}NO_7P$	517.64	10.7	518.3	184.0*, 104.1, 86.0	32, 28, 40
1-arachidoyl-*sn-glycero*-3-phosphocholine [C]	Lyso-PC (20:0)	$C_{28}H_{58}NO_7P$	551.74	17.8	552.3	184.0*, 104.1, 86.0	28, 28, 40
1-eicosenoyl-*sn-glycero*-3-phosphocholine [C]	Lyso-PC (20:1)	$C_{28}H_{56}NO_7P$	549.72	15.9	550.3	184.0*, 104.1, 86.0	28, 28, 40
1-eicosadienoyl-*sn-glycero*-3-phosphocholine [C]	Lyso-PC (20:2)	$C_{28}H_{54}NO_7P$	547.70	14.2	548.3	184.0*, 104.1, 86.0	28, 28, 40
1-eicosatrienoyl-*sn-glycero*-3-phosphocholine [C]	Lyso-PC (20:3)	$C_{28}H_{52}NO_7P$	545.69	12.9	546.3	184.0*, 104.1, 86.0	28, 28, 40
1-arachidonoyl-*sn-glycero*-3-phosphocholine [C]	Lyso-PC (20:4)	$C_{28}H_{50}NO_7P$	543.67	11.9	544.3	184.0*, 104.1, 86.0	28, 28, 40
1-eicosapentaenoyl-*sn-glycero*-3-phosphocholine [C]	Lyso-PC (20:5)	$C_{28}H_{48}NO_7P$	541.66	10.4	542.3	184.0*, 104.1, 86.0	28, 28, 40
1-docosapentaenoyl-*sn-glycero*-3-phosphocholine [C]	Lyso-PC (22:5)	$C_{30}H_{52}NO_7P$	569.71	13.1	570.3	184.0*, 104.1, 86.0	28, 28, 40
1-docosahexaenoyl-*sn-glycero*-3-phosphocholine [C]	Lyso-PC (22:6)	$C_{30}H_{50}NO_7P$	567.69	11.7	568.3	184.0*, 104.1, 86.0	28, 28, 40
Lysophosphatidylethanolamines							
1-palmitoyl-sn-*glycero*-3-phosphoethanolamine [D]	Lyso-PE (16:0)	$C_{21}H_{44}NO_7P$	453.55	13.3	454.3	313.3*, 436.3, 62.0	16, 12, 12
1-palmitoleoyl-sn-*glycero*-3-phosphoethanolamine [D]	Lyso-PE (16:1)	$C_{21}H_{42}NO_7P$	451.53	11.3	452.3	311.3*, 434.3, 62.0	16, 12, 12
1-stearoyl-sn-*glycero*-3-phosphoethanolamine [E]	Lyso-PE (18:0)	$C_{23}H_{48}NO_7P$	481.60	15.8	482.3	341.3*, 464.3, 62.0	16, 12, 12
1-oleoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (18:1)	$C_{23}H_{46}NO_7P$	479.59	14.0	480.3	339.3*, 462.3, 62.0	16, 12, 12
1-linoleoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (18:2)	$C_{23}H_{44}NO_7P$	477.57	12.2	478.3	337.3*, 460.3, 62.0	16, 12, 12
1-linolenoyl-*sn-glycero*-3-phosphoethanolamine [F]	Lyso-PE (18:3)	$C_{23}H_{42}NO_7P$	475.56	10.6	476.3	335.3*, 458.3, 62.0	16, 12, 12
1-eicosenoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (20:1)	$C_{25}H_{50}NO_7P$	507.64	16.1	508.3	367.3*, 490.3, 62.0	16, 12, 12
1-eicosadienoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (20:2)	$C_{25}H_{48}NO_7P$	505.62	14.5	506.3	365.3*, 488.3, 62.0	16, 12, 12
1-eicosatrienoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (20:3)	$C_{25}H_{46}NO_7P$	503.61	13.1	504.3	363.3*, 486.3, 62.0	16, 12, 12
1-arachidonoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (20:4)	$C_{25}H_{44}NO_7P$	501.59	12.2	502.3	361.3*, 484.3, 62.0	16, 12, 12
1-docosatetraenoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (22:4)	$C_{27}H_{48}NO_7P$	529.65	14.1	530.3	389.3*, 512.3, 62.0	16, 12, 12
1-docosapentaenoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (22:5)	$C_{27}H_{46}NO_7P$	527.63	13.4	528.3	387.3*, 510.3, 62.0	16, 12, 12
1-docosahexaenoyl-sn-*glycero*-3-phosphoethanolamine [F]	Lyso-PE (22:6)	$C_{27}H_{44}NO_7P$	525.61	11.9	526.3	385.3*, 508.3, 62.0	16, 12, 12

[IS] Internal Standard.
[A,B,C,D,E,F] Serum concentration was calculated with Lyso-PC (16:0), Lyso-PC (18:0), Lyso-PC (20:0), Lyso-PE (16:0), Lyso-PE (18:0), Lyso-PE (18:1) calibration curve, respectively.
* Transitions for quantification.

USA). The chromatographic section comprised a degasser, a binary pump, a thermostatted autosampler (held at 4 °C) and a thermostatted column compartment. The mobile phase was 0.2% (v/v) acetic acid in water (solvent A) and 0.2% (v/v) acetic acid in methanol (solvent B). The flow rate was 0.6 mL/min. An injection volume of 2 μL was loaded onto a Zorbax SB-Aq (1.8 μm particle size, 2.1 mm internal diameter × 50 mm length) analytical column held at 60 °C and equipped with a Zorbax SB-C8 Rapid Resolution Cartridge (3.5 μm, 2.1 × 30 mm) guard column, also from Agilent Technologies. Chromatographic separation was performed using a linear gradient of 2–98% B over 13 min, followed by a 3-min solvent B hold and 1 min to return to initial conditions. Thereafter, a post-time of 5 min was applied. To identify the greatest possible number of Lyso-PLs, the mass spectrometer was operated in both positive (+ESI) and negative (-ESI) ionization modes. The ESI source settings were as follows: nebulizer pressure 45 psi; desolvation gas flow rate 11 L/min at 325 °C; source temperature and gas flow rate, 200 °C and 14 L/min, respectively; capillary voltage 4 kV; and fragmentor 140 V. Data were acquired using MassHunter Data Acquisition (Agilent Technologies, version 6.0) over the 100–1200 *m/z* range at a rate of 1.5 spectra/s. Dynamic mass axis calibration was accomplished through continuous infusion of a reference mass solution: 121.0509 and 922.0098 *m/z* for +ESI and 119.0363 and 980.0164 *m/z* for −ESI. Molecules were tentatively identified by comparison of the exact mass with published data. The confirmation of the metabolites as Lyso-PC and Lyso-PE molecular species was achieved with the spectrometer operating in MS and MS/MS modes and by applying collision energies of 10, 20 and 40 V. In addition, six commercially available standards were analyzed to verify the identity of the lipids. All identified serum Lyso-PLs were subsequently quantified by UHPLC-QqQ.

2.4. Sample preparation for exhaustive lyso-PL quantification

After determination of the target Lyso-PLs, the sample treatment process was optimized. Lyso-PLs are found in a broad range of concentrations in the circulation. Consequently, it was necessary to use two slightly different procedures (named *Low Sample Volume* and *High Sample Volume*) to treat the serum samples and quantify all compounds.

The *Low Sample Volume* (LSV) procedure was developed to quantify the most abundant circulating Lyso-PLs. Briefly, 5 μL of serum and 4 μL of IS (20 μM) were added to 71 μL of cold methanol containing BHT (1 μM) as an antioxidant. The mixture was homogenized by vortexing (1 min) and ultrasonication (30 s). The samples were then incubated on ice for 10 min and centrifuged at 20,000*g* and 4 °C for 10 min. The supernatants were collected in dark-glass vials and analyzed immediately.

Additionally, *High Sample Volume* (HSV) extraction was developed for the quantification of the remaining Lyso-PLs. This procedure yielded serum extracts with higher concentrations of the desired analytes than the LSV method. In summary, 50 μL of serum and 2.5 μL of IS (20 μM) were added to 197.5 μL of cold methanol containing BHT (1 μM), vortexed (1 min) and ultrasonicated (30 s). Then, the samples were placed on ice for 10 min and centrifuged (22,000*g*, 10 min, 4 °C). The supernatants were dried under nitrogen flow and re-dissolved in 50 μL of water/isopropanol/acetonitrile (4:3:3 v/v/v) prior to injection.

2.5. Lyso-PL quantification: UHPLC- + ESI–MS/MS

Quantitative analysis of the target compounds was performed by coupling the UHPLC 1290 to a QqQ 6490 Series mass spectrometer operating in AJS +ESI (Agilent Technologies, Palo Alto, CA, USA). Solvent A consisted of water/isopropanol/acetonitrile/500 mM ammonium acetate (89:5:5:1 v/v/v/v), whereas solvent B was isopropanol/acetonitrile/500 mM ammonium acetate (50:49:1 v/v/v). A sample volume of 2 μL was applied to a reversed-phase column (Acquity UPLC BEH C8, 1.7 μm, 2.1 × 150 mm) (Waters Corporation, Milford, MA, USA) held at 50 °C with a 0.3 mL/min flow of 30% B. The analytes were separated using linear gradient elution to 75% B over 20 min, followed by a linear increase in solvent B to 100% over 1 min and isocratic 100% B for 4 min. The chromatographic system was returned to the initial conditions in 2 min, followed by a 2-min equilibration prior to the subsequent injection. The total run time was 29 min. The ionization source parameters were optimized using MassHunter Optimizer (Agilent Technologies, version 6.0) as follows: nebulizer gas (nitrogen) with a pressure of 25 psi; a gas flow of 12 L/min at 240 °C; a sheath gas flow of 12 L/min at 350 °C; a capillary voltage of 4.5 kV; and a nozzle voltage of 500 V. To obtain the highest abundances of the selected product ions, the fragmentor and cell accelerator voltages were also optimized and set to 380 and 5 V, respectively. The other operating parameters for the targeted quantification of Lyso-PLs are described in Table 1, including the optimal collision energies for each transition, which were determined by direct injection of standard solutions with 0.5 mL/min flow of solvent A/solvent B (1:1 v/v). $[M + H]^+$ ions were used as parent ions. For each analyte, the most abundant transition was selected for the subsequent quantification; the other two transitions were used for qualitative purposes. To further increase the sensitivity of the method, dynamic Multiple Reaction Monitoring (MRM) was selected as acquisition mode. The MRM transition list was dynamically created using a window of 1 min around the expected retention times (RTs). The cycle time was fixed to 750 ms, and the dwell time ranged from 27.50 to 373.92 ms.

Standard calibration curves were established for the quantitative analysis of endogenous Lyso-PLs. Increasing concentrations of the standards were diluted with constant final volumes of water/isopropanol/acetonitrile (4:3:3 v/v/v) in the presence of the IS. The standard concentrations ranged from 0 to 5 mg/L, whereas the IS was added at a final concentration of 450 or 325 μg/L depending on the later procedure of extraction. Then, the mixtures were extracted and analyzed using the same procedure as for the serum samples. Peak detection and integration were performed using MassHunter Quantitative Analysis (Agilent Technologies, version 6.0). Calibration curves for each standard were generated by plotting the peak abundance ratios (analyte/IS) versus the concentration ratios (analyte/IS) and fitting to a linear regression. As described in Table 1, the concentration of each circulating Lyso-PLs was calculated by using the closest related calibration curve, according to a previously described procedure [24]. For the two described extractions of the sample, the method detection and quantification limits (MDL and MQL, respectively) were calculated based on the respective instrumental limits of detection (3 times the signal/noise ratio) and quantification (10 times the signal/noise ratio).

2.6. Quality parameters

Other quality parameters were determined to validate and evaluate the suitability of the developed quantitative method. The percentages of recovery, precision, repeatability, accuracy and matrix effect were determined by spiking pooled serum samples from ST- or CAF-fed animals with standard solutions. Moreover, two different concentration levels of Lyso-PLs (0.1 and 1 mg/L) were studied. The recovery percentages were calculated by comparing the resulting abundances of a standard added before or after the extraction of the pooled samples. The method variation was analyzed in triplicate and considered as the relative standard deviation (% RSD) of the concentration in spiked serum samples that were randomly distributed intraday (precision) and on three different days (repeatability). The method accuracy was assessed by comparing the mean concentration of three spiked, pooled, pre-treated samples with the value of the commercial standard at this concentration. Finally, the matrix effects were determined as the ratio of the difference between the abundances obtained by spiking pooled samples after extraction and the blank pooled sample versus the abundance of the diluted standard at the same concentration.

2.7. Statistical evaluation

Statistical analysis was performed with Statistical Package for Social Sciences (IBM SPSS Statistics, version 19.0). The serum concentration of Lyso-PLs is expressed as the mean ± standard error (SEM). The differences among the two animal groups were assessed using Student's *t*-test. A two-tailed value of $p < 0.05$ was considered statistically significant. Principal component analysis (PCA) and multivariate biomarker validation using receiver operating characteristic (ROC) curves were performed using the online software MetaboAnalyst 3.0 [29].

3. Results

3.1. Development of an optimized methodology for the evaluation of serum Lyso-PLs

After characterization of the lysophospholipidome in rat serum, eighteen Lyso-PCs and thirteen Lyso-PEs were tentative identified. The chromatographic peaks eluted within a range of 10.8–12.7 min (data not shown). The Lyso-PCs were identified in the +ESI analysis with *m/z* values between 468.3048 and 570.3512, whereas the Lyso-PEs were detected in both ionization modes. During the process of Lyso-PE identification, we observed that one of the major product ions in the +ESI fragmentation corresponded to the loss of the phosphorylethanolamine group, with a mass of 141 ($C_2H_8NO_4P$). In −ESI, although the polar head of Lyso-PEs is easily ionizable, with an *m/z* 140, the acyl chain is the predominant anionic fragment [8,30]. Thus, different ions from the fragmentation of Lyso-PEs can be observed in each ionization mode. As an example, Fig. 1 shows the MS/MS spectra of a Lyso-PE species at a given collision energy in both ionization modes. Due to the ability of Lyso-PEs and their characteristic fragments to be ionized in both modes, we decided to quantify all identified Lyso-PLs in a unique +ESI–MS/MS analysis performed by UHPLC-QqQ.

The development of the quantitative method started with the determination of the most abundant Lyso-PL transitions and their collision energies (Table 1). For all analytes, $[M+H]^+$ ions were selected as precursor ions. Consistent with the observations of Liebisch et al., the fragmentation of Lyso-PCs generated common product ions with the following *m/z* values: 184.0 (the phosphocholine ion); 104.1 (the choline ion); and 86.0 (the choline subsequently fragmented) [24]. By contrast, the optimal fragmentation patterns of Lyso-PEs required lower collision energies and differed from each other since they tend to lose the phosphorylethanolamine group and a molecule of water [30]. Only *m/z* 62.0 (the ethanolamine ion) was preserved in all Lyso-PEs because it is the only ion that does not come from the radical chain, which has a variable mass. As Table 1 indicates, the abundant transitions $[M+H]^+ > 184$ and $[M+H]^+ > [M-141+H]^+$ were used for the quantification of Lyso-PCs and Lyso-PEs, respectively. By monitoring these quantitative transitions in a mixture of four standards, the solvent gradient was optimized using a 0.3 mL/min flow held at 50 °C (Fig. 2). Starting with the chromatographic conditions described in Donovan et al. [31] (Fig. 2A), the proportion of solvents (dashed line) was modified to achieve baseline separation between the different standards and distinction of less abundant isomeric forms (Fig. 2C). In that final chromatogram, all of the peaks displayed suitable efficiency and symmetry. Lyso-PC (16:0) eluted first, followed by Lyso-PE (18:1) and Lyso-PCs (18:0) and (20:0) in that order. Hence, the elution of the Lyso-PLs depended on the length and number of double bonds of the acyl chain rather than the nature of the polar head group. In addition, the *sn1*-Lyso-PLs were retained by the column longer than the *sn2*-isomers, as shown previously [25]. For further analysis of multiple samples, the 100% B hold was extended to 4 min to ensure clearance of the chromatographic column. In addition, the MRM scan type was switched to dynamic mode. Thus, the transitions were monitored only around the expected RT allowing to reach a suitable sensitivity for the quantification of the multiple molecular species of Lyso-PLs identified in serum. The RT selected for the acquisition of each analyte was the RT

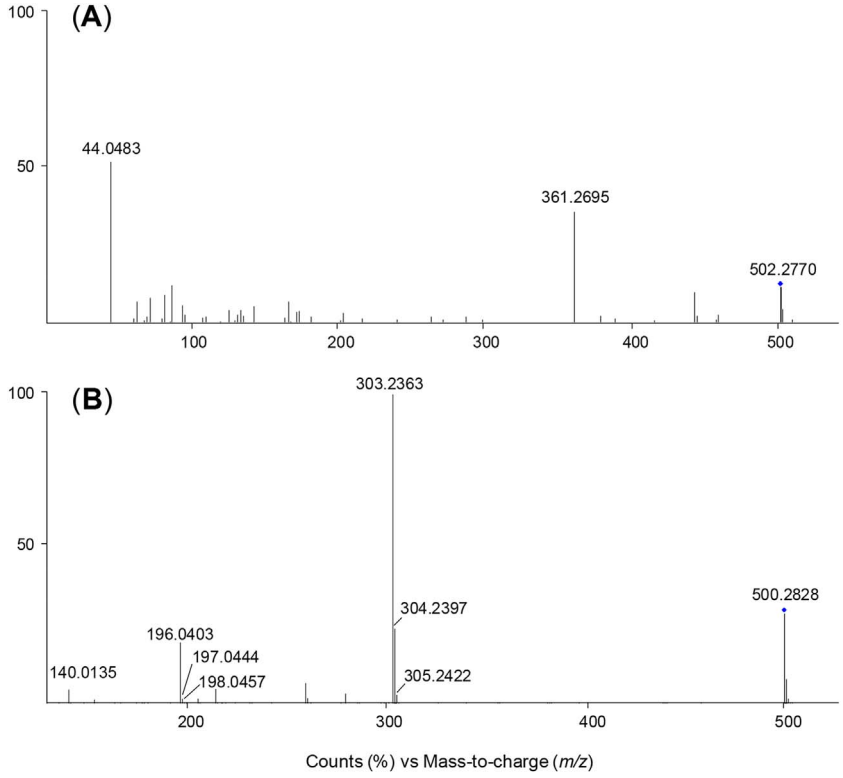

Fig. 1. ESI–MS/MS spectra acquired by UHPLC-QTOF corresponding to Lyso-PE (20:4) at a collision energy of 20 V. (A) In positive ionization mode, the major peaks identified were the parent ion (*m/z* 502), the ethylamine ion (*m/z* 44) and the ion corresponding to loss of the phosphorylethanolamine head group (*m/z* 361). (B) In negative mode, the parent ion (*m/z* 500), the arachidonic acid side chain (*m/z* 303), the released phosphorylethanolamine group (*m/z* 140) and the propylene glycol phosphorylethanolamine ion (*m/z* 196) were identified.

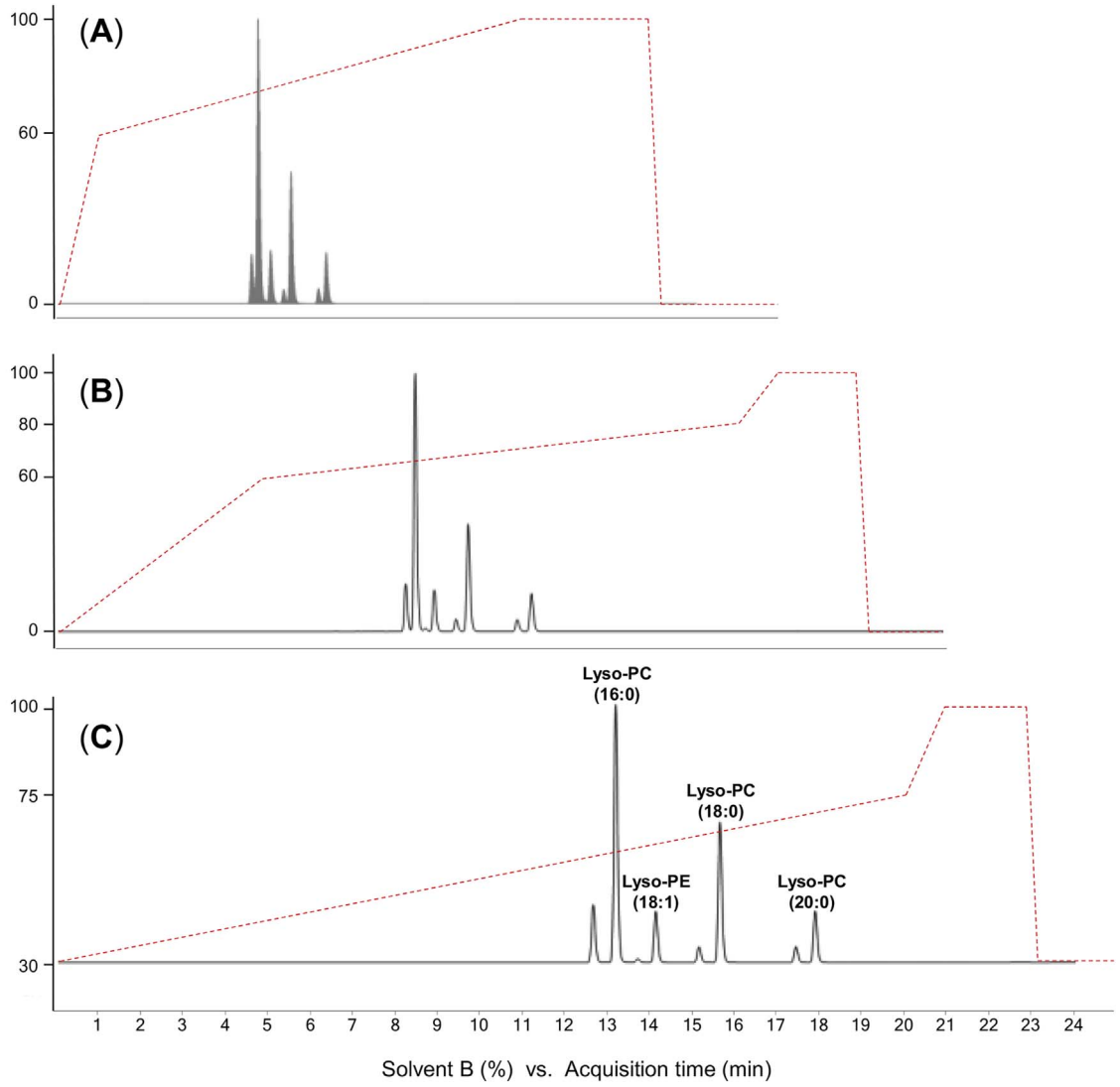

Fig. 2. Optimization of the solvent gradient. Total ion chromatograms from UHPLC-QqQ analysis of Lyso-PL standards at different run times and proportions of the solvents. (A) Chromatographic conditions obtained from Donovan et al. (B) Intermediate conditions. (C) Optimized conditions.

of the second peak ± 0.5 min, that is, *sn1*-Lyso-PL. Representative chromatograms from the Lyso-PL analyses of both a standard solution and a serum sample are shown in Figs. 3 and 4, respectively.

As shown in Fig. 3, the sensitivity levels were greater for the analysis of Lyso-PCs than for Lyso-PEs. The signals from Lyso-PEs were approximately 25% of those from the Lyso-PCs. This difference is related to the highest values of slope obtained in the calibration curves of Lyso-PCs and is in agreement with the limits of the UHPLC-+ ESI–MS/MS method (Table 2), which are generally greater for Lyso-PE quantification than for Lyso-PC. The slopes of the Lyso-PC and Lyso-PE standard curves were very similar, including that of the unsaturated Lyso-PE (18:1), and displayed a variation coefficient of 6% and 17%, respectively. The calibration curves for the Lyso-PLs were linear over the concentration ranges analyzed (Table 2), and all equations displayed excellent data fit ($R^2 > 0.999$). The samples could not be prepared in the biological matrix since the constitutive levels of the serum compounds were too high. Consequently, the parameters describing method performance (Table 3) could not be evaluated for the most concentrated circulating forms, Lyso-PCs (16:0) and (18:0) (Fig. 4A).

After testing various proportions of sample and methanol (data not shown), 16- and 1.25-fold dilutions with respect to the initial serum volume were selected to cover the whole range of circulating levels of Lyso-PLs. Table 4 displays the specific identified Lyso-PLs analyzed from each different extraction. Lyso-PCs were mainly quantified in LSV extracts, and Lyso-PEs were mainly quantified after the HSV procedure. The recoveries, intra and interday variations, accuracies and matrix effects of the quantitative method are shown in Table 3. For the LSV extraction, all analyzed parameters gave satisfactory results, and no considerable differences were noted between the pooled sera of ST- and CAF-fed rats. The recoveries varied between 98 and 117%, and the% RSD ranged from 0.8 to 9.4 and 2.7 to 6.4 for intraday and interday studies, respectively. The optimum recoveries (98–105%) and the largest variations were observed for the highest spiked concentration of 1 mg/L, but the method accuracy remained nearly 100% at this concentration, and the matrix effect was minimal for both levels of concentration. The performance of the Lyso-PE (18:1) standard could be determined for the HSV treatment only because this form represents the minor circulating Lyso-PL among the used standards. Consequently, its quality results should be extrapolated to the analysis of the other, even less abundant Lyso-PEs in quantification. Whereas the recovery and variations of Lyso-PE (18:1) were similar in both extractions, the percentages of accuracy and, in particular, the matrix effect were worse for HSV than for the LSV procedure. Therefore, the HSV extraction must be used only to assist the complete quantification of Lyso-PLs by enabling the measurement of those with the lowest levels, mainly

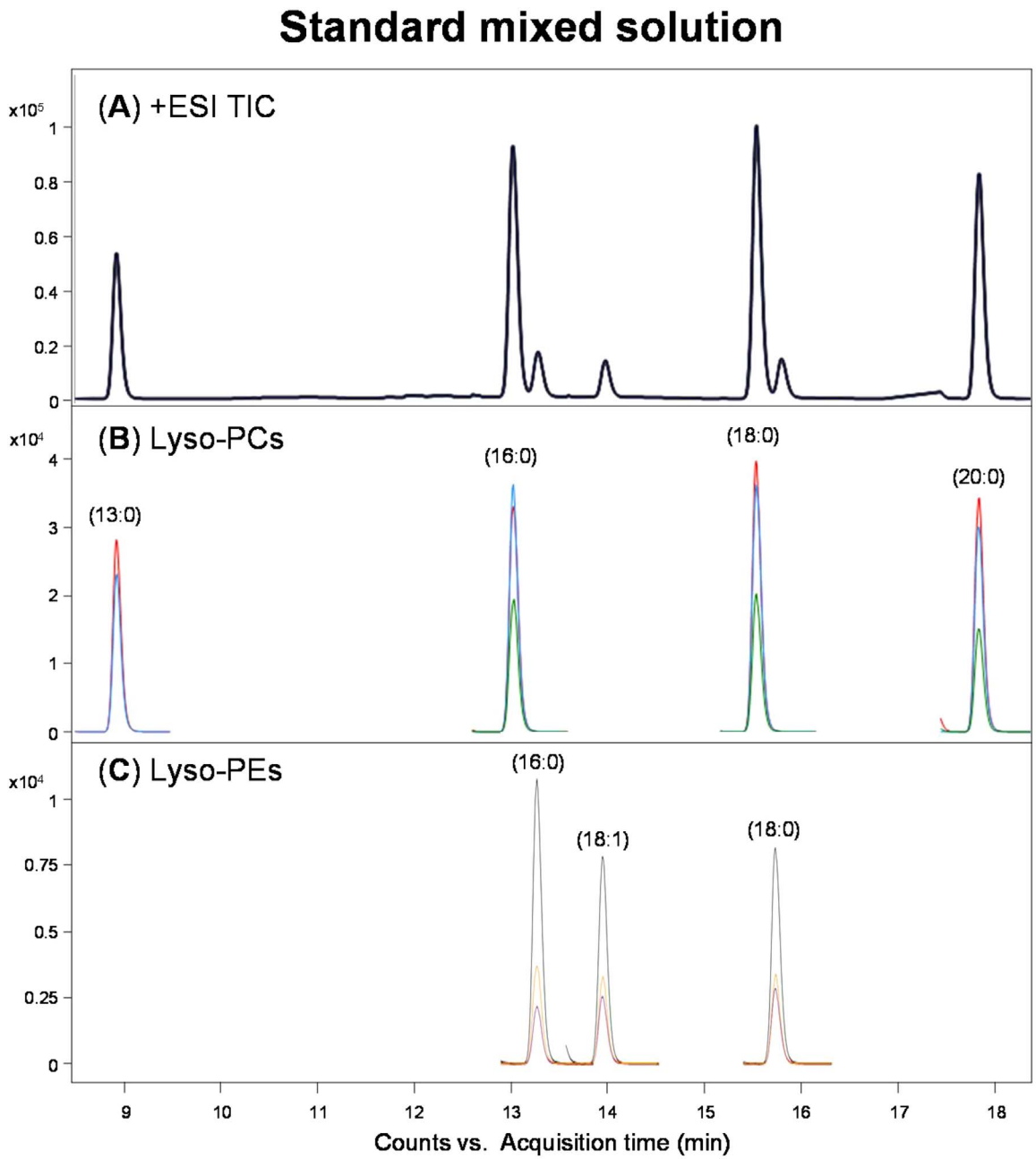

Fig. 3. Transition abundances at an intermediate point of the standard curve of Lyso-PLs. (A) Total ion chromatogram and (B, C) extracted ion chromatograms of Lyso-PCs and Lyso-PEs, respectively. The standards were diluted with water/isopropanol/acetonitrile (1 mg/L) in the presence of the internal standard (0.45 mg/L) and processed similarly to the samples. The acquisition mode was dynamic MRM with a RT window of 1 min.

Lyso-PEs. Thus, the MDL and MQL for Lyso-PEs were approximately 0.3 and 1.1 nmol/L for the HSV procedure and 1.3 and 4.3 for LSV, respectively (Table 2).

3.2. Circulating levels of Lyso-PLs found in rats fed different diets

After validation, the analytical method was used for the quantification of Lyso-PL levels in rat serum. Table 1 and Fig. 4 show the chromatographic and spectrometric results of the quantitative method in a rat serum sample. Three transitions were monitored for each analyte. Endogenous Lyso-PLs eluted in increasing order of carbon atoms, and Lyso-PLs of identical length eluted by decreasing number of double bonds in the side chain. Because of the association between endogenous Lyso-PLs and the intake of diets rich in fats [12,13], circulating levels were compared between animals fed two different diets (ST or CAF) (Table 4). Following the order of abundance, the rat serum was particularly rich in Lyso-PCs containing the acyl chains (18:0) > (16:0) ≫ (20:4) > (18:2) > (18:1). In general, the circulating amounts of Lyso-PEs were lower, but the distribution of their abundances was similar to that of Lyso-PCs, and the saturated Lyso-PEs with 18 and 16 carbon atoms were by far the most abundant species. The results also showed numerous significant changes in Lyso-PCs and Lyso-PEs among both dietary groups. It is remarkable that the two subclasses of lipids exhibited similar trends depending on the structure of the hydrocarbon chain. This trend could be clearly observed in the significantly increased (16:1), (18:1), (20:3) and (20:4) and decreased (18:2) and (20:2) Lyso-PL levels in CAF- compared to ST-fed rats. These results indicated that the developed UHPLC- + ESI–MS/MS method can be successfully applied for the quantification of endogenous changes in Lyso-PL levels in response to chronic intake of CAF.

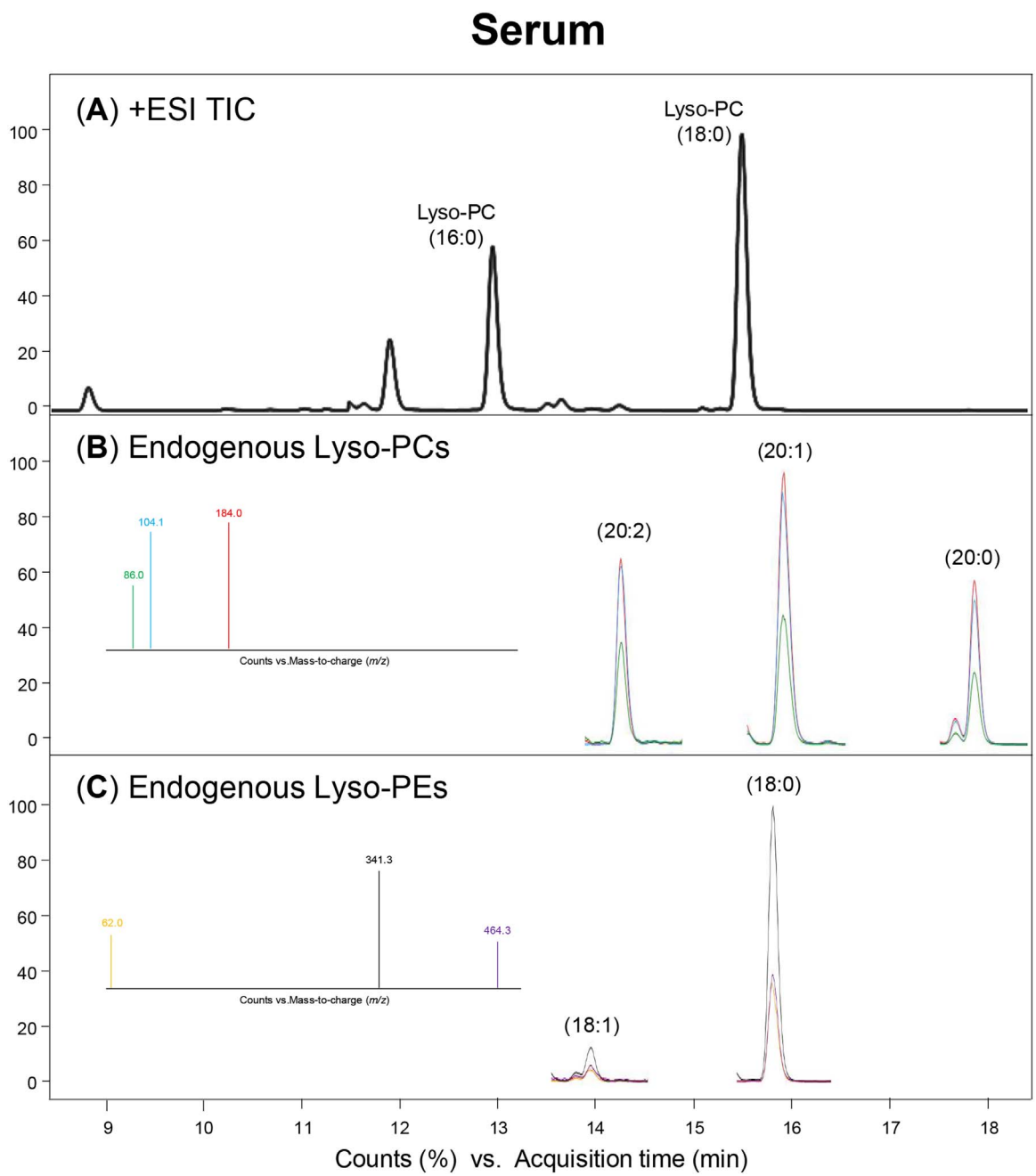

Fig. 4. Transition abundances of circulating Lyso-PLs. (A) Total ion chromatogram from Lyso-PL analysis of a serum sample showing the most concentrated forms. (B, C) Extracted ion chromatograms of some endogenous Lyso-PCs and Lyso-PEs, respectively. The daughter ions from the joint fragmentation of Lyso-PCs and the spectra of a specific Lyso-PE containing the (18:0) acyl chain are also shown. The acquisition mode was dynamic MRM with a RT window of 1 min.

3.3. Lyso-PL examination as a suitable biomarker of nutritional phenotype

To simplify the large amount of data, multivariate analysis based on PCA and ROC curve evaluation including the thirty-one quantified Lyso-PLs was performed (Fig. 5). The unsupervised PCA analysis revealed clear clustering between the two dietary groups that explained 70% of the variance when the scores of the first two principal components were employed (Fig. 5A). Furthermore, the predictive capacity of the method was assessed using ROC curve analysis (Fig. 5B). The results indicated excellent accuracy (100%) of the model, with an area under the curve (AUC) of 1. An expanded version of the multivariate analysis can be found in the Supplementary material. Our results demonstrated that the comprehensive examination of circulating Lyso-PL levels represents an excellent biomarker of the nutritional phenotype of CAF-fed rats, thus highlighting the need for a method specifically developed to quantify Lyso-PLs.

4. Discussion

Direct flow injection coupled to mass spectrometry has been applied by many researchers for the quantification of lyso compounds [17,24,32]. Most of them use TLC before sample injection to separate the lipids into classes [17,24], but this methodology has certain disadvantages. The use of chromatographic columns allows more efficient separation of lipid compounds and, consequently, high peak resolution [33–35]. The UHPLC- + ESI–MS/MS method described here assisted the separation and quantification of eighteen Lyso-PCs and thirteen Lyso-PEs of endogenous origin, minimizing ion suppression between different molecular species and matrix components. This is particularly important in the analysis of circulating Lyso-PLs, which are

Table 2
Calibration curves, regression coefficients (R^2), working linear ranges and method limits of detection and quantification (MDL and MQL) for the analysis of Lyso-PCs and Lyso-PEs.

Compound[a]		Calibration curve	R^2	Working linear range (nM)	MDL[b] (nM)	MQL[b] (nM)
Lyso-PC	**(16:0)**	y = 0.7295x − 0.0135	0.999	0.1–10,088	0.48	1.60
	(18:0)	y = 0.7595x + 0.0090	1.000	0.1–9548	0.30	1.01
	(20:0)	y = 0.6729x + 0.0064	0.999	0.3–9062	1.50	5.00
Lyso-PE	**(16:0)**	y = 0.2005x + 0.0007	1.000	0.2–6615	1.06/0.24	3.53/0.81
	(18:0)	y = 0.1524x + 0.0007	1.000	0.2–6229	1.11/0.35	3.69/1.15
	(18:1)	y = 0.1495x − 0.0001	0.999	0.3–4170	1.67/0.38	5.56/1.26

The bold is used to highlight the axes of the tables.

[a] Lipid nomenclature: Monoacylglycerophospholipids such as Lyso-PCs and Lyso-PEs are composed of acyl chains of different lengths and unsaturation degrees. The first value shown in parentheses represents the number of carbon atoms of the acyl chain, whereas the second value denotes the number of double bonds.

[b] Method detection and quantification limits are expressed in nmol/L of fresh sample. The limits were calculated for the quantification of Lyso-PCs and Lyso-PEs from 5 μL of serum (LSV procedure). Low abundant circulating Lyso-PEs were quantified starting from 50 μL of fresh sample (HSV procedure). Lyso-PE limits from HSV extracts are shown after those from LSV (LSV/HSV).

present in a wide concentration range. The optimized parameters used in our UHPLC system even allowed baseline separation of isomeric forms. Although this work focused on the determination of 1-acyl-Lyso-PLs (*sn1*-Lyso-PLs), it would be possible to apply the method to quantitative studies of both isomers because the *sn2*-isomer always elutes first. In agreement with the observations of Lee et al., the Lyso-PLs eluted according to the length of the acyl chain and the degree of saturation rather than the nature of their polar group [30].

In our study, Lyso-PLs of the same family had similar slopes, particularly those containing acyl chains of equal length, regardless of the number of double bonds (Table 2). This is in accordance with the results obtained by Liebisch et al. when comparing Lyso-PCs of different chain lengths [24]. Based on these findings and the lack of commercially available standards for some endogenous Lyso-PLs, circulating unsaturated Lyso-PLs were quantified using the closest saturated standard curve. Other researchers have conducted detailed evaluations of Lyso-PLs in rodent serum [8,25], but the present method permits the quantification of a greater number of Lyso-PCs and Lyso-PEs. The disadvantages of those methods mainly pertained to lyso forms containing ethanolamine as polar group, which are often present in lower circulating amounts than Lyso-PCs, with serum levels of several hundred micromolar [2,25]. According to Bollinger et al., the most abundant serum Lyso-PCs are those with saturated acyl chains of 18 and 16 carbons, as well as those with (18:2), (18:1) and (22:6) side chains, albeit in much lower quantities [8]. However, we also observed that Lyso-PC (20:4) was further concentrated in serum than (18:2), probably due to differences between rodent species (Table 4). Another metabolomics study conducted in rats showed that the serum level of Lyso-PC (20:4) is similar to that of Lyso-PC (18:1) and decreased when the diet was supplemented with a natural product with hypocholesterolemic effects [2]. This finding suggests that Lyso-PC (20:4) could be particularly influenced by the diet composition. In our study, Lyso-PC (20:4) was one of the quantified Lyso-PCs whose serum levels increased significantly in rats fed the pro-dyslipidemic CAF diet. In fact, CAF induced dysregulations in the levels of the majority of Lyso-PCs and Lyso-PEs in the present study. These results are comparable to those obtained in a previous work in which we identified several metabolites, including Lyso-PLs, whose circulating levels differed between rats fed ST and CAF [14]. These targeted and non-targeted studies are consistent since the serum amounts of Lyso-PCs containing (16:1) and (17:1) acyl chains increased and those with (20:1) and (20:2) acyl chains decreased in CAF-fed animals. The present methodology enables the identification of many more significant changes related to Lyso-PLs compared with non-targeted techniques.

The solvent chosen for treating the serum samples, methanol, was effective in the extraction of the majority of polar lipids present in biological samples and was compatible for ESI–MS/MS analysis [33,34]. Other procedures commonly used for lipid extraction include modifications of the Folch [36] or Bligh and Dyer [37] methods. These traditional techniques involve the formation of two organic phases and promote the increased enrichment of the chloroform phase with hydrophobic lipids in contrast to Lyso-PLs. Alternative methodologies have recently emerged, such as the methyl-*tert*-butyl ether (MTBE) [17] or butanol-methanol (BUME) [38] procedures, but these chloroform-free lipid extractions are more time-consuming than the methanol treatment. The recoveries of nearly 100% indicate that our procedure is adequate for the analysis of choline and ethanolamine containing Lyso-PLs without requiring a tedious extraction process and using low

Table 3
Quality parameter percentages of the quantitative method used for the determination of Lyso-PCs and Lyso-PEs.

Compound	Spiked concentration (mg/L)	Recovery		Precision[a]		Repeatability[b]	Accuracy		Matrix effect	
		ST	CAF	ST	CAF	CAF	ST	CAF	ST	CAF
Lyso-PC (20:0)	**0.1**	114	109	0.8	3.1	3.0	94	86	89	88
	1	98	101	9.1	6.2	6.4	105	99	81	85
Lyso-PE (16:0)	**0.1**	113	115	2.7	4.5	3.7	111	115	84	75
	1	101	103	7.5	5.8	4.5	113	99	77	82
Lyso-PE (18:0)	**0.1**	117	117	2.9	2.6	2.8	118	116	67	61
	1	105	105	8.0	6.5	5.2	120	115	70	78
Lyso-PE (18:1)	**0.1**	109	112	2.3	3.0	2.7	115	105	83	74
	1	99/93	102/98	9.4/8.1	6.1/6.2	4.9/6.2	96/71	87/79	75/50	80/57

The bold is used to highlight the axes of the tables.

The percentages of recovery, precision, repeatability, accuracy and matrix effect were determined by spiking pooled serum samples from standard (ST)- or cafeteria (CAF)-fed animals with standard Lyso-PLs. Two concentration levels (0.1 and 1 mg/L) were assessed. The constitutive concentrations of Lyso-PCs (16:0) and (18:0) in serum were too high to calculate any parameters. For similar reasons, the method quality parameters were evaluated only for the LSV pre-treatment of the sample, except in the Lyso-PE (18:1) analysis, for which the estimation of the HSV procedure could also be performed at the highest level of the standard concentration (LSV/HSV).

[a] RSD (%, n = 3) of intraday variation.

[b] RSD (%, n = 9) of interday variation (3 days).

Table 4
Circulating levels of the identified Lyso-PLs expressed in micromolarity (μM).

Lyso-PL class	Acyl chain	Animal group (n = 5)		p-value	Lyso-PL class	Acyl chain	Animal group (n = 5)		p-value
		ST	CAF				ST	CAF	
Choline	**(14:0)**	2.076 ± 0.056	2.907 ± 0.222	0.007	**Ethanolamine**				
	(15:0)	1.503 ± 0.100	1.237 ± 0.060	0.052					
	(16:0)	154.173 ± 0.529	151.653 ± 2.456	0.345		**(16:0)**	4.561 ± 0.279	4.615 ± 0.057	0.860
	(16:1)	2.302 ± 0.099	4.281 ± 0.462	0.003		**(16:1)** [HSV]	0.030 ± 0.002	0.047 ± 0.002	< 0.001
	(17:0)	3.322 ± 0.223	2.605 ± 0.171	0.034					
	(17:1) [HSV]	0.111 ± 0.007	0.254 ± 0.008	< 0.001					
	(18:0)	221.317 ± 15.612	231.646 ± 11.577	0.609		**(18:0)**	9.198 ± 0.380	10.508 ± 0.509	0.073
	(18:1)	10.806 ± 0.211	21.415 ± 1.665	< 0.001		**(18:1)**	1.111 ± 0.060	1.320 ± 0.060	0.039
	(18:2)	31.985 ± 1.111	20.759 ± 0.266	< 0.001		**(18:2)**	1.059 ± 0.026	0.616 ± 0.097	0.006
	(18:3)	0.413 ± 0.011	0.374 ± 0.083	0.665		**(18:3)** [HSV]	0.023 ± 0.001	0.009 ± 0.002	< 0.001
	(20:0)	0.506 ± 0.019	0.293 ± 0.020	< 0.001					
	(20:1)	1.328 ± 0.121	0.833 ± 0.070	0.008		**(20:1)** [HSV]	0.048 ± 0.003	0.053 ± 0.009	0.599
	(20:2)	0.569 ± 0.028	0.163 ± 0.017	< 0.001		**(20:2)** [HSV]	0.033 ± 0.003	0.016 ± 0.003	0.002
	(20:3)	0.806 ± 0.104	1.586 ± 0.099	0.001		**(20:3)** [HSV]	0.027 ± 0.001	0.038 ± 0.003	0.028
	(20:4)	48.629 ± 2.998	58.608 ± 1.804	0.021		**(20:4)**	1.059 ± 0.046	1.626 ± 0.144	0.006
	(20:5)	0.129 ± 0.046	0.274 ± 0.051	0.069					
						(22:4) [HSV]	0.050 ± 0.002	0.041 ± 0.004	0.089
	(22:5)	0.536 ± 0.125	0.738 ± 0.058	0.197		**(22:5)** [HSV]	0.041 ± 0.004	0.054 ± 0.008	0.155
	(22:6)	6.587 ± 1.235	6.176 ± 0.355	0.763		**(22:6)**	0.346 ± 0.043	0.343 ± 0.011	0.937

The bold is used to highlight the axes of the tables.
Rats were fed standard chow (ST) or cafeteria diet (CAF) for 2 months. The serum levels of Lyso-PLs were quantified at the end of the experiment after an overnight fast (12 h). The quantitative analysis of Lyso-PLs required the use of 5 μL of serum for the LSV procedure. As indicated in the table, the determination of minor Lyso-PLs required the use of 50 μL of sample for the HSV extraction. The data are presented as means ± SEM (n = 5 per group). Statistical comparisons between the two groups of animals were performed using Student's *t*-test. A two-tailed value of $p < 0.05$ was considered statistically significant.

sample volumes (5–50 μL). In addition, the simplicity of the procedure allowed good reproducibility between analyses, which is essential for the quantitative evaluation of multiple samples.

The validation study also showed excellent accuracies and slight matrix effects in the spiked samples, indicating that this methodology enabled real quantification of lipids in serum. The lack of difference in quality parameters between ST and CAF pooled samples suggests that this method is also useful for accurate comparisons of different dietary groups and for the evaluation of Lyso-PLs as biomarkers of nutrition and disease. The results obtained in sera of ST- and CAF-fed rats demonstrated that the overall sensitivity and precision obtained with the developed method allowed the quantification of endogenous changes in circulating Lyso-PLs. Furthermore, multivariate analysis showed that Lyso-PL examination is an excellent biomarker for the prolonged intake of a diet rich in fat and carbohydrates in rats. Interestingly, this type of diet has been associated with the development of metabolic syndrome in rodents and humans [39,40]. Thus, serum Lyso-PL evaluation may be regarded as a quantitative measure of the nutritional phenotype of CAF-fed rats, a holistic concept that integrates the effects of the diet with health status [41].

We conclude that the simple methodology for the collection and treatment of serum samples, which does not require lipid pre-fractionation, along with the great results for the quality parameters of the technique support the use of UHPLC- + ESI–MS/MS analysis of Lyso-PCs and Lyso-PEs for accurate comprehensive diagnosis of lipid disorders. The present method is expected to be further convenient for the quantitative profiling of Lyso-PLs in other interesting biological fluids and tissues.

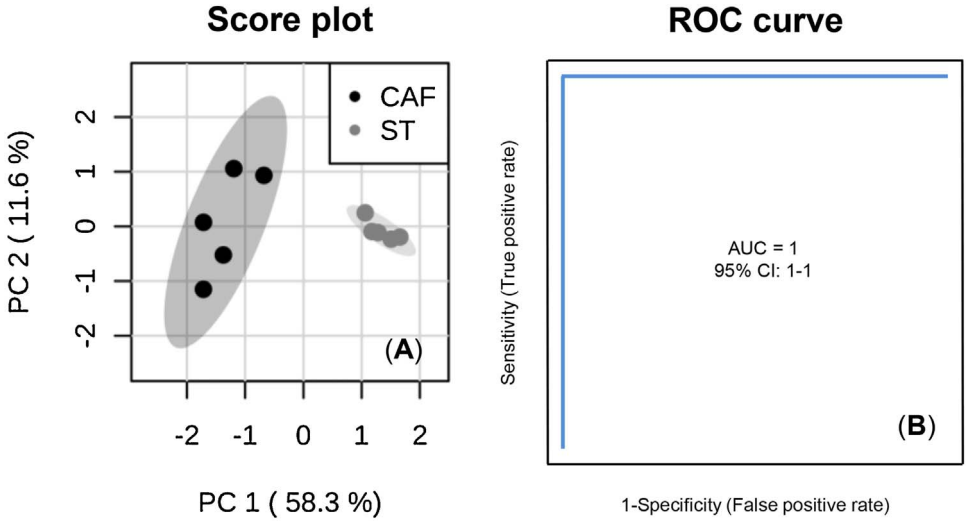

Fig. 5. Multivariate analysis of the Lyso-PL biomarker model. (A) PC1/PC2 scores plot from principal component analysis of rats fed standard chow (ST) or cafeteria diet (CAF) (n = 5 per group). The percentages in parentheses indicate the variance explained by each component. The ellipses mark the 95% confidence interval (CI). (B) ROC curve analysis exploring the suitability of the multivariate biomarker. The precision of the model was measured using the area under the curve (AUC) and the corresponding 95% CI.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2017.04.028.

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