Profiling and quantification of aminophospholipids based on chemical derivatization coupled with HPLC-MS

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Abstract In this study, a novel strategy based on acetone stable-isotope derivatization coupled with HPLC-MS for profiling and accurate quantification of aminophospholipids (phosphatidylethanolamine and phosphatidylserine) in biological samples was developed. Acetone derivatization leads to alkylation of the primary amino groups of aminophospholipids with an isopropyl moiety; the use of deuterium-labeled acetone (d6-acetone) introduced a 6 Da mass shift that was ideally suited for profiling and quantification analysis with high selectivity and accuracy. After derivatization, significantly increased column efficiency for chromatographic separation and detection sensitivity for MS analysis of aminophospholipids was observed. Furthermore, an accuracy quantification method was developed. Aminophospholipids in biological samples were derivatized with d0-acetone; while more than two aminophospholipid standards were selected for each class of aminophospholipid and derivatized with d6-acetone, which were then used as the internal standards to typically construct a calibration curve for each class to normalize the nonuniformity response caused by the differential fragmentation kinetics resulting from the distinct chemical constitution of individual aminophospholipid species in the biological samples. The excellent applicability of the developed method was validated by profiling and quantification of aminophospholipids presented in liver samples from rats fed with different diets.

Supplementary key words high-performance liquid chromatography-mass spectrometry • polyphenols • acetone

Phospholipids (PLs) are the main constituents of biological membranes and play important functional, structural, and metabolic roles (1). PLs consist of a glycerol backbone esterified with two fatty acids at the sn-1 and sn-2 positions. The backbone’s sn-3 position is occupied by a phosphate group attached to a polar head of different nature. Aminophospholipids [phosphatidylethanolamine (PE) and phosphatidylserine (PS)] belong to the category of PLs. PE and PS account for approximately 20% and 3–15% of total PLs in eukaryotic cells, respectively. They play a critical role in regulating various biological processes, including intra- and inter-cellular signaling, phagocytic recognition, apoptotic cell clearance, cell division, angiogenesis, and vascular remodeling under normal physiological conditions in eukaryotic cells (2). Furthermore, aminophospholipids are associated with various human diseases, including cancer, diabetes, obesity, and neurodegeneration (3, 4). Hence, profiling of the individual aminophospholipid molecular species in cellular components is critical to link physiological or pathophysiological changes to the underlying biochemistry and thus target therapeutic interventions. However, precise profiling of aminophospholipids is not easy because of the sheer number and the high complexities of their structures.

Direct infusion MS equipped with an ESI source holds much promise for the characterization of PLs (5). Through effective intra-source separation of predetermined groups of lipid classes according to their intrinsic electrical propensities, lipid extracts can be analyzed directly without preseparation. However, the disadvantages of the direct infusion MS method are ion suppression for lipid species with low proton affinities, negative impact of unavoidable matrix effects on the analysis of minor content of lipid species and serious contamination to

Abbreviations: CID, collision-induced dissociation; EPI, enhanced product ion; IPA, isopropanol; LOQ, limit of quantification; MRM, multiple reaction monitoring; NaBH4, sodium cyanoborohydride; NL, neutral loss; NLS, neutral loss scan; PE, phosphatidylethanolamine; PL, phospholipid; PS, phosphatidylserine; QNLs, quadruple neutral loss scan; RSD, relative standard deviation.

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the mass spectrum. Therefore, eliminating endogenous interference and improving the ionization efficiency and detection sensitivity of PLs would be particularly valuable, especially for lipid species with low proton affinities and minor content in biological samples (such as PE and PS). HPLC-MS with the column eluent injected directly into the mass spectrometer is most widely approached for identification of PL molecular species and their quantitation. It is well-suited to analyze lipids in the extremely low abundance regime where the salutary aspects of increased analyte concentration during peak elution, differential elution times for lipids with different chemical structures, and discrimination of confounding mass overlaps that are currently difficult or have intractable problems for direct infusion approaches (6). Using multiple reaction monitoring (MRM) with a mass spectrometer, multiple diagnostic fragmentation patterns have shown a potential to minimize the false discovery rates in the low abundance regime. However, the disadvantages of LC is that peak tailings on reverse-phase LC often yield inferior separations and low detection sensitivity of some PLs (such as PS). And for the quantification of aminophospholipids as a complex mixture of closely related compounds, using HPLC-MS raises significant problems with respect to sensitivity and accuracy because of the nonuniformity response caused by the differential fragmentation kinetics of individual aminophospholipid species. The differential fragmentation kinetics results from the distinct chemical constitution (including acyl chain lengths and unsaturation) of individual species and can lead to species-dependent mass spectra after collision-induced dissociation (CID) (7, 8). The use of isotope internal standards of each analyte ensures high accuracy of quantitative measurement during HPLC-MS analysis. However, commercially available isotope internal standards of aminophospholipids are extremely limited and expensive. Alternatively, at least two internal standards are generally required for each class of aminophospholipid, and a calibration curve is typically constructed using the internal standards to quantify the species of the entire class. The selection of the internal standards for each class should well represent the chemical structures that span the entire class of interest, but should not be present in the biological samples. However, there are large numbers of aminophospholipids present in biological samples, while commercial standards are only available for a limited range, which may require being synthesized in the laboratory.

The advantages of integrating chemical derivatization with MS analysis include improved selectivity and sensitivity. Aminophospholipids and Oalkenyl ether double bonds of plasmalogen lipids are shifted in mass by using a “fixed charge” sulfoniumion containing S,S’-dimethylthiobutanoyl-N-hydroxysuccinimide (DMBNHS) ester and iodine/methanol reagent, respectively, to resolve the overlapping of these lipid classes (9). Han et al. (10) presented a one-step in situ derivatization strategy using fluorenylmethoxylcarbonyl chloride (Fmoc-Cl) to identify and quantify PE and lysoPE molecular species from lipid extracts of biological tissues with significantly improved detection sensitivity. Recently, 18-crown-6 ether has been used as a derivatization agent for the derivatization of PE species and the MS signal intensities of derived PEs were improved by more than 10-fold (11).

The pattern change of the lipid species in a lipidome has been widely used in relative quantification measures by integrating derivatization with MS analysis. In this method, two samples, one from a control condition and the other from an experimental condition, are metabolically or chemically labeled. A set of four (D0, D4, D6, and D10) deuterium-enriched 4-(dimethylamino) benzoic acid (DMABA) N-hydroxysuccinimide (NHS) ester reagents were developed that react with the primary amine group of PE lipids to analyze all subclasses of DMABA-labeled PE with a common precursor ion scan (12). We previously successfully developed the acetone stable isotope derivatization with double neutral loss (NL) scan technology to profiling of PE based on shotgun ESI tandem-quadrupole MS analysis (13). Although derivatization can improve the selectivity and detection sensitivity, a series of derivatization reagents is still required. The excess derivatization reagents should be removed before the sample injection into the mass spectrometer. Otherwise, the excess reagents would reduce the signals of the analytes and probably damage the mass spectrometer. It is really necessary to develop a fast, efficient, and reliable method for derivatization-based qualitative and quantitative analysis of aminophospholipids.

In contrast to the relative quantification method, absolute quantification determines the mass levels of individual lipid molecular species, which is critical for the elucidation of the biochemical mechanisms responsible for the changes and the pathway/network analysis. However, absolute quantification is often impractical due to a limited range of commercially available standards and the large numbers of aminophospholipids present in biological samples (13, 14). It is obviously impractical to use thousands of stable isotope-labeled internal standards for quantitative analyses of the sheer number and high complexity of lipids in a biological sample. Recently, we developed an absolute quantification method for determination of PL molecular species in biological samples. Two or more PL standards were selected for each PL class and derivatized with TMSCHN₂, which were then used as the internal standards for construction of a calibration curve to normalize the nonuniformity response resulting from the distinct chemical constitution of individual PL species in the biological samples (15). It is a promising methodology for profiling and accurate quantification of complex lipid molecules in biological samples.

In this study, a novel strategy based on acetone stable-isotope derivatization coupled with HPLC-MS for profiling and accurate quantification of aminophospholipids in biological samples was developed. Acetone derivatization leads to alkylation of the primary amino groups of aminophospholipids with an isopropyl moiety; the use of deuterium-labeled acetone (d₆-acetone) introduced a 6 Da mass shift that was ideally suited for profiling and quantification analysis with high selectivity and accuracy. After derivatization, significantly increased column efficiency for
chromatographic separation and detection sensitivity for MS analysis of aminophospholipids was observed. Furthermore, an accuracy quantification method was developed. Aminophospholipids in biological samples were derivatized with d0-acetone; while more than two aminophospholipid standards were selected for each class of aminophospholipids and derivatized with d6-acetone, which were then used as the internal standards to typically construct a calibration curve for each class to normalize the nonuniformity response caused by the differential fragmentation kinetics resulting from the distinct chemical constitution of individual aminophospholipid species in the biological samples. The innovation enlarges the choice range of internal standards, for it obviates selecting standards absent in the biological samples, which makes it easy to choose many standards representing the chemical structures of the entire class to be used as internal standards. Its excellent applicability was validated by profiling and quantification of aminophospholipids presented in liver samples from rats fed with different diets.

MATERIALS AND METHODS

Materials
Sodium cyanoborohydride (NaBH3CN), d6-acetone (99.9 atom percent deuterium), and HybridSPE cartridges (50 mg, volume 2 ml) were obtained from Sigma-Aldrich (St. Louis, MO). HPLC-grade hexane, acetic, isopropanol (IPA), chloroform (CHCl3), methanol, ammonium hydroxide (>25%), formic acid (>99%), acetic acid (>99%), and ammonium acetate were purchased from CNW (Düsseldorf, Germany). Sodium chloride and all other solvents and chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. and were of analytical grade. The water used throughout the study was purified on a Milli-Q apparatus (Millipore, Bedford, MA).

Aminophospholipid standards (see the supplemental information) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). All aminophospholipid standards were dissolved in methanol/chloroform (1:1, v/v) at a concentration of 1 mg/ml as a premixed solution and stored at −20°C in glass tubes.

Acetone derivatization
The reactions of acetone (light labeling) and d6-acetone (heavy labeling) with aminophospholipids are shown in Fig. 1. In brief, the dried aminophospholipid extracts or the aminophospholipid standards were dissolved in methanol at a concentration of approximately 1 nmol/ml NaBH3CN (the reducing reagent) was freshly prepared before using with d0- or d6-acetone at a concentration of 3 μg/μl. After mixing 100 μl of aminophospholipids (10 nmol/ml), 300 μl of acetone containing 3 μg/μl of NaBH3CN, and methanol in a 5 ml glass tube, the reaction was performed at 40°C for 50 min. Subsequently, the reaction mixture was carefully swirled for several seconds and then immediately dried under a stream of nitrogen. Afterward, the residue was reconstituted in 1 ml of methanol/chloroform (1:1, v/v) and subjected to HPLC-MS analysis.

LC-MS analysis
LC separation was performed on a Shimadzu LC-20AD HPLC system (Tokyo, Japan) equipped with two 20AD pumps, a SIL-20AC autosampler, a CTO-20A thermostat column compartment, and a CMB-20A and DGU-20A3R degasser. An Eclipse Plus C18 column (100 × 4.6 mm inner diameter, 1.8 μm particle size; Agilent Technologies, Wilmington, DE) was used for separation. Solvent A [IPA/methanol/water (5/1/4, v/v/v) with ammonium acetate (10 mM) and acetic acid (0.1%, v/v)] and solvent B [IPA/water (99/1, v/v) with ammonium acetate (10 mM) and acetic acid (0.1%, v/v)] were employed as mobile phase. The following gradient was used: 0–5 min 0% B (100% A), 5–20 min 0–40% B (100–60% A), 20–30 min 40–100% B (60–0% A), 30–38 min 100% B (0% A), 38–40 min 100–0% B (0–100% A). Flow rate, 0.4 ml/min; injection volume, 10 μl; column temperature, 50°C. The retention time of each peak was obtained from the chromatogram.

A major issue that usually arises in chemical derivatization is that the excess derivatization reagents are difficult to remove and show high ionization efficiency, which not only interferes with the analysis but also pollutes the mass spectrometer. Thus, the excess derivatization reagent should be removed before MS analysis. In this study, both d0- and d6-acetone are easily volatilized, which will not interfere with the analysis. However, after derivatization, the excess reducing reagents (NaBH3CN) are difficult to remove. If liquid-liquid extraction was used for removing the excess NaBH3CN, the step should be repeated five times to completely get rid of the remaining NaBH3CN, which requires a large amount of organic solvent, is time-consuming, and is not practical for routine use (12). Therefore, a convenient, rapid, and stable system for removing excess derivatization reagents is required. A 09049017 10-port valve (Valco Instruments Co., Houston, TX) coupled to the Eclipse Plus C18 column with a two-position actuator control module configuration of the HPLC-valve was used as an auto-switcher for removing excess NaBH3CN. The two-position (position A and position B) actuator control module (VICI Valco Instruments Co., Inc.) was used to automatically control the 10-port valve to alternately switch to position A and position B. Within 4.5 min, the 10-port valve was set to switch to position A and the early eluate of excess salt NaBH3CN was removed as waste; after that, the 10-port valve was set to switch to position B and the eluate of analytes was directed to the mass spectrometer for analysis.

A 4000 Q-Trap mass spectrometer (Sciex, Toronto, ON, Canada) combined with HPLC was used for analysis. MS conditions were as follows: EI mode, positive; curtain gas pressure, 137.9 kPa; collision gas, medium; nebulizer current, 27.58 kPa; temperature, 500°C; scan mode, MRM-enhanced product ion (MRM-EPI); scan rate, 4000 µs; curtain gas, 35 psi; ion source gas 1 (GS1), 50 psi; ion source gas 2 (GS2), 55 psi; interface heater, on; declustering potential, 90 V; collision energy, 35 V and 55 V; collision energy spread, 5 V; collision cell exit potential, 17 V; mass range: enhanced mass scan, m/z 400–1,100; enhanced product ion (EPI), m/z 50–1,000. The EPI scan mode was applied for qualitative analysis, and the MRM-EPI mode was used for quantification of aminophospholipids. MRM parameters, declustering potential, and collision energy are listed in supplemental Table S1. Data acquisitions were performed using Analyst 1.6.1 software (Applied Biosystems). Multiquant software (Applied Biosystems) was used to quantify all aminophospholipids.

Animal experiments
Twenty-four male Sprague-Dawley rats (Sino-British Sippr/Bk, Shanghai, China) initially weighing 150–180 g were used in this study. The rats were housed individually and maintained at a controlled ambient temperature (20–22°C), humidity (45–50%), and under diurnal conditions (light-dark: 0800–2000) with access to laboratory chow and tap water ad libitum. After being acclimated for 1 week, the rats were randomly distributed into four experimental groups of six animals each and fed for 12 weeks. The experimental groups were distributed as follows: control group (C, n = 6), fed with Research Diets Inc. D12492 [60 kcal% fat (high-fat]
diet); rapeseed polyphenol intervention group (S, n = 6) (supplemented with 200 mg rapeseed polyphenols per kilogram high-fat diet); grape seed polyphenol intervention group (R, n = 6) (supplemented with 200 mg grape seed polyphenols per kilogram high-fat diet); mixed rapeseed polyphenol and grape seed polyphenol intervention group (F, n = 6) (supplemented with a mixture of 100 mg rapeseed polyphenols and 100 mg grape seed polyphenols per kilogram high-fat diet). After 12 weeks, rats were euthanized by asphyxiation with CO2. The livers were excised quickly, perfused with ice-cold PBS to remove blood, blotted with Kimwipes (Kimberly-Clark, Roswell, GA) to remove excess buffer, and then immediately freeze-clamped at the temperature of liquid N2. All tissue samples were stored at −80°C until lipid extraction. The animal procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals. And all experimental procedures were approved by the Oil Crops Research Institute Council on Animal Care Committee, Chinese Academy of Agricultural Sciences.

Lipid extraction and purification

Lipid extraction was performed using the Bligh and Dyer method with some modifications (16, 17). Briefly, liver tissues were ground with a pestle in a mortar containing liquid nitrogen. Liver tissue of about 50 mg was weighed and homogenized with 1 ml of normal saline in a 1.5 ml centrifuge tube by a homogenizer and transferred into a disposable glass tube. Then, 4 ml of extraction solvent (chloroform/methanol/water, 2:1:1, v/v/v) were added to the liver homogenate. The sample was vortexed for 15 min and then centrifuged at 11,000 g for 5 min. The subnatant was transferred into a new glass tube. The extraction process was repeated twice, and the subnatant was combined. The combined extract was dried under a nitrogen stream, capped, and stored at −20°C.

To avoid matrix effect and ion suppression, a HybridSPE-PL cartridge (50 mg, 2 ml volume; Sigma-Aldrich/Supelco, Bellefonte, PA) was used to purify and enrich aminophospholipids in liver samples. Under acidic conditions, aminophospholipids with phosphate moieties behave as strong Lewis bases (electron donors), which can interact with Zr atoms coated on the surface of the HybridSPE-PL column; under basic conditions, the bound aminophospholipids can be eluted with a basic solution. The SPE process is based on the steps in our previously published article (15). The HybridSPE-PL column was first activated with 3 ml of methanol, and then the lipid extract reconstituted in 300 µl of methanol with 4% (v/v) formic acid was loaded onto the SPE column. Afterward, the SPE column was washed with 1 ml of methanol with 4% (v/v) formic acid and then 1 ml of methanol in sequence. After that, the aminophospholipids trapped on the HybridSPE-PL column (PE and PS) were eluted with six consecutive 1 ml aliquots of methanol with 5% (v/v) ammonium hydroxide. The fractions were combined and dried under a stream of nitrogen and reconstituted in methanol for derivatization.

Qualitative analysis

For qualitative analysis of the aminophospholipid species present in the liver tissues of rats, a pooled rat liver sample was prepared by taking an equal amount of each liver sample from 24 male Sprague-Dawley rats. After extraction, the purified aminophospholipids from the rat livers were then divided into two aliquots equally, which were labeled with d0-acetone and d6-acetone, respectively. Then d0- and d6-acetone-labeled samples were mixed at 1:1 (v/v) and subjected to LC-quadruple neutral loss scan (QNLS)-MS analysis. Four characteristic neutral fragments (NL 183 and 189 Da or 227 and 233 Da) were generated from d0- and d6-acetone-labeled target compounds in CID, respectively, and were used to profile aminophospholipids. Peak-pair data were extracted from the two ion chromatograms (183 and 189 Da or 227 and 233 Da, respectively) according to a mass shift of 6 Da (i.e., d6-acetone labeled − d0-acetone labeled = 6 Da), and only peak pairs with the same retention time and intensities were assigned to the potential candidates of aminophospholipids. The numbers of total carbon atoms and double bonds in the aliphatic chains of the aminophospholipid molecules were deduced.
through detection of $m/z$ values of labeled aminophospholipid extracts through NL scan (NLS). And then the fatty acid composition of individual aminophospholipid molecules was further determined and confirmed by EPI scan in negative ion mode.

**Quantitative analysis**

Figure 2 shows the workflow of the sample processing and relative quantification analysis of aminophospholipids based on isotope labeling. In this strategy, aminophospholipid extracts in liver samples from rats fed with the polyphenol diets and the normal control group were converted to heavy-labeled and light-labeled samples, respectively. The light- and heavy-labeled sample solutions were mixed (1:1, v/v) and followed by HPLC-MS analysis with MRM scan in positive ion mode. The peak areas of heavy-labeled aminophospholipid species were extracted and ratiometrically compared with the peak areas of the corresponding light-labeled aminophospholipids to obtain the results of relative quantitative analysis.

Figure 2 also shows the workflow of the sample processing and absolute quantification analysis of aminophospholipids based on isotope labeling. Aminophospholipid extracts from liver samples were light labeled, while aminophospholipid standards in each class of aminophospholipids representing the chemical structures of the entire class, including PE (12:0_12:0), PE (16:0_18:1), PE (22:6_22:6), PS (12:0_12:0), PS (16:0_18:1), and PS (22:6_22:6) (with concentrations of 2.00, 2.00, 2.00, 1.50, 1.50, and 1.50 nmol/ml, respectively), were heavy labeled and used as internal standards. The heavy-labeled standards were spiked into the light-labeled aminophospholipid extracts from liver samples. For the accurate quantification of the species in the entire class, a

![Data acquisition](image)

**Fig. 2.** Workflow of the sample processing and quantification analysis of aminophospholipids based on isotope labeling.
calibration curve was typically constructed using the internal standards to normalize the nonuniformity response of individual aminophospholipid species in the liver samples.

An example of the absolute quantification of PE species is described. First, PE (12:0_12:0), PE (16:0_18:1), and PE (22:6_22:6) standards with the concentration of 0.50 nmol/ml were heavy-labeled and spiked into light-labeled PE extracts from liver samples to be used as the internal standards (Std1, Std2, and Std3, respectively). The known concentrations of the added Std1, Std2, and Std3 were recorded as C1, C2, and C3, respectively (C1, C2, and C3 did not need to be exactly equal). m1, m2, and m3 were the m/z of Std1, Std2, and Std3, respectively. After LC-MS analysis with MRM scan, the peak areas for Std1, Std2, and Std3 at the addition concentration of C1, C2, and C3 were recorded as P1, P2, and P3, respectively. A calibration curve of the m/z value of the internal standard (i.e., C1, C2, and C3) versus the corresponding peak areas of P1, P2, and P3 was constructed. If C1, C2, and C3 were equal to each other, then P1, P2, and P3 were used to construct the calibration curve. If C1, C2, and C3 were not equal to each other, then the peak areas of Std2 and Std3 should be normalized to the peak area that represents the concentration of C1. After normalization, the peak areas P2’ and P3’ represent the peak areas of Std and Std at the same concentration (C1) as that of Std, respectively, i.e., P2’ = (C1/C2) P2 and P3’ = (C1/C3) P3. P1, P2’, and P3’ were used to construct the calibration curve. The data for the internal standard signal were fitted with the best straight line (least-squares fit) between the three standards, and the formula for the trend line was obtained.

The PE species in the liver samples were accurately quantified. We take one PE molecule in liver samples as an example: the target PE molecule was recorded as A and the concentration of A was recorded as C0. After LC-MS analysis, the peak area of light-labeled A was recorded as P0. The relative m/z of heavy-labeled A was recorded as M0. M1 was substituted into the formula of the calibration curve fitted by the internal standards. The calculated peak area can be obtained and recorded as P, which represents the theoretical peak area of A at the same concentration C1 as that of Std. In theory, C0/P = C1/P0; thus, the concentration of target compound (C1) is equal to (C0/P0)/P.

Quantification of PS species was identical with the aforementioned principles. PS (12:0_12:0), PS (16:0_18:1), and PS (22:6_22:6) standards with the concentration of 0.50 nmol/ml were heavy-labeled and used as the internal standards to quantify the PS species in liver samples.

Data analysis
Data acquisition and processing were conducted using AB SCIEX Analyst 1.6.2 software (Applied Biosystems, Foster City, CA). All results are expressed as mean ± standard deviation, and statistical analysis was conducted by a one-way ANOVA in comparison to control.

RESULTS AND DISCUSSION
Optimization of derivatization conditions
To achieve efficient and quantitative labeling of aminophospholipids with d0-acetone, the reaction conditions were optimized using standard PE (12:0_12:0) and PS (12:0_12:0) as a model and NaBH4CN as a reducing agent. The amount of reducing agent, concentration of the reaction mixture, reaction temperature, and reaction time for d0-acetone derivatization were successively investigated. The intensity of the derived and underived aminophospholipids was used to calculate the derivatization efficiency.

The effect of acetone dosage is shown in supplemental Fig. S1A. The derivatization efficiency increased as the acetone dosage increased from 0 to 300 µl/0.1 µg PE/PS, increasing sharply in the range of 0–100 µl/0.1 µg PE/PS. The derivatization efficiency reached a plateau when the acetone dosage was >300 µl/0.1 µg PE/PS. Therefore, 300 µl/0.1 µg PE/PS was used for the alkylation reaction.

As shown in supplemental Fig. S1B, the derivatization efficiency increased as the amount of NaBH4CN increased from 0 to 5,000 µg/100 µl d0-acetone and then decreased slightly at higher concentrations. The highest derivatization efficiency was reached with 5,000 µg NaBH4CN/100 µl d0-acetone. Therefore, 5,000 µg NaBH4CN/100 µl d0-acetone was used for the following experiment.

In theory, because elevated temperature helps to overcome the energy barrier, a relatively high reaction temperature would be beneficial for increasing the rate of reaction and enhancing derivatization efficiency. However, higher temperatures lead to oxidation and decomposition of lipids and their derivatives. Therefore, the reaction temperature was explored over the range of 20–70°C (supplemental Fig. S1C). The results show that derivatization efficiency increased from 20°C to 40°C, and then decreased at temperatures >40°C. Thus, 40°C was selected as the reaction temperature for further study.

The effect of reaction time (1–70 min) on the derivatization efficiency of d0-acetone labeling was also examined. Supplemental Fig. S1D illustrates that the derivatization efficiency reached a plateau with a reaction time of 50 min, suggesting that 50 min was sufficient for the derivatization of aminophospholipids by d0-acetone.

To achieve efficient and quantitative labeling of aminophospholipids with d6-acetone, the reaction conditions were also optimized, which was relatively similar to that mentioned above, except for the replacement of d0-acetone with d6-acetone. With the optimized reaction conditions, the reaction efficiency reached 97% for both d0- and d6-acetone.

MS behavior of aminophospholipids after acetone derivatization
To investigate the fragmentation behavior of acetone-labeled aminophospholipids in CID, the acetone-labeled aminophospholipid standards were analyzed with a product ion scan. The product ion mass spectra of PE (12:0_12:0) before derivatization and after labeling with d0- and d6-acetone are shown in Fig. 3A, B, and C, respectively. The fragmentation pattern shows the presence of a fragment ion corresponding to the NL of 141 Da from unmodified PE (12:0_12:0). Peaks at m/z 580.4, 622.2, and 628.2 represent [M+H]+-type ions of unmodified PE (12:0_12:0) and d0- and d6-acetone-labeled PE (12:0_12:0), respectively (Fig. 3A–C). After labeling with d0- and d6-acetone, ions corresponding to the loss of 183 and 189 Da neutral fragments (Fig. 3B and C, respectively) were observed. The product ion mass spectra of PS (12:0_12:0) before derivatization and after labeling with d0- and d6-acetone are shown in

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Fig. 3. Elucidation of fragmentation patterns of aminophospholipids with representative PE and PS standards. Product ion mass spectra of PE (12:0_12:0) and PS (12:0_12:0) before and after derivatization: unmodified PE (12:0_12:0) (A), light-labeled PE (12:0_12:0) (B), heavy-labeled PE (12:0_12:0) (C), unmodified PS (12:0_12:0) (D), light-labeled PS (12:0_12:0) (E), heavy-labeled PS (12:0_12:0) (F).

Fig. 3D, E, and F, respectively. The fragmentation pattern shows the presence of a fragment ion corresponding to the NL of 185 Da from unmodified PS (12:0_12:0). Peaks at m/z 624.2, 666.6, and 672.4 represent [M+H]+-type ions of unmodified PS (12:0_12:0) and d0- and d6-acetone-labeled PS (12:0_12:0), respectively (Fig. 3D–F). After labeling with d0- and d6-acetone, ions corresponding to the loss of 227 and 233 Da neutral fragments (Fig. 3E and F, respectively) were observed. The results imply that the fragmentation mainly occurred between the polar group and backbone of each class of aminophospholipids, and as expected, a 42 Da mass increase was observed after d0-acetone labeling; after d6-acetone labeling, a 48 Da mass increase was observed. Because the product ions were converted and fixed at m/z [M+H]+-183 and [M+H]+-189 or [M+H]+-227 and [M+H]+-233 for the d0- and d6-acetone labeled PE and PS, respectively, the MRM transitions of precursor ions could be easily acquired. MRM parameters, declustering potential, and collision energy are listed in supplemental Table S1.

Enhancement of detection sensitivity upon acetone labeling

Equal quantities of PE and PS standards (0.10 nmol/ml) with and without acetone derivatization were analyzed by LC-MRM-MS. It can be seen that the detection sensitivity of PS before and after labeling (Fig. 4C, D) is about a factor of ten lower than that of PE before and after labeling (Fig. 4A, B). Compared with unlabeled PE and PS (Fig. 4A, C), after acetone derivatization, significantly increased column efficiency and detection sensitivity for analysis were observed (Fig. 4B, D). Especially for PS, acetone labeling can significantly improve the separation efficiency and reduce peak tailing; the column efficiency of labeled PS increased about 10-fold and the detection sensitivity of labeled PS increased about 15-fold relative to that of unlabeled ones. Hence, after alkylation of primary amino groups of PEs and PSs by acetone labeling, the aminophospholipids with isopropyl moiety could be readily protonated under acidic conditions, which therefore could enhance ionization efficiency. The results imply that acetone derivatization can effectively improve the detection sensitivity of aminophospholipids, especially aminophospholipids with low proton affinities, such as PS. Acetone derivatization thus holds promise as a valuable analytical tool for low-abundance PS in biological samples.

To evaluate the linearity of the method, acetone-labeled aminophospholipid standard solutions at concentrations ranging from 0.1–1,000 pmol/ml were used to construct calibration curves by plotting the peak area against the concentration of labeled aminophospholipid standards. Satisfactory correlation coefficients were obtained (0.9977 to 0.9998). The LODs and limits of quantification (LOQs) were determined at a concentration where the signal-to-noise ratios were 3 and 10, respectively. Table 1 shows the LOQs, LODs, and the improvement of detection sensitivities of aminophospholipid standards after acetone derivatization. It can be seen that, after derivatization, the detection sensitivities for PE and PS were improved by about 8.56 and 16.06, respectively, which suggests that acetone derivatization can markedly improve the detection sensitivity of aminophospholipids.

The MRM mode is widely used in targeted metabolomics in LC-MS analysis, which has good repeatability, sensitivity, and broad dynamic range. For comparison, the d0-acetone-labeled aminophospholipid standards were also analyzed by direct infusion-NLS-MS, which is commonly used for lipid analysis (12, 15). Table 2 shows the LODs, LOQs, and
relative standard deviations (RSDs) of labeled aminophospholipid standards obtained by LC-MRM-MS and direct infusing-NLS-MS. The results show that the LODs improved by about 3- to 5-fold by using LC-MRM-MS compared with the direct infusing-NLS-MS method. Furthermore, by using LC-MRM-MS, significantly lower RSDs (1.46–3.65%) were observed compared with the RSDs (7.36–11.26%) obtained by using direct infusing-NLS-MS. Hence, the LC-MRM-MS method can significantly improve the detection sensitivity, selectivity, and accuracy, and is beneficial to profiling the aminophospholipids in biological samples.

Development and validation of the relative quantification method

Supplemental Fig. S2 shows the extracted ion chromatograms of 1:1 mixtures of d0- and d6-acetone-labeled aminophospholipids from rat livers analyzed with the LC-MS method. We can see that the light- and heavy-labeled aminophospholipids have the same retention time and peak area, enabling accurate identification and quantification by eliminating the MS response fluctuation and mutual interference from the two labeled samples.

To further illustrate the linearity and dynamic range, measurements corresponding to peak area ratios for aminophospholipids at different d0:d6-acetone proportions were plotted against their respective actual molar ratios, as shown in supplemental Fig. S3. The results show a decent linearity between the experimental peak area ratios and actual molar ratios over the range studied. Within this range, the correlation efficiency ($R^2$) was 0.9995 and 0.9978, demonstrating the feasibility of the suggested strategy for relative quantification of aminophospholipids. A 100-fold linear dynamic range over concentration...

**TABLE 1.** The LOQ, LOD, linearity range, and decreased folds of detection limit of aminophospholipid standards after d0-acetone derivatization by LC-MRM-MS analysis

| PLs          | LOQ (pmol/ml) | LOD (pmol/ml) | Linearity Range (pmol/ml) | Decreased Folds of Detection Limit |
|--------------|---------------|---------------|----------------------------|-----------------------------------|
| PE (12:0_12:0) | 0.37          | 0.12          | 0.37–1,000                 | 7.86                               |
| PE (16:0_18:1) | 0.42          | 0.11          | 0.42–1,000                 | 8.10                               |
| PE (18:1_18:1) | 0.44          | 0.13          | 0.44–1,000                 | 8.44                               |
| PE (20:4_20:4) | 0.53          | 0.22          | 0.53–1,000                 | 9.28                               |
| PE (22:0_22:6) | 0.58          | 0.25          | 0.58–1,000                 | 9.33                               |
| PS (12:0_12:0) | 0.76          | 0.20          | 0.76–1,000                 | 15.14                              |
| PS (16:0_18:1) | 0.8           | 0.33          | 0.80–1,000                 | 15.81                              |
| PS (18:1_18:1) | 0.79          | 0.34          | 0.79–1,000                 | 16.23                              |
| PS (20:4_20:4) | 0.85          | 0.32          | 0.85–1,000                 | 16.44                              |
| PS (22:0_22:6) | 0.88          | 0.30          | 0.88–1,000                 | 16.81                              |

Fig. 4. Extracted ion chromatograms of PE and PS standards before and after acetone derivatization. PE (12:0_12:0), PE (16:0_16:0), and PE (18:1_18:1) before derivatization (A), PE (12:0_12:0), PE (16:0_16:0), and PE (18:1_18:1) after derivatization (B), PS (12:0_12:0), PS (16:0_16:0), and PS (18:1_18:1) before derivatization (C), PS (12:0_12:0), PS (16:0_16:0), and PS (18:1_18:1) after derivatization (D).
Development and validation of the absolute quantification method

In order to investigate the accuracy of the developed absolute quantification method based on acetone stable isotope derivatization, six PE standards and six PS standards at concentrations of 0.50 nmol/ml were light labeled with d0-acetone. Three PE standards [PE (12:0_12:0), PE (16:0_18:1), and PE (22:6_22:6)] and three PS standards [PS (12:0_12:0), PS (16:0_18:1), and PS (22:6_22:6)] with the same concentration of 0.50 nmol/ml were heavy labeled with d6-acetone, which were then used as internal standards and spiked into the solution of light-labeled PE and PS standards (target PE and PS species). When only one internal standard was used for quantification, heavy-labeled PE (16:0_18:1) and PS (16:0_18:1) were selected as the internal standards. In theory, \( \frac{C_{IS}}{C_A} = \frac{P_{IS}/P_{IS}}{P_{1}/P_{1}} \), where \( P_{IS} \) and \( P_{1} \) are the peak area of the heavy-labeled internal standard and the light-labeled analytes, respectively; \( C_{IS} \) and \( C_A \) are the known concentration of the internal standard and the unknown concentration of analytes, respectively. Thus, the concentration of the analyte can be calculated by using \( C_A = C_{IS}(P_{IS}/P_{1}) \).

When two internal standards were used for quantification, heavy-labeled PE (12:0_12:0), PE (22:6_22:6) and PS (12:0_12:0), PS (22:6_22:6) were selected as the internal standards because their \( m/z \) values cover the range for the target PE and PS species, respectively. Two calibration curves of the \( m/z \) value of heavy-labeled PE (12:0_12:0), PE (22:6_22:6) and PS (12:0_12:0), PS (22:6_22:6) (i.e., \( M_I \) and \( M_A \)) versus their corresponding peak areas (i.e., \( P_I \) and \( P_A \)) at the same concentration (\( C_{IS} \)) were established, respectively. The internal standard signal data were fitted with the best straight line (least-squares fit) between the two standards, and the formula for the trend line was obtained. The target PE and PS species were recorded as \( A \), and the concentration of \( A \) was recorded as \( C_A \). The peak area of light-labeled \( A \) was recorded as \( P_{1} \), and the relative \( m/z \) of heavy-labeled \( A \) was recorded as \( M_A \). \( M_A \) was substituted into the formula of the calibration curve fitted with the two internal standards and three internal standards, respectively. The calculated peak area could be obtained and recorded as \( P \), which represents the theoretical intensity of \( A \) at the concentration (\( C_{IS} \)) of \( P_I \) and \( P_P \) or \( P_{IS} \) and \( P_P \). In theory, \( \frac{C_{IS}}{C_A} = \frac{P}{P_A} \); thus, the concentration of the target compound is expressed as \( C_A = C_{IS}(P/A) \).

Table 3 shows the results of accuracy comparison and verification of the aforementioned three quantification methods. When only one heavy-labeled internal standard was used for quantification, large discrepancies between the measured (0.30–0.49 nmol/ml) and actual (0.50 nmol/ml) concentrations of the light-labeled PE and PS species were observed. The larger the differences in the number of double bonds and the carbon chain lengths were between the internal standard and target PE and PS species, the less accurate the quantification results were, and vice versa. The quantification accuracy could be improved significantly if multiple internal standards were used. When two or three heavy-labeled internal standards were used for quantification, the majority of measured concentrations of PE and PS species were comparable to the actual concentrations. The reason is that PE and PS species with various acyl chains of differential chain length and unsaturation are related to their \( m/z \), so that selecting internal standards with different acyl chains of differential chain length and unsaturation can be used to calibrate these impacts on quantification of aminophospholipids. Thus, by selection of internal standards with the range of carbon chain lengths and degrees of unsaturation of the target analytes, the negative effects of these properties on the quantification can be corrected.

**Figure 5A** and D show the chromatograms of light-labeled PEs (0.50 nmol/ml) and light-labeled PSs (0.50 nmol/ml), respectively. It can be seen that, even at the same concentration, different peak areas of different
Aminophospholipid species were observed. The larger the differences in m/z values were, the larger the difference in the peak area of aminophospholipid species was, which can be compensated by an appropriate calibration curve. Calibration curves were fitted with the m/z values of heavy-labeled PE (12:0_12:0), PE (22:6_22:6) and heavy-labeled PS (12:0_12:0), PS (22:6_22:6) (two internal standards); or heavy-labeled PE (12:0_12:0), PE (16:0_18:1), PE (22:6_22:6) and heavy-labeled PS (12:0_12:0), PS (16:0_18:1), PS (22:6_22:6) (three internal standards) and their corresponding peak areas (Fig. 5B, E). Figure 5C and F depict the chromatograms of light-labeled PEs (0.50 nmol/ml) and light-labeled PSs (0.50 nmol/ml) after modification by the calibration curve fitted with the three internal standards, respectively. It can be seen that, after calibration, the peak areas of light-labeled PE and PS species with the same concentration were close to each other.

Identification of aminophospholipid species present in rat liver tissues

Quadruple NLS mode was used in profiling of aminophospholipids. In this respect, an equal volume of the pooled

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**TABLE 3. Accuracy comparison and verification of three different quantification methods by using single internal standard, double internal standards, and three internal standards, respectively**

| PLs          | Actual Concentration (nmol/ml) | Absolute quantification results (nmol/ml) |
|--------------|-------------------------------|-----------------------------------------|
|              |                               | Single Internal Standard | Double Internal Standards | Three Internal Standards |
| PE (12:0_12:0) | 0.50                          | 0.49 ± 0.09                  | 0.49 ± 0.07               | 0.50 ± 0.06               |
| PE (16:0_18:1) | 0.50                          | 0.48 ± 0.10                  | 0.50 ± 0.02               | 0.50 ± 0.08               |
| PE (17:0_17:0) | 0.50                          | 0.41 ± 0.12                  | 0.53 ± 0.04               | 0.51 ± 0.07               |
| PE (18:1_18:1) | 0.50                          | 0.38 ± 0.07                  | 0.53 ± 0.03               | 0.51 ± 0.08               |
| PE (20:4_20:4) | 0.50                          | 0.32 ± 0.02                  | 0.50 ± 0.10               | 0.50 ± 0.08               |
| PE (22:6_22:6) | 0.50                          | 0.32 ± 0.15                  | 0.48 ± 0.07               | 0.49 ± 0.05               |
| PS (12:0_12:0) | 0.50                          | 0.49 ± 0.07                  | 0.50 ± 0.03               | 0.50 ± 0.02               |
| PS (16:0_18:1) | 0.50                          | 0.46 ± 0.09                  | 0.50 ± 0.04               | 0.50 ± 0.03               |
| PS (17:0_17:0) | 0.50                          | 0.41 ± 0.02                  | 0.53 ± 0.08               | 0.52 ± 0.07               |
| PS (18:1_18:1) | 0.50                          | 0.37 ± 0.07                  | 0.53 ± 0.04               | 0.52 ± 0.04               |
| PS (20:4_20:4) | 0.50                          | 0.32 ± 0.02                  | 0.49 ± 0.03               | 0.49 ± 0.03               |
| PS (22:6_22:6) | 0.50                          | 0.30 ± 0.15                  | 0.48 ± 0.07               | 0.48 ± 0.06               |

Single internal standard: PE (16:0_18:1) or PS (16:0_18:1); double internal standards: PE (12:0_12:0) and PE (22:6_22:6) or PS (12:0_12:0) and PS (22:6_22:6); three internal standards: PE (12:0_12:0), PE (16:0_18:1) and PE (22:6_22:6) or PS (12:0_12:0), PS (16:0_18:1) and PS (22:6_22:6).
rat liver samples was labeled with d0- and d6-acetone, respectively. Then the light- and heavy-labeled samples were mixed and analyzed by LC-QNLS-MS. The LC-QNLS-MS approach consisted of four NLSs (NL 183 and 189 Da, or NL 227 and 233), which generated two individual ion chromatograms corresponding to the neutral fragments of d0- and d6-acetone-labeled aminophospholipids, respectively. Peak pairs data were extracted from the two ion chromatograms (NL 183 and 189 Da, or NL 227 and 233) according to a mass shift of 6 Da (i.e., d6-acetone labeled − d0-acetone labeled = 6 Da), and only peak pairs with the same retention times and intensities were assigned to be the candidates of aminophospholipids (Fig. 6A, B). For example, the peak intensities and retention times were the same in the extracted ion chromatograms at \( m/z \) of 762.5/768.5 under NL of 183 and 189 Da, and \( m/z \) of 832.5/838.5 under NL of 227 and 233 Da from d0- and d6-acetone-labeled samples (Fig. 6A, B), which can be deduced to be PE (34:0) and PS (36:1), respectively, according to LIPID MAPS.

The structures of the assigned candidates of aminophospholipids were further elucidated by EPI scan under negative ion modes. Figure 6C and D show the mass spectrum of d0-acetone-labeled PE (34:0) and PS (36:1), respectively. Two primary fragment ions (255.2 Da and 283.2 Da) were obtained from EPI scanning of 760.5 Da in negative ion mode (Fig. 6C), which confirmed that the fatty acid composition of PE (34:0) was PE (16:0_18:0). And as shown in Fig. 6D, two primary fragment ions (281.2 Da and 283.2 Da) were obtained from EPI scanning of 830.5 Da in negative ion mode, which confirmed that the fatty acid composition of PS (36:1) was PS (18:1_18:0).

Using the LC-QNLS-MS method, 71 potential aminophospholipid species (49 PEs and 22 PSs) were found in the rat liver sample (supplemental Table S2). A major advantage of the method is that the targeted analytes can be easily identified by extracting the peak pair data even if the retention time changed due to the use of a different analytical instrument.

Quantification of aminophospholipid species present in rat liver tissues

For relative quantification of aminophospholipids, rat liver samples from the control group (C, high-fat diet) were light labeled, and those from the rapeseed polyphenol...
intervention group (S), grape seed polyphenol intervention group (R), and mixed rapeseed polyphenol and grape seed polyphenol intervention group (F) were heavy labeled, respectively. The light-labeled and heavy-labeled samples were mixed at equal ratios, respectively, and then analyzed by HPLC-MRM-MS/MS. The results were obtained by dividing the peak area of heavy-labeled derivatives by that of light-labeled derivatives (supplemental Table S3). If the levels of aminophospholipid species of the control and polyphenol intervention groups are the same, then d6/d0 = 1. Thus, d6/d0 − 1 was used to represent relative changes in the levels of significantly changed aminophospholipid species as shown in Fig. 7. It can be seen that the vast majority of liver aminophospholipid species in the control group were close to the horizontal axis (|d6/d0 − 1| = 0). Compared with control group C (high-fat diet), 19 kinds of aminophospholipid species were significantly above the horizontal axis in the polyphenol intervention groups S, R, and F, including 10 kinds of PE species (PE32:1, PE34:3, PE36:3, PE42:7, PE44:12, PE44:9; plasmalogen PE36:1, PE38:0, PE40:5, PE40:4) and 9 kind of PS species (PS34:1, PS36:4, PS36:3, PS36:2, PS36:1, PS38:6, PS38:5, PS40:6, PS40:4). In contrast, one PE molecule (plasmalogen PE36:5) was significantly below the horizontal axis (Fig. 7). Most of the similar changes in lipid compositions were generally observed for the rapeseed polyphenol intervention group (S), grape seed polyphenol intervention group (R), and mixed rapeseed polyphenol and grape seed polyphenol intervention group (F), except that greater changes were observed in group R and F compared with that in group S.

A previous study discovered that PE32:1, PE34:3, PE36:3, and PS38:6 decreased significantly in the livers of rats fed with high-fat diet (18), suggesting that these aminophospholipid species may serve as an indicator of fatty liver disease. In this study, we found that, after polyphenol dietary intervention, most of the declined aminophospholipid species had increased, especially in groups R and F; the levels of PE and PS species were significantly increased, indicating that rapeseed polyphenols and especially grape seed polyphenols may have a role in improvement of fatty liver metabolic disease.

For accurate quantification of aminophospholipids, three heavy-labeled standards in each aminophospholipid class representing the chemical structures for the entire class were used as internal standards. Heavy-labeled standards were spiked into the light-labeled aminophospholipid extracts from the rat liver samples. A calibration curve based on the internal standards was constructed for each

![Fig. 7.](image-url) Significantly upregulated and downregulated aminophospholipid species from the liver of rats fed with different diets, analyzed by relative quantification after acetone derivatization. Control group (C, high-fat diet), rapeseed polyphenol intervention group (S), grape seed polyphenol intervention group (R), and mixed rapeseed polyphenol and grape seed polyphenol intervention group (F).
aminophospholipid class for calibration. The accurate quantification results are listed in supplemental Table S4. For confirmation, theoretical relative quantification values were also calculated by dividing the accurate quantification data for aminophospholipids from the polyphenol intervention groups by those from control groups. It can be seen from supplemental Table S5 that most of the ratios of accurate quantification data for aminophospholipid species were consistent with the relative quantification values. These results further demonstrate the reliability of the accurate quantification method.

Supplemental Fig. S4 shows the content of total PEs and PSs in the livers of rats fed with different diets. It can be seen that the total content of PEs (7,912.34–8,642.44 nmol/g) was much higher than that of PSs (469.39–758.12 nmol/g) in four groups of rat liver (more than 10 times). Studies in the literature have reported that the content of PE and PS decreased in the livers of rats fed with high-fat diet (18). In this study, we found that, as compared with control group C (high-fat diet, total PEs 7,912.34 nmol/g and total PSs 469.39 nmol/g), the total PE and PS content in polyphenol intervention groups S (PEs 8,093.21 nmol/g and total PSs 725.92 nmol/g), R (PEs 8,642.44 nmol/g and total PSs 758.12 nmol/g), and F (PEs 8,448.09 nmol/g and total PSs 570.19 nmol/g) increased significantly (supplemental Fig. S4), indicating that after polyphenol intervention, the content of aminophospholipids in the liver of rats fed with high-fat diet was significantly increased, which was closer to that in the livers of normal rats. Rapseed polyphenols and grape seed polyphenols have a role in improvement of metabolic diseases, such as nonalcoholic fatty liver disease and cardiovascular disease (19), which are consistent with the results of this study. Furthermore, by comparison of the polyphenol intervention groups S, R, and F, it was found that the increase of PE and PS content in the R group was the most obvious (supplemental Fig. S4), which may indicate that the effect of grape seed polyphenols (R group) was better than that of rapseed polyphenols (S group) and mixed rapseed polyphenols/grape seed polyphenols (F group).

In addition, from the perspective of fatty acid composition in aminophospholipids, supplemental Fig. S3 shows that the content of total EPA and DHA of aminophospholipids increased in rats liver of polyphenol intervention groups S (EPA 83.36 nmol/g and DHA 691.24 nmol/g), R (EPA 93.46 nmol/g and DHA 729.73 nmol/g), and F (EPA 92.65 nmol/g and DHA 734.08 nmol/g) by comparing with the content of EPA and DHA in the control high-fat diet group C (EPA 79.17 nmol/g and DHA 670.89 nmol/g). And the total EPA and DHA content of aminophospholipids in polyphenol intervention groups R and F increased significantly. Research shows that the protective effects of α-linolenic acid in nonalcoholic fatty liver disease are attributed to EPA and DHA synthesis (20, 21). There are multiple factors regulating EPA and DHA levels (22), and higher levels of these metabolites may contribute to the depletion of their precursor, α-linolenic acid (20, 21).

In conclusion, we developed a novel method based on acetone stable-isotope derivatization coupled with HPLC-MS for profiling and accurate quantification of aminophospholipids in biological samples. Acetone derivatization leads to alkylation of the primary amino groups of aminophospholipids with an isopropyl moiety, the use of deuterium-labeled acetone (d6-acetone) introduced a 6 Da mass shift that was ideally suited for profiling and quantification analysis with high selectivity and accuracy. After derivatization, significantly increased column efficiency for chromatographic separation and detection sensitivity for MS analysis of aminophospholipids was observed. Furthermore, an accuracy quantification method was developed. Aminophospholipids in biological samples were derivatized with d6-acetone; while more than two aminophospholipid standards were selected for each class of aminophospholipid and derivatized with d6-acetone, which were then used as the internal standards to typically construct a calibration curve for each class to normalize the nonuniformity response caused by the differential fragmentation kinetics resulting from the distinct chemical constitution of individual aminophospholipid species in the biological samples. Its excellent applicability was validated by profiling and quantification of aminophospholipids presented in liver samples from rats fed with different diets. The method developed in this article is a promising methodology for profiling and accurate quantification of complex lipid molecules in biological samples.

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