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Graphical abstract

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Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease

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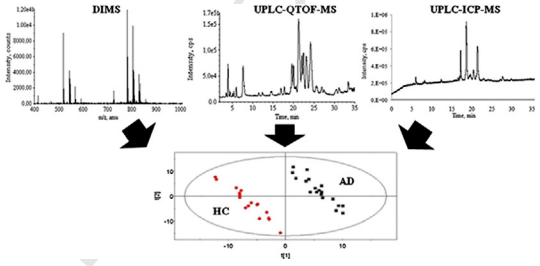

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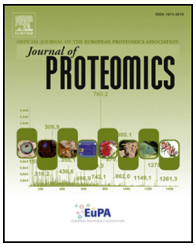

Highlights

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Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease

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- Combined metabolomic-profiling approach allows broader study of phospholipids.
- Different phospholipid species are altered in Alzheimer's disease.
- Alterations depend on the phospholipid class and fatty acid composition.
- Abnormal phospholipid metabolism in Alzheimer has a multifactorial origin.

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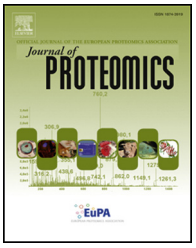

Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease☆

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ABSTRACT

Alzheimer's disease is closely related to abnormal metabolism of phospholipids from neural membranes, so that the study of their dyshomeostasis could be of great interest for the discovery of potential biomarkers for diagnosis and disease monitoring. In this work, it has been developed a metabolomic multi-platform for the characterization of phospholipid alterations occurring in serum from Alzheimer's disease patients. For this purpose, we performed a metabolomic screening by direct infusion mass spectrometry and profiling analysis by reversed phase ultra-high performance liquid chromatography with complementary detection by molecular and atomic mass spectrometry, which allowed combining the high-throughput capability of shotgun metabolomics and the targeted character of profiling approaches. Thus significant changes were detected in the levels of several molecular species of phosphatidylcholines, phosphatidylethanolamines, plasmenylcholines, plasmenylethanolamines and different classes of lysophospholipids, which provided a global vision of the possible factors triggering membrane breakdown. In this sense, alterations of phospholipids metabolism appears to have a multifactorial origin involving overactivation of phospholipases, increased anabolism of lysophospholipids, peroxisomal dysfunction, imbalances in the levels of saturated/unsaturated fatty acids contained in the structure of phospholipids and oxidative stress.

Abbreviations: AD, Alzheimer's disease; HC, healthy control; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PPE, plasmenylethanolamine; PPC, plasmenylcholine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPPC, lysoplasmenylcholine; PLA_2, phospholipase A_2; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; DHA, docosahexaenoic acid; AA, araquidonic acid; APP, amyloid precursor protein; Aβ, β amyloid; CSF, cerebrospinal fluid; DIMS, direct infusion mass spectrometry; RP, reversed phase; UPLC, ultra-high performance liquid chromatography; QTOF-MS, quadrupole-time-of-flight mass spectrometry; ESI, electrospray ionization; ICP-MS, inductively coupled plasma mass spectrometry; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in the projection.

☆ This article is part of a Special Issue entitled: Environmental and structural proteomics.

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Biological significance

This work represents the first comprehensive characterization of serum phospholipids alterations in relation to Alzheimer's disease, by combining shotgun metabolomics and phospholipids profiling through different analytical approaches.

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1. Introduction

Phospholipids play a central role in the biochemistry of all living organisms, constituting the lipid bilayer that serves as a structural barrier to protect cells and subcellular components from external conditions, and being required for the proper function of integral membrane proteins, receptors, and ion channels [1]. Moreover, phospholipids also act as storage depot of a complex meshwork of lipid mediators, such as eicosanoids, lysophospholipids, platelet activating factors or diacylglycerides, which exert a diverse array of effects on cellular functional activities including neural cell homeostasis, immune responsiveness, oxidative stress, and neuroinflammation [2]. Thus, defects in lipid metabolism are involved in numerous human diseases, particularly from the central nervous system because of its high lipid content, so changes in brain phospholipids levels may lead to many neurological disorders, including bipolar disorders, schizophrenia and neurodegenerative diseases, as for example Alzheimer's and Parkinson's disease [3]. Alzheimer's disease (AD) is the most common neurodegenerative disorder among older people, with a complex etiology in which multiple pathologic processes are involved [4]. Thereby, although major hallmarks of AD are the formation of senile plaques and neurofibrillary tangles, related to aberrant processing of amyloid precursor protein (APP) by secretases leading to the deposition of amyloid β, and hyperphosphorylation of tau protein [5], lipids also may be linked to pathogenesis of Alzheimer. In this sense, both APP and secretases are integral membrane proteins, so lipid surroundings play an important regulatory role in APP processing and formation of Aβ peptides [6]. In addition, genomic studies have demonstrated that the ε4 allele of apolipoprotein E (apoE), a lipid transport protein, is a major risk factor for late-onset AD, so apoE-mediated lipid alterations appear to be an important trigger of AD [7].

For these reasons, numerous studies have attempted to characterize the changes in the lipid profile occurring in the brain of AD individuals. These alterations have been traditionally associated with abnormal metabolism of brain phospholipids leading to breakdown of cellular membranes, so that post mortem studies of human AD brain usually showed decreased total levels of phospholipids [8] and accumulation of their degradation products [9]. However, confusing results are observed when specific changes in individual phospholipids are considered. Decreased levels of total phosphatidylethanolamine (PE) [10–13] and phosphatidylinositol (PI) [12–14] have been previously reported, while phosphatidylserine (PS) was found significantly increased [11,15]. In addition, considerably reductions have been described for plasmalogens, in both ethanolamine (PPE) [15,16] and choline (PPC) species [17]. In contrast, findings about phosphatidylcholine (PC) levels are less clear since different studies found its content unchanged [11,12], increased [18] or decreased [10] in specific brain regions. On the other hand, only a few studies attempted to monitor phospholipid alterations in cerebrospinal fluid (CSF) or blood samples, accessible *in vivo* to investigate possible diagnostic markers of neurodegeneration. Mulder et al. found a significant reduction of total CSF phospholipids [19], but recently PI, PE and PC levels were found unchanged in the CSF from AD patients representing different stages of the disease [20,21]. In other study, unchanged PC levels and decreased lysoPC/PC ratio have been reported in the CSF from individuals with AD [22], while different water-soluble PC metabolites increased, suggesting that AD development is accompanied with increased phospholipid hydrolysis [23]. In case of blood samples, circulating plasmenylethanolamine (PPE) levels were observed to be significantly decreased in serum from clinically and pathologically diagnosed AD subjects [24], while by using a 2D-phospholipidomics method was possible to distinguish plasma samples of AD patients from those of elderly controls [25]. Therefore, it can be concluded that analysis of phospholipids offers a great potential for biomarker discovery in AD, but a comprehensive characterization of the phospholipid profile of AD individuals versus controls needs to be addressed.

Metabolomics presents a high potential in health survey and biomarker discovery, because changes in specific groups of metabolites may be sensitive to pathogenically relevant factors. Thus, metabolomics is emerging as a powerful tool for characterization of complex phenotypes affected by both genetic and environmental factors [26]. Nevertheless, metabolomic fingerprinting often lacks of robustness, so targeted or profiling approaches may be useful techniques for validation purposes, with the necessary specificity, precision, accuracy, linearity, sensitivity, recovery and stability in the presence of potentially interfering compounds [27]. In this study, shotgun metabolomics of serum samples was performed by direct infusion mass spectrometry (DIMS) for the screening of phospholipids involved in neurodegenerative processes associated with AD. Furthermore, a targeted analytical approach focused on phospholipids was optimized, based on separation of different species by reversed phase ultra-high performance liquid chromatography (RP-UPLC) and complementary detection by molecular and atomic mass spectrometry. Liquid chromatography coupled to mass spectrometry is widely employed for metabolomic and profiling analyses due to its high resolution and sensitivity, fast analysis and good potential for biomarker identification [28]. However, the high complexity of samples makes necessary to develop selective methods by targeting particular classes of compounds. In this sense, the hyphenation of inductively coupled plasma mass spectrometry (ICP-MS) to LC has been previously described for the sensitive and selective detection of phospholipids, enabling their quantification by phosphorous-tagging without the use of

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structurally matched standards [29,30]. Thus, the combination of different mass spectrometry-based metabolomics strategies, from untargeted to targeted, allows us to shed light on the dyshomeostasis of different classes of phospholipids in AD.

2. Material and methods

2.1. Subjects and serum collection

Blood samples were obtained by venipuncture of the antecubital region after 8 h of fasting. All samples were collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system, previously cooled in refrigerator. The samples were immediately cooled and protected from light for 30 min to allow clot retraction, and centrifuged (3500 rpm for 10 min). The serum was divided into aliquots in Eppendorf tubes and frozen at −80 °C until analysis. Subjects of the study were volunteers over 65 years recruited by the Neurologic Service of Hospital Juan Ramón Jiménez (Huelva, Spain), all residents in the province of Huelva to minimize heterogeneity due to dietary and environmental factors. All subjects underwent blood tests at the time of blood extraction, and other relevant data were collected such as smoking or presence of co-morbidities. Although individual values are not recorded, there were no significant differences among the groups. Patients were newly diagnosed of sporadic AD according to the criteria of the NINCDS-ADRDA [31], and only subjects that had not yet received any type of medication were included in the study ($N = 19$, age 78.0 ± 4.8, male/female 8/11). On the other hand, healthy controls (HC) were studied by neurologists to confirm the absence of neurological disorders, whom have not more than two reported cases of AD in their families ($N = 17$, age 70.6 ± 4.2, male/female 7/10). The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of the University of Huelva.

2.2. Instrumentation

Mass spectrometry experiments, both metabolomic and profiling ones, were performed in a quadrupole-time-of-flight mass spectrometry system (QTOF-MS), model QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA), using the electrospray (ESI) source. For accurate mass measurement, the TOF mass analyzer was daily calibrated using renin as standard. The samples were directly introduced into the mass spectrometer using an integrated apparatus pump and a 1000 μL volume Hamilton syringe for shotgun metabolomic analysis. On the other hand, profiling of phospholipids was carried out by ultra-high performance liquid chromatography in an Accela LC system (Thermo Fisher Scientific) equipped with autosampler and quaternary pump, using a reversed-phase column (Hypersil Gold C18, 2.1 × 50 mm, 1.9 μm). Finally, elemental detection of phospholipids was performed by ICP-MS, using the Agilent 7500ce collision/reaction cell system (Agilent Technologies, Tokyo, Japan), with oxygen 21% in argon as optional gas. Instrumental conditions were daily optimized by using a tuning aqueous solution containing Li, Co, Y, and Tl at 1 μg L^{-1}.

2.3. Metabolomic analysis

Serum metabolomics was performed by extracting individual samples in a two-stage sequential procedure, followed by analysis with high resolution tandem mass spectrometry using ESI source in both positive and negative ionization modes. For the extraction of metabolites, 100 μL of serum was mixed with 400 μL of methanol/ethanol (50%) and stirred for 5 min, followed by centrifugation at 4000 rpm for 10 min at 4 °C. The supernatant is transferred to another tube, and the precipitate is kept for further treatment. Then, supernatant was dried under nitrogen stream and the resulting residue reconstituted with 80:20 methanol/water (polar extract). On the other hand, the precipitate isolated in the first step was extracted with 400 μL of chloroform:methanol (50%) by stirring during 5 min, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. Finally, the resulting supernatant was taken to dryness under nitrogen stream and reconstituted with 100 μL of 60:40 dichloromethane:methanol (lipophilic extract). For analysis by mass spectrometry, 0.1% formic acid was added to the extracts when positive ionization mode was used. In the case of negative ionization, it is not necessary the addition of any reagent. Samples were introduced into the mass spectrometer at 5 μL min^{-1} flow rate using the integrated pump. Data were obtained in both positive and negative ionization modes, acquiring full scan spectra for 0.2 min in the m/z range 400–1000 with 1.005 s scan time. In positive mode, the ion spray voltage (IS) was set at 3300 V, and high-purity nitrogen was used as curtain and nebulizer gas at flow rates about 1.13 L min^{-1} and 1.56 L min^{-1}, respectively. The source temperature was fixed at 60 °C, with a declustering potential (DP) of 60 V and a focusing potential (FP) of 250 V. For ESI(−), only few parameters were modified respect ESI(+) method, with an IS voltage at −4000 V, a DP of −100 V and an FP of −250 V. To acquire MS/MS spectra, nitrogen was used as collision gas. Samples were prepared and analyzed randomly to minimize the sources of variation that differ from the inherent biochemical composition, and to avoid a bias related to the order of injection.

2.4. Profiling of phospholipids

For targeted analysis of serum phospholipids, species were separated by RP-UPLC and detected by complementary molecular and atomic mass spectrometry. Individual samples were treated following a modification of Zhao procedure for the extraction of lysophospholipids and phospholipids from blood samples [32] by an extremely simple and reproducible method. For this, 50 μL of serum was mixed with 500 μL of methanol and vortexed for 5 min. The mixture is centrifuged at 10,000 rpm for 10 min at 4 °C, and supernatant is dried under nitrogen stream and reconstituted with 50 μL of methanol. Chromatographic separations were performed in a reversed-phase column thermostatized at 60 °C, with an injection volume of 5 μL. Solvents were delivered at a flow rate 0.5 mL/min, using water with 10 mM ammonium acetate (solvent A) and methanol (solvent B). The gradient elution program was: 0 min, 50% B; 2 min, 65% B; 15 min, 85% B; 15–25 min, 85% B, 35 min, 100% B. Finally, system returns to initial conditions in 2 min, and column is equilibrated for

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1 min with 50% B before the next run. MS operated in positive and negative polarities, acquiring full scan spectra in the m/z range 400–1000 with 1.005 s scan time. The IS voltage was set at 5000 V and −2500 V, and high-purity nitrogen was used as curtain, nebulizer and heater gas at flow rates about 1.48 L min^{-1}, 1.56 L min^{-1} and 5.5 L min^{-1}, respectively. The source temperature was fixed at 400 °C, with a DP of 60 V/−100 V, and an FP of ± 250 V. To acquire MS/MS spectra, nitrogen was used as collision gas. For selective detection of phosphorus-containing compounds, the flow from UPLC column was introduced in the ICP-MS, equipped with a MicroMist nebulizer and platinum sampling and skimmer cones. To prevent deposition of carbon from organic mobile phase on the interface cones, an optional gas (21% oxygen in argon) was applied through a T-piece connecting spray chamber and torch. Furthermore, such phosphorus is a difficult element to analyze using quadrupole ICP-MS due to polyatomic isobaric interferences (i.e. $^{14}N^{16}O^{1}H$, m/z 31); the addition of oxygen enabled the detection of this element via reaction of the phosphorus ions with oxygen resulting in the formation of oxides ($^{47}PO^{+}$), which can be monitored free of isobaric interferences. Thus, detection was carried out by recording m/z ratio 47, with a dwell time of 0.3 s per point. The forward power was set at 1500 W, and the gas flow rates were fixed at 15 L min^{-1} for plasma gas, 1 L min^{-1} for auxiliary gas, 1 L min^{-1} for carrier gas, 0.15 L min^{-1} for makeup gas and 40% for optional gas (of carrier gas flow rate). Spray chamber temperature was fixed at −5 °C and sample depth (torch-interface distance) at 7 mm. Samples were prepared and analyzed randomly to minimize the sources of variation that differ from the inherent biochemical composition and to avoid a bias related to the order of injection.

2.5. *Data processing*

Metabolomic data were submitted to peak detection by Markerview™ software (Applied Biosystems) in order to filter the mass spectrometry results based on the total ion current and to carry out the reduction into a two-dimensional data matrix of spectral peaks and their intensities. For this, the peak search was done with a mass tolerance of 0.1 Da, and a minimum response of 10 counts was considered for filtering.

Missing values were substituted with the mean of the non-missing values across all samples for that peak [33], and finally data were normalized according to the total area sum. In the case of hyphenated approaches, data processing proceeds through multiple stages, including filtering and feature detection (or peak picking), for the removal of noise and accurate peak detection and peak matching across samples; alignment and normalization, for the correction of drifts in retention time and intensity between samples, respectively [34]. For this purpose, we employed freely available software XCMS, included in the R 2.13.0 platform (http://www.r-project.org). MS files were converted into mzXML format using the msConvert tool (ProteoWizard) and subsequently extracted using the matchedFilter method. This algorithm slices data into extracted ion chromatograms on a fixed step size (default 0.1 m/z), and then each slice is filtered with matched filtration using a second-derivative Gaussian as the model peak shape [35]. The XCMS parameters were optimized according to the characteristics of data sets obtained in order to find the maximum number of peaks. Finally, the settings applied were S/N threshold 2 and full width at half-maximum 10. After peak extraction, grouping and retention time correction of peaks (alignment) was accomplished in three iterative cycles with descending bandwidth (bw) from 10 to 1 s. Then, imputation of missing values was performed by returning to the raw spectral data and integrating the areas of the missing peaks which are below the applied signal-to-noise ratio threshold, using the fillPeaks algorithm. For statistical data normalization, the locally weighted scatter plot smoothing normalization method was used, which adjusts the local median of log fold changes of peak intensities between samples in the data set to be approximately zero across the whole peak intensity range [36]. Finally, data were submitted to logarithmic transformation, in order to stabilize the variance of results. The preprocessed data, from both metabolomic and profiling approaches, were then exported as a .csv file for further data analysis by multivariate procedures.

2.6. *Data analysis*

In order to find differences between the groups of study, data was processed by partial least squares discriminant analysis (PLS-DA) in SIMCA-P™ software (version 11.5, UMetrics AB, Umeå, Sweden). Before performing PLS-DA, data are usually scaled and transformed in order to minimize the technical variability between individual samples to extract the relevant biological information from these data sets [37]. For this, data was submitted to Pareto scaling, for reducing the relative importance of larger values, and logarithmic transformation, in order to approximate a normal distribution. Altered phospholipids in AD were selected according to the Variable Importance in the Projection, or VIP (a weighted sum of squares of the PLS weight, which indicates the importance of the variable in the model), considering only variables with VIP values higher than 1.5, indicative of significant differences among groups. In addition, groups' comparison was conducted by one-way analysis of variance with Bonferroni correction for multiple testing, using the STATISTICA 8.0 software (StatSoft, Tulsa, USA). Only p values below 0.05 were regarded as statistically significant.

2.7. *Identification of phospholipids*

A preliminary identification of significant compounds was made matching the experimental accurate mass with those available in metabolomic databases (METLIN and LIPIDMAPS), using a mass accuracy of 30 ppm. Then, different classes of phospholipids were confirmed based on characteristic fragmentation patterns described in literature [38,39]. Choline-containing phospholipids were detected as protonated ($[M + H]^+$), sodiated ($[M + Na]^+$) and potassiated ($[M + K]^+$) ions in positive ion mode, while in negative polarity these lipids formed demethylated ions ($[M\text{-}CH_3]^-$) of chlorinated adducts ($[M + Cl]^-$). On the other hand, more abundant ions for ethanolamine species were $[M + H]^+$ and $[M\text{-}H]^-$, in positive and negative modes respectively. PCs and lysophosphatidylcholines (LPCs) presented characteristic ions in positive ionization mode at m/z 184.07, 104.10 and 86.09, and two typical fragments due to the loss of trimethylamine (m/z 59)

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and phosphocholine (m/z 183, 205 or 221, if the counterion is proton, sodium or potassium). In contrast, the product-ion spectra of ethanolamines were dominated by $[M + H - 141]^+$ arising from elimination of the phosphoethanolamine moiety. Finally, in negative mode, these distinctive signals were found at 168.04 and 196.07, for choline and ethanolamine derived lipids, respectively. Furthermore, the fragmentation in the glycerol backbone and release of the fatty acyl substituents enabled the identification of individual species of phospholipids, as previously described [40].

3. Results

3.1. *Metabolomic fingerprints and phospholipid profiles from serum*

The use of a high-throughput approach based on DIMS provided comprehensive metabolomic profiles, by means of an exhaustive extraction of metabolites in a two-step procedure and complementary analysis by ESI(+)/ESI(–) modes (Fig. 1). A large number of peaks can be observed, distributed in different clusters associated with structurally related compounds. In addition, it is remarkable the higher complexity of spectra from lipophilic extracts, corroborating the requirement of a second extraction step to recover highly non-polar lipids adsorbed in protein precipitate. Therefore, shotgun metabolomics highlights as a powerful technique for fast screening of serum metabolites, allowing obtaining broad metabolic information.

However, shotgun analyses suffer from significant matrix effects, which can lead to ion suppression of minor species by more abundant phospholipids and reduction in sensitivity [41]. Thus, the use of a chromatographic separation enables the analysis of phospholipids at a level of detail that is not achievable by mass spectrometry alone, complementing the preliminary metabolomic results. The high multiplicity of phospholipidome makes impossible the complete chromatographic separation of thousands of molecular species, so complex profiles were obtained (Fig. 2A) that need advanced tools for automated peak recognition, integration and alignment of peaks from different, as explained under Materials and methods section. On the other hand, it has been demonstrated the high potential of elemental mass spectrometry for monitoring phosphorus-containing compounds (Fig. 2B), reducing the complexity of phospholipid profiles. In this way, the combination of elemental and molecular profiling allows to simplify the search of phospholipid matching signals from both chromatograms.

3.2. *Altered phospholipids in AD*

Multivariate statistics was used to determine the capability of phospholipids for discriminating between AD patients and HC. For this purpose, PLS-DA was performed in order to build a predictive model for classification of samples, which allows the visualization of groupings and trends between different groups of samples through the representation of scores plots of two score vectors [42]. The PLS-DA model built with our data set provided a perfect separation between Alzheimer and control cases (Fig. 3), demonstrating the potential of serum phospholipids as biomarkers for diagnosis of AD.

Thus, numerous altered phospholipids were identified using the VIP metric as well as one-way ANOVA, as described under the Material and methods section. These statistically significant phospholipids are summarized in Tables 1–3, including the experimental mass, the ionization mode used for detection, the diagnostic ions employed for the identification, and the fold change (AD/HC ratio) expressed as mean ± standard deviation.

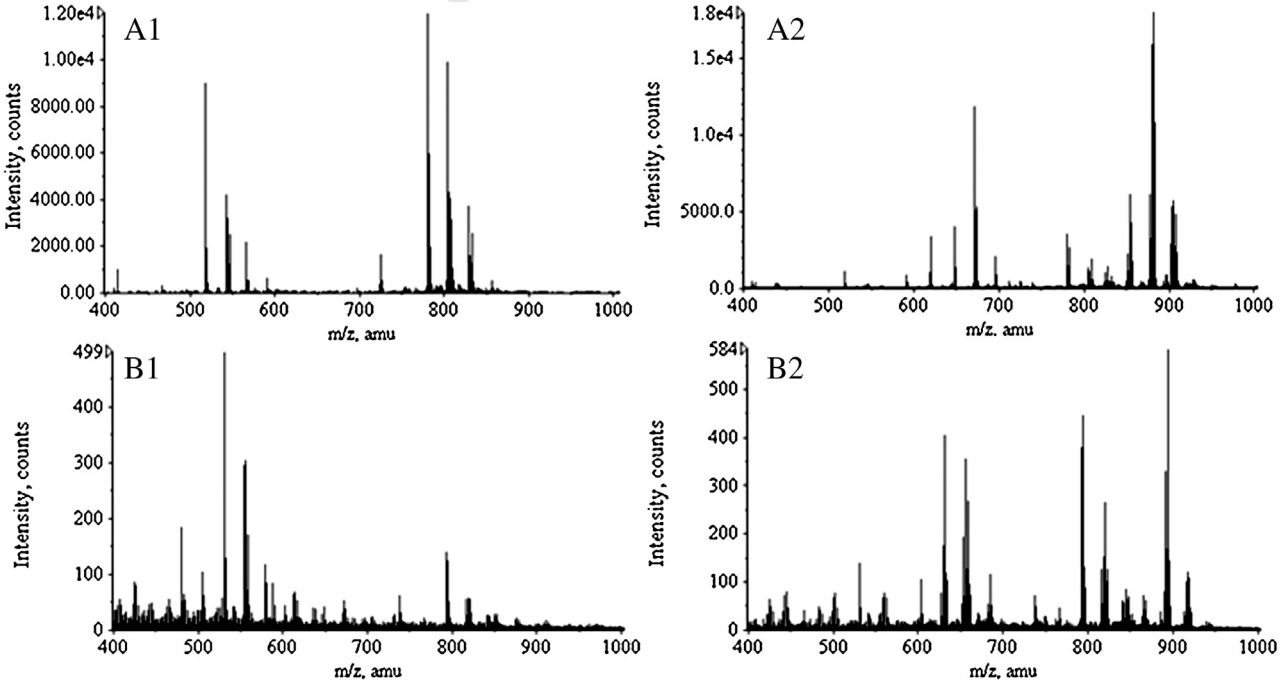

Fig. 1 – Mass spectra from serum samples for ESI+ (A) and ESI– (B), with polar (1) and lipophilic (2) extracts.

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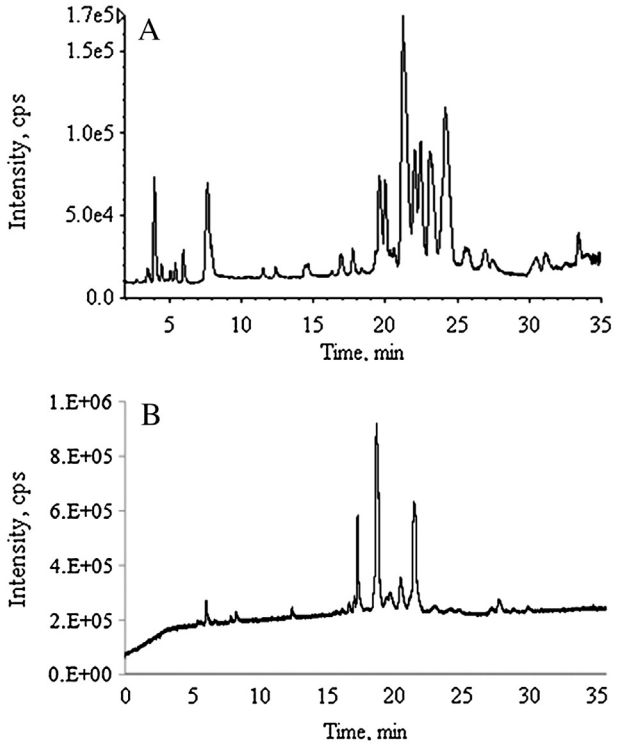

Fig. 2 – Phospholipid profiles from serum samples obtained by UPLC-ESI-QTOF-MS (A) and UPLC-ICP-MS (B).

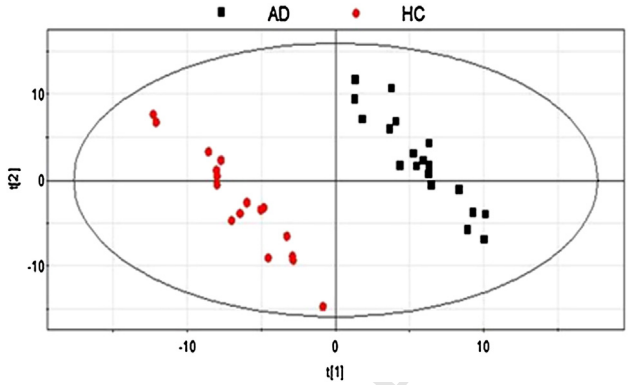

Fig. 3 – Scores plot for the PLS-DA model.

As can be observed, important dyshomeostasis occurs in major phospholipid classes, lipids derived from choline and ethanolamine. Lysophospholipid levels were reduced in serum from AD patients (Table 1), including choline (LPC), plasmenylcholine (LPPC) and ethanolamine (LPE) species. In the case of PCs, a different trend is observed depending on the type of fatty acid linked to the molecular moiety, while all PEs detected were decreased in AD samples (Table 2). Finally, a decrease in total levels of plasmalogens was observed in our results (Table 3), in both PPE and PPC compounds.

4. Discussion

The combination of fingerprinting and profiling methodologies denoted numerous phospholipids that could be used as potential biomarkers for AD and may help to understand the biochemical processes associated with neurodegeneration. Abnormalities in lipids from membrane are well known processes previously described in brains from AD and other neurodegenerative diseases. These alterations have been traditionally associated with abnormal metabolism of brain phospholipids leading to breakdown of cellular membranes, principally related to overactivation of phospholipase A_2 (PLA_2), but there are also evidences of the role of phospholipases C and D, although they have been much less studied [43]. The postulated mechanism of this pathological breakdown supposes a complex sequence of cellular events in hypoxic neurons, involving release of glutamate and influx of large amounts of calcium into neurons, which finally induce overactivation of catabolic enzymes including phospholipases [44]. As a consequence, this abnormal metabolism results in important biochemical changes in brain, which are reflected in peripheral serum (Tables 1–3), as discussed below.

4.1. *Lysophospholipids*

Lysophospholipids are transiently generated during the remodeling of phospholipids by the action of phospholipase A_2, but they are rapidly acylated with acyl-CoA in the deacylation–reacylation cycle for the maintenance of the normal and essential neural membrane composition [45]. However, it is recognized that lysophospholipids are not simply intermediates in metabolism of glycerolipids, but they serve as mediators in multiple neuronal pathways involved in neurobiology of AD [46]. In this sense, significant impairments in metabolism of lysophospholipids have been described in AD brain attributed to overactivation of lysophospholipid acyltransferase [47], which recycles lysophospholipids produced in membrane breakdown into phospholipids, and lysophospholipase [48,49], which converts lysophospholipids into lysophosphatidic acid. Thus, previous studies reported lower total LPC concentration in brain [50], CSF [22] and plasma [51] of AD patients, reflecting an alteration in the metabolism of choline-containing phospholipids. In this work, this rationale was corroborated by decreased levels of different species of LPC (Table 1), but we also observed the similar trend for LPE and LPPC species, only once described for LPE(18:1) in AD plasma [25]. Therefore, it might be assumed that anabolic stimulation of lysophospholipids metabolism is not only produced in choline-containing compounds but also occurs in other families of compounds such as PEs and PPC.

4.2. PCs

In the present work, alterations in serum PC levels depended on the type of fatty acid linked to the molecular moiety, decreasing polyunsaturated fatty acid (PUFA) PCs and increasing saturated fatty acid (SFA) containing ones (Table 2). Fatty acid composition of membrane lipids influences their biophysical properties, including fluidity, permeability and charge. Thereby, the loss of potentially important unsaturated fatty acids such as docosahexaenoic (DHA) and araquidonic

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Table 1 – Lysophospholipids identified as potential biomarkers of Alzheimer's disease.

Compound	Mass (Da)	Ion mode	Identification	Fold change
LPE(16:0)	453.286	P	454.30 $[M + H]^+$; 313.27	0.98 ± 0.36
LPE(18:2)	477.286	P	478.31 $[M + H]^+$; 337.27	0.67 ± 0.31
LPE(18:1)	479.301	P, N	P: 480.32 $[M + H]^+$; 339.29 N: 478.29 $[M-H]^-$; 281.25; 196.07	0.77 ± 0.34
LPC(16:0)	495.332	P	496.34 $[M + H]^+$; 313.27; 184.07; 104.10; 86.09	0.76 ± 0.31
LPPC(18:1)	505.353	P	528.33 $[M + Na]^+$; 184.07; 104.10; 86.09	0.75 ± 0.26
LPPC(18:0)	507.369	P	530.35 $[M + Na]^+$; 184.07; 104.10; 86.09	0.73 ± 0.28
LPC(18:2)	519.332	P	520.33 $[M + H]^+$; 337.27; 184.07; 104.10; 86.09	0.88 ± 0.24
LPC(18:1)	521.348	P, N	P: 522.35 $[M + H]^+$; 544.33 $[M + Na]^+$; 560.31 $[M + K]^+$; 339.29; 184.07; 104.10; 86.09 N: 556.31 $[M + Cl]^-$; 281.25; 168.04	0.65 ± 0.27
LPC(18:0)	523.364	P, N	P: 524.36 $[M + H]^+$; 562.33 $[M + K]^+$; 341.31; 184.07; 104.10; 86.09 N: 558.33 $[M + Cl]^-$; 283.26; 168.04	0.73 ± 0.21
LPC(20:5)	541.317	P, N	P: 564.31 $[M + Na]^+$; 359.26; 184.07; 104.10; 86.09 N: 526.29 $[M-Me]^-$; 576.29 $[M + Cl]^-$; 301.22; 168.04	0.81 ± 0.33
LPC(22:6)	567.332	P	590.33 $[M + Na]^+$; 385.27; 184.07; 104.10; 86.09	0.79 ± 0.41

acid (AA), with a high presence in neurons, appears to contribute to membrane damage in AD pathogenesis [52]. In this sense, numerous previous studies reported reduced levels of PUFA-containing phospholipids, both in brain and plasma from patients with AD [53–55], and particularly in PC species [17,56]. However, this is the first time that is described a parallel increase of PC containing saturated and short chain fatty acids in serum from AD patients. This altered fatty acid profile in individual phosphocholines might suggest a deregulation in the biosynthesis, turnover and acyl chain remodeling of phospholipids, in accordance with an increased breakdown and resynthesis of phospholipid due to overactivation of PLA_2 [57]. In this way, we can conclude that not only hydrolysis of phospholipids due to overactivation of phospholipases causes membrane destabilization processes, but also imbalances in the levels of saturated/unsaturated fatty acids contained in the structure of phospholipids could be involved in this abnormal membrane behavior.

4.3. PE

PEs are the second major class of brain phospholipids, with a content of PUFAs significantly higher than PC [58]. Unlike confusing findings about PC levels, PEs and/or PE-derived PUFAs are usually decreased in brain [10–13,17] and plasma [56] from AD patients, as confirmed in our experimental data (Table 2). This decrease of PEs suggests the implication of oxidative stress in the progressive degradation of brain phospholipids in AD, since they are rich in readily oxidizable AAs and DHAs. Oxidative stress is recognized as a primary factor in pathogenesis of Alzheimer due to the high-metabolic rate of brain, which makes it particularly susceptible to reactive oxygen species (ROS) [59]. Because of the weak antioxidant defense mechanisms of the brain tissue, the high concentration of PUFAs usually serves as a substrate for lipid peroxidation, generating aldehydes such as 4-hydroxynonenal which in turn can oxidize proteins and inhibit glycolysis [60]. Thus, it could be hypothesized that, in the membrane destabilization process, oxidative stress is an important player in degradation of PUFA-containing phospholipids, which may contribute to the neuropathology of AD brain.

4.4. Plasmalogens

Plasmalogens are major constituents of neural membranes forming part of myelin sheath, but in addition they present other metabolic functions, such as reservoir of PUFAs for the production of prostaglandins and thromboxanes, antioxidant properties, and precursor of an analog of platelet-activating factor [61]. Deficiency of brain PPE, which constitute up to 70% of total plasmalogens, has been traditionally associated with AD development [15,16,62,63], which is finally reflected in lower levels of circulating PPEs in CSF [64] and serum [24]. Different mechanisms have been proposed for the reduction of PPE in AD brain, related to decreased rate of synthesis and increased rate of degradation. In the first place, deficits in PPE may be correlated to peroxisomal dysfunction, since the enzymes required for the creation of the 1-O-alkyl bond are found exclusively in peroxisomes. Thereby, substantial peroxisome-related alterations have been described in AD brains, inducing the accumulation of very long chain fatty acids and decreased synthesis of plasmalogens and DHA [65]. On the other hand, the key enzyme involved in the turnover and degradation of PPE, the plasmalogen-selective phospholipase A_2, is markedly increased in brains from Alzheimer disease patients [66]. In addition, PPE molecular species consist predominantly of polyunsaturated acyl chains, which make them a primary target for ROS [63]. Thus, decrease observed in PPE species (Table 3), principally containing AA and DHA, could be related to enzymatic and non-enzymatic factors, involved in altered catabolism and anabolism of these lipids. Similarly, besides the reduction of PPE species, we also observed a parallel decrease of PPC (Table 3). Although much less studied, decreased PPC concentrations have been observed previously in brain from AD patients [17,50], and like PPE species, PPCs were rich in highly unsaturated fatty acids, pointing again to the importance of oxidative stress in membrane instability and synaptic loss in AD.

5. Conclusion

The combination of high-throughput metabolomic screening by DIMS and profiling by reversed phase ultra-high performance

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Table 2 – Phospholipids identified as potential biomarkers of Alzheimer's disease.

Compound	Mass (Da)	Ion mode	Identification	Fold change
Phosphatidylcholines				
PC(16:1/16:1)	729.53	P	752.52 $[M + Na]^+$; 693.44 $[M\text{-}N(CH_3)_3 + Na]^+$; 547.47; 311.26; 184.07; 104.10; 86.09	1.19 ± 0.38
PC(16:1/16:0)	731.55	P	754.55 $[M + Na]^+$; 695.47 $[M\text{-}N(CH_3)_3 + Na]^+$; 549.50; 313.27; 311.26; 184.07; 104.10; 86.09	1.57 ± 1.11
PC(16:0/16:0)	733.56	P	756.55 $[M + Na]^+$; 697.48 $[M\text{-}N(CH_3)_3 + Na]^+$; 551.51; 313.27; 184.07; 104.10; 86.09	1.43 ± 0.58
PC(16:1/18:3)	753.53	P	776.53 $[M + Na]^+$; 792.50 $[M + K]^+$; 717.45 $[M\text{-}N(CH_3)_3 + Na]^+$; 733.43 $[M\text{-}N(CH_3)_3 + K]^+$; 571.48; 311.26; 335.26; 184.07; 104.10; 86.09	1.27 ± 0.45
PC(16:0/18:3)	755.55	P	778.54 $[M + Na]^+$; 794.51 $[M + K]^+$; 719.46 $[M\text{-}N(CH_3)_3 + Na]^+$; 735.44 $[M\text{-}N(CH_3)_3 + K]^+$; 573.49; 313.27; 335.26; 184.07; 104.10; 86.09	1.39 ± 0.53
PC(16:0/18:2)	757.56	P, N	P: 780.54 $[M + Na]^+$; 796.52 $[M + K]^+$; 721.45 $[M\text{-}N(CH_3)_3 + Na]^+$; 737.44 $[M\text{-}N(CH_3)_3 + K]^+$; 575.50; 313.27; 337.27; 184.07; 104.10; 86.09 N: 792.55 $[M + Cl]^-$; 742.54; 255.23; 279.23; 168.04	1.40 ± 0.52
PC(16:0/18:1)	759.58	P, N	P: 782.56 $[M + Na]^+$; 798.54 $[M + K]^+$; 723.47 $[M\text{-}N(CH_3)_3 + Na]^+$; 739.45 $[M\text{-}N(CH_3)_3 + K]^+$; 577.52; 313.27; 339.29; 184.07; 104.10; 86.09 N: 794.56 $[M + Cl]^-$; 744.55; 255.23; 281.25; 168.04	1.24 ± 0.41
PC(16:0/18:0)	761.59	P	784.57 $[M + Na]^+$; 725.49 $[M\text{-}N(CH_3)_3 + Na]^+$; 579.54; 313.27; 341.31; 184.07; 104.10; 86.09	1.26 ± 0.54
PC(16:0/20:5)	779.55	P, N	P: 802.54 $[M + Na]^+$; 743.46 $[M\text{-}N(CH_3)_3 + Na]^+$; 597.49; 313.27; 359.26; 184.07; 104.10; 86.09 N: 814.52 $[M + Cl]^-$; 764.53; 255.23; 301.22; 168.04	0.72 ± 0.33
PC(18:2/18:2)	781.56	P, N	P: 804.55 $[M + Na]^+$; 820.54 $[M + K]^+$; 745.47 $[M\text{-}N(CH_3)_3 + Na]^+$; 761.46 $[M\text{-}N(CH_3)_3 + K]^+$; 599.50; 337.27; 184.07; 104.10; 86.09 N: 816.55 $[M + Cl]^-$; 766.56; 279.23; 168.04	1.42 ± 0.61
PC(18:2/18:1)	783.58	P, N	P: 806.56 $[M + Na]^+$; 822.55 $[M + K]^+$; 747.48 $[M\text{-}N(CH_3)_3 + Na]^+$; 763.47 $[M\text{-}N(CH_3)_3 + K]^+$; 601.51; 339.29; 337.27; 184.07; 104.10; 86.09 N: 818.56 $[M + Cl]^-$; 768.56; 281;25; 279.23; 168.04	1.37 ± 0.48
PC(18:1/18:1)	785.59	P	808.58 $[M + Na]^+$; 824.56 $[M + K]^+$; 749.50 $[M\text{-}N(CH_3)_3 + Na]^+$; 765.47 $[M\text{-}N(CH_3)_3 + K]^+$; 603.52; 339.29; 184.07; 104.10; 86.09	1.20 ± 0.43
PC(18:0/18:0)	789.62	P	828.57 $[M + K]^+$; 769.49 $[M\text{-}N(CH_3)_3 + K]^+$; 607.48; 341.31; 184.07; 104.10; 86.09	1.33 ± 0.98
PC(18:2/20:5)	803.54	N	838.50 $[M + Cl]^-$; 788.51; 279.23; 301.22; 168.04	0.92 ± 0.39
PC(16:0/22:6)	805.56	P, N	P: 828.56 $[M + Na]^+$; 769.48 $[M\text{-}N(CH_3)_3 + Na]^+$; 623.52; 313.27; 385.27; 184.07; 104.10; 86.09 N: 840.53 $[M + Cl]^-$; 790.54; 255.23; 327.23; 168.04;	0.68 ± 0.48
PC(16:0/22:5)	807.58	N	842.54 $[M + Cl]^-$; 792.54; 255.23; 329.25; 168.04	0.83 ± 0.42
PC(18:1/20:4)	807.58	P	830.56 $[M + Na]^+$; 771.47 $[M\text{-}N(CH_3)_3 + Na]^+$; 625.51; 339.29; 361.27; 184.07; 104.10; 86.09	0.73 ± 0.32
PC(18:1/20:3)	809.59	P	810.59 $[M + H]^+$; 751.52 $[M\text{-}N(CH_3)_3 + H]^+$; 627.52; 339.29; 363.29; 184.07; 104.10; 86.09	0.81 ± 0.34
PC(18:0/20:3)	811.61	N	846.59 $[M + Cl]^-$; 796.58; 283.26; 305.25; 168.04	0.86 ± 0.45
PC(18:0/22:6)	833.59	P	856.58 $[M + Na]^+$; 797.51 $[M\text{-}N(CH_3)_3 + Na]^+$; 651.53; 341.31; 385.27; 184.07; 104.10; 86.09	0.57 ± 0.24
Phosphatidylethanolamines				
PE(16:1/20:5)	735.48	P	736.51 $[M + H]^+$; 595.49; 311.26; 359.26	0.85 ± 0.35
PE(16:0/20:5)	737.50	P	738.53 $[M + H]^+$; 597.49; 313.27; 359.26	0.68 ± 0.25
PE(18:1/20:5)	763.52	P	764.54 $[M + H]^+$; 623.51; 339.29; 359.26	0.95 ± 0.26
PE(18:1/20:4)	765.53	P, N	P: 766.54 $[M + H]^+$; 625.52; 339.29; 361.27 N: 764.55 $[M\text{-}H]^-$; 281.25; 303.23; 196.07	0.86 ± 0.27
PE(18:2/22:6)	787.52	P	788.53 $[M + H]^+$; 647.51; 337.27; 385.27	0.84 ± 0.27
PE(18:1/22:6)	789.53	P	790.55 $[M + H]^+$; 649.51; 339.29; 385.27	0.83 ± 0.25
PE(18:0/22:6)	791.55	P	792.56 $[M + H]^+$; 651.53; 341.31; 385.27	0.87 ± 0.24
PE(22:6/22:5)	837.53	P	838.54 $[M + H]^+$; 697.52; 385.27; 387.29	0.92 ± 0.38
PE(22:5/22:4)	841.56	P	842.57 $[M + H]^+$; 701.55, 387.29, 389.31	0.75 ± 0.40

liquid chromatography with complementary detection by molecular and atomic mass spectrometry has been demonstrated as a suitable approach to characterize phospholipid alterations occurring in serum from AD patients. Major changes were observed in molecular species of PCs, PE, PPC, PPE and different classes of lysophospholipids, indicating the great importance of membrane breakdown processes in pathogenesis of this neurodegenerative disorder. Furthermore, results obtained suggest a multifactorial origin for this abnormal phospholipid metabolism, in which could be involved overactivation of phospholipases, increased anabolism of lysophospholipids, peroxisomal dysfunction, imbalances in the levels of saturated/unsaturated fatty acids contained in the structure of phospholipids and oxidative stress. Finally, it should be noted that these alterations, some of them not previously described, could be investigated as potential biomarkers for Alzheimer diagnosis, due to the use of an easily available biological sample such as blood serum.

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Table 3 – Plasmalogens identified as potential biomarkers of Alzheimer's disease. (+) up-regulated in AD, (–) down-regulated in AD.

Compound	Mass (Da)	Ion mode	Identification	Fold change
Plasmenylethanolamines				
PPE(16:0/20:5)	721.50	N	720.51 $[M-H]^-$; 301.22, 196.07	0.83 ± 0.36
PPE(16:0/20:4)	723.52	N	722.52 $[M-H]^-$; 303.23; 196.07	0.80 ± 0.38
PPE(18:1/18:2)	725.54	N	724.53 $[M-H]^-$; 279.23; 196.07	0.90 ± 0.38
PPE(18:1/18:1)	727.55	N	726.54 $[M-H]^-$; 281.25, 196.07	0.89 ± 0.34
PPE(16.0/22.6)	747.52	N	746.53 $[M-H]^-$; 327.23; 196.07	0.77 ± 0.31
PPE(18:1/20:4)	749.54	N	748.54 $[M-H]^-$; 303.23; 196.07	0.70 ± 0.32
PPE(18:0/20:4)	751.55	N	750.56 $[M-H]^-$; 303.23; 196.07	0.75 ± 0.33
PPE(18:1/22:6)	773.54	N	772.55 $[M-H]^-$; 327.23; 196.07	0.76 ± 0.26
PPE(18:0/22:6)	775.55	N	774.55 $[M-H]^-$; 327.23; 196.07	0.68 ± 0.19
Plasmenylcholines				
PPC(16:0/22:6)	789.57	P	812.57 $[M + Na]^+$; 753.49 $[M-N(CH_3)_3 + Na]^+$; 607.52; 385.27; 184.07; 104.10; 86.09	0.82 ± 0.25
PPC(18:1/20:4)	791.58	N	826.57 $[M + Cl]^-$; 776.58; 303.23; 168.04	0.88 ± 0.24
PPC(18:0/20:4)	793.59	N	828.58 $[M + Cl]^-$; 778.58; 303.23; 168.04	0.80 ± 0.29
PPC(18:1/22:6)	815.58	P	816.58 $[M + H]^+$; 757.50 $[M-N(CH_3)_3 + H]^+$; 633.50; 385.27; 184.07; 104.10; 86.09	0.84 ± 0.23
PPC(18:0/22:6)	817.59	P	818.60 $[M + H]^+$; 759.53 $[M-N(CH_3)_3 + H]^+$; 635.52; 385.27; 184.07; 104.10; 86.09	0.90 ± 0.28

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