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2019

Huang, Q., Lei, H., Dong, M., Tang, H., & Wang, Y. (2019). Quantitative analysis of 10 classes of phospholipids by ultrahigh-performance liquid chromatography tandem triple-quadrupole mass spectrometry. The Analyst, 144(13), 3980–3987. doi:10.1039/c9an00676a

https://hdl.handle.net/10356/143894

https://doi.org/10.1039/C9AN00676A

ARTICLE

Quantitative analysis of 10 classes of phospholipids by ultrahigh-performance liquid chromatography tandem triple-quadrupole mass spectrometer

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Phospholipids are the main constituents of biological membranes and their biological function has been increasingly recognized. Therefore, there is an unmet need to develop methods capable of quantifying a wide range of phospholipids with high sensitivities and high throughput. We employed an ultrahigh-performance liquid chromatography system coupled to a triple-quadrupole mass spectrometer (UHPLC-MS) and developed a method that can quantitatively analyze 10 major classes of phospholipid in biological samples in 11 mins. These are phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, sphingomyelin, lyso-phosphatidic acid, lyso-phosphatidylcholine and lyso-phosphatidylethanolamine. The limit of detection (LOD) and limit of quantitation (LOQ) are 0.04-33 pmol/mL and 0.1-110 pmol/mL, respectively. The method takes three steps: first and second steps identified phospholipid structures in a mixture containing aliquots of all the samples using the combinations of multiple reaction monitoring (MRM), product ion scan and retention time in the positive and negative ion mode. These steps enable identification of phospholipids presented in the samples and provided information for efficient sample analysis in the final step of sample quantitative analysis. We have developed fast and sensitive label-free quantitation with normalization of acyl chain length to achieve more accurate quantification. The method developed was applied to analyze 6 different biological samples (plasma, cells and tissues) for applicability validation, where a total of 308 phospholipid species across 10 phospholipid classes were identified and 295 phospholipid species were quantified. The method is highly efficient, sensitive, and is universally applicable.

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Introduction

Phospholipids are known to be the main constituents of biological membranes and their biological function has been recognized in recent decades.[1, 2] Additionally, phospholipids can act as cell signaling molecules in signal transduction,[3, 4] and influence the function and metabolism of lipoprotein.[5, 6] Phospholipids can be divided into glycerophospholipids (GP) and sphingolipids (SP). Glycerophospholipids consist of two esterified nonpolar acyl chain (sn-1 and sn-2 positions) and a phosphoric acid polar head. Depending on the functional groups of phosphoric head, glycerophospholipids can form phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA). If presented with one of the esterified nonpolar acyl chains (sn-1 or sn-2 positions), glycerophospholipids can form a range of lyso-glycerophospholipids. Another category of phospholipid is sphingolipids, the basic unit of which is ceramide or dihydroceramide and when the hydroxyl group of ceramides is replaced by phosphorylcholine, ceramide or dihydroceramide forms sphingomyelin (SM).

Due to the complexity and diversity of phospholipids (different polar head, the length of acyl chain and the number of unsaturation), the phospholipids detection has been a focused attention. Previously, colorimetry and spectrophotometry are used to detect total phospholipids.[7, 8] Thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) can detect the concentrations of each class of phospholipids, but it cannot identify the molecular structure of phospholipids.[9, 10] Untargeted method employed for many lipidomic studies involves the direct infusion mass spectrometry (MS)[11-13]. The untargeted analysis is simple, fast and high throughput. However, the untargeted assay can result in ion suppression with too many compounds, which can interfere the minor species within a sample matrix, leading to reduced sensitivity and inaccuracy in quantification.[14, 15] Another drawback of the untargeted assay is that it cannot distinguish the isomers.[16] The high-performance liquid chromatography (HPLC) tandem mass spectrometry (HPLC-MS) method is a well-developed and widely used method for detecting phospholipids.[17-19] The HPLC-MS approach can reduce the matrix effects and improve isomers separations and sensitivity.[20] However, the HPLC-MS approach is time consuming, it can take hours in some of the analysis, hence not applicable to large sample-set analysis.[21] Therefore, new improved method for detecting phospholipids with high accuracy, high sensitivity and high throughput is needed for large human cohort studies.

In this study, we developed a targeted analysis method that is capable of detecting 10 main classes of phospholipids using an ultrahigh-performance liquid chromatography system coupled to a triple-quadrupole mass spectrometer (UHPLC-MS). The 10 classes of phospholipids include PA, PC, PE, PG, PI, PS, SM, lyso-phosphatidic acid (LPA), lyso-phosphatidylcholine (LPC) and lyso-phosphatidylethanolamine (LPE). Qualitative and quantitative analysis of phospholipids were achieved in three steps. First, phospholipid species were screened on pooled samples by MRM mode, which detected all the possible precursor ions. This procedure identified the precursor ions that were presented in the samples, so that in the second step, which was structure identification step, we only perform MS/MS spectra on the precursor ions that were presented in the samples. By combination of the first and second step, we were able to pinpoint the numbers of phospholipid species that needed to be quantified in given sample set. Therefore, in the final step of sample analysis, only selective MRM detection was required for the quantification of phospholipids. In addition, relative response of phospholipids with different acyl chain lengths to MS was taken into consideration for quantification. Our newly developed method is capable of simultaneously detecting and quantifying 10 classes of phospholipids with detection sensitivity of femtomole range within 11 minutes. The method was tested for the suitability for wide-ranges of samples, including plasma, cell and tissue, proving the applicability of the method.

Materials and methods

Reagents and Chemicals

HPLC grade ammonium acetate and formic acid were purchased from Sigma-Aldrich (St. louis, MO, USA). HPLC grade chloroform was purchased from Duksan Pure Chemicals (Seoul, Korea), methanol was obtained from ThermoFisher Scientific (Beijing, China), and deionized water was prepared with an Elix Advantage system (Waters, Millipore, MA, USA).

Phosphatidic acid (from egg yolk lecithin), phosphatidylcholine (from egg yolk), phosphatidylethanolamine (from egg yolk), phosphatidylglycerol (from egg yolk lecithin), phosphatidylinositol (from bovine liver), phosphatidylserine (from bovine brain) and sphingomyelin (from chicken egg yolk) were obtained from Sigma-Aldrich (St. louis, MO, USA). Phospholipid standards including PC (14:0/14:0), PC (16:0/18:1),

PC (18:1/18:1), PC (18:0/18:0), PE (14:0/14:0), PE (16:0/16:0), PE (18:1/18:1), PG (18:1/18:1), PG (18:0/18:0), PS (16:0/18:1), PA (16:0/16:0) and PI (18:0/20:4) were purchased from Sigma-Aldrich (St. louis, MO, USA). PC (17:0/20:4), PC (21:0/22:6), PE (17:0/20:4), PE (21:0/22:6), PG (17:0/20:4), PG (21:0/22:6), PS (17:0/20:4), PS (21:0/22:6), PA (17:0/20:4), PA (21:0/22:6), PI (17:0/20:4), PI (21:0/22:6) and ten internal standards (IS) PC (17:0/14:1), PE (17:0/14:1), PG (17:0/14:1), PS (17:0/14:1), PA (17:0/14:1), PI (17:0/14:1), LPC (17:1), LPE (17:1), LPA (17:1), SM (d18:1/17:0) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All standards are dissolved in methanol/chloroform (v/v = 1:1) solution. The standards were prepared at 1 mg mL^{-1} concentrations in the appropriate solvent, stored at -20℃, and diluted appropriately before analysis. The internal standards were prepared at 10 μg mL^{-1} concentrations and stored at -20℃ before sample preparation. The nomenclature of phospholipids described by LIPID MAPS (http://www.lipidmaps.org/).

Sample preparation

Ten plasma samples from healthy people were collected from volunteers, informed consent form was obtained from each of participant. A549 (human alveolar epithelial carcinoma) was purchased from American Type Culture Collection, and 16HBE (normal human bronchial epithelial cells) was purchased from Peking University Health Science Center (China). Both A549 and 16HBE cells were cultured in dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine plasma (FBS) at 37℃ and 5% CO_2. Adipocyte formed by inducing the differentiation of 3T3L1 mouse embryo fibroblasts based on the method of Jonathan G. Boucher et al.[22] The cells were digested by trypsinization and washed with cold PBS 3 times, then stored at -80℃ until extraction. 10 normal rat plasma and liver samples were taken from SD rats.

Phospholipids were extracted based on the method described by Bligh and Dyer.[23] Briefly, 80 μL plasma or 10 mg cells or 10 mg tissues for quantitative analysis were transferred into 2 mL eppendorf tube, then 10 internal standards (100 ng of each) was added. The plasma and cells were added with 1 mL of $CHCl_3$ / CH_3OH (v/v = 1:1), while the tissues were extracted with 1 mL of $CHCl_3$ / CH_3OH (v/v = 1:1) using Tissuelyzer II (QIAGEN TissueLyser II, Germany) for 90 s at 20 Hz. The mixture was then added with 0.45 mL deionized water and vortexed followed by centrifugation at 11, 060 g for 10 min at 4℃. The bottom layer containing phospholipids were collected whereas the top layer was subjected to the same extraction procedure. The samples collected from the two extractions were pooled and re-dissolved in 80 μL $CHCl_3$ / CH_3OH (v/v = 1:1) after removing solvent. The treatment of mixed sample (approximately 1 mL of mixed plasma sample or 100 mg of cells or tissues) for qualitative analysis is consistent with the sample preparation for quantitative analysis. The extracted samples were stored at -20℃ for the UHPLC-MS/MS analysis.

UHPLC-MS conditions

An Agilent 1290 ultrahigh-performance liquid chromatography system coupled to a 6460 triple-quadrupole mass spectrometer equipped with a dual AJS electrospray ionization (ESI) source (Agilent Technologies, Inc, USA) was used to analyze the complex mixture of extracted phospholipids. Data were collected in ESI positive and negative ion modes in separate runs.

The final method for phospholipids analysis employed a ZORBAX Eclipse plus C18 (2.1 x 100mm, 1.8μm) column from Agilent. A binary isocratic elution with 98%B was applied, with water for solvent A and methanol for solvent B. Both solvent A and B included 0.01% formic acid and 5mM ammonium acetate. The column temperature was 50℃, the injection volume was 2 μL, and the flow rate was 0.5 mL/min. Capillary voltage was set at 4.0 kV (positive ion mode) and 3.5 kV (negative ion mode), respectively. Sheath gas flow was set at 8 L/min. The final quantitative method only runs for 7 minutes and 4 minutes in positive and negative ion mode for a sample, respectively. The fragmentor, collision energy and other experimental conditions were optimized for each class of phospholipid.

Data analysis

All the MRM spectra and MS/MS spectra data were processed manually by Mass Hunter Qualitative software (Agilent, B.06.00) and Mass Hunter Quantitative software (Agilent, B.06.00).

The concentrations of phospholipid species were calculated from their relative abundances related to the internal standard of each phospholipid class, and then normalized by the wet weight or volume of the samples. Principal component analysis (PCA) was used to analyze the quantitative data of phospholipids among different kinds of samples. PCA was performed with SIMCA-P+ (v12.0, Umetrics, Sweden) and the trend of clustering and distribution of the data could be seen in the map of PCA score.

Results and discussion

Choice of column and optimization of UHPLC-MS conditions

Reversed-phase (RP) HPLC is often used to separate the lipid species based on the carbon number and the double bond number of acyl chains,[21, 24, 25] while the normal-phase HPLC is often used to separate the classes of lipids.[26] In addition, the peak width, analysis time and solvent consumption were greatly reduced with the development of UHPLC.[24, 27, 28] Therefore, RP-UHPLC-MS system was used to analyze the phospholipids in our research. In order to select the appropriate columns, 3 columns including Agilent ZORBAX Eclipse plus C18 (2.1×100 mm, 1.8 μm), Agilent ZORBAX Eclipse plus C8 (2.1×150 mm, 1.8 μm), Phenomenex Kinetex 2.6u C18 (2.1×100 mm) were tested with the mixed phospholipid standards. The positive ion mode was used for PC, PE and SM, while the negative ion mode was used for PA, PG, PI and PS. Finally, the Agilent ZORBAX Eclipse plus C18 column was selected for the analysis, because the separation was better for both positive and negative ion modes (Fig. 1 and Fig. 2).

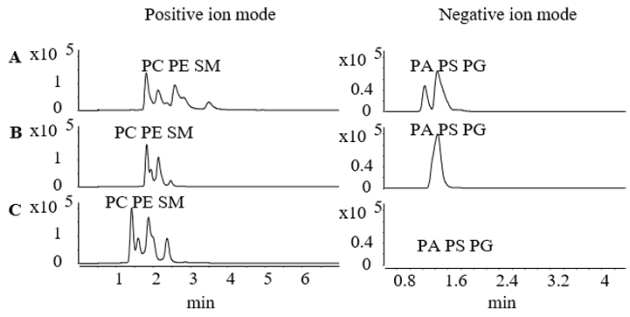

Fig. 1. Choice of column. The mobile phase A (water) and B (methanol) was used to analyze the mixed phospholipid standards. Both A and B included 5mM ammonium acetate and 0.01% formic acid. The positive ion mode detected PC, PE and SM, the negative ion mode detected PA, PG, PI and PS. A binary isocratic elution with 98% B was applied, the column was held at 45 ℃ and the separation was allowed at a flow rate of 0.4 mL/min. **A:** Agilent ZORBAX Eclipse plus C18 (2.1×100 mm, 1.8 μm); **B:** Agilent ZORBAX Eclipse plus C8 (2.1×150 mm, 1.8 μm); **C:** Phenomenex Kinetex 2.6u C18 (2.1×100 mm).

We employed 10 internal standards for the optimization of mobile phase by combinations of 3 kinds of mobile phase: (1) mobile phase A (water) and mobile phase B (methanol), (2) mobile phase A (water / 5mM ammonium acetate) and mobile phase B (methanol / 5mM ammonium acetate), (3) mobile phase A (water/ 5mM ammonium acetate / 0.01% formic acid) and mobile phase B (methanol / 5mM ammonium acetate / 0.01% formic acid). The positive ion mode was used for PC (17:0/14:1), PE (17:0/14:1), LPC (17:1), LPE (17:1) and SM (d18:1/17:0), while the negative ion mode was used for PA (17:0/14:1), PG (17:0/14:1), PI (17:0/14:1), PS (17:0/14:1) and LPA (17:1). The results showed that phospholipids could be separated well using the mobile phase A (water / 5mM ammonium acetate / 0.01% formic acid) and B (methanol / 5mM ammonium acetate / 0.01% formic acid) both in positive and negative ion modes (Fig. S-1). Our method employed the same mobile phase for both positive and negative ion mode in order to improve throughput of phospholipid analysis. Other parameters of liquid chromatography and mass spectrometric were also optimized (data were not shown).

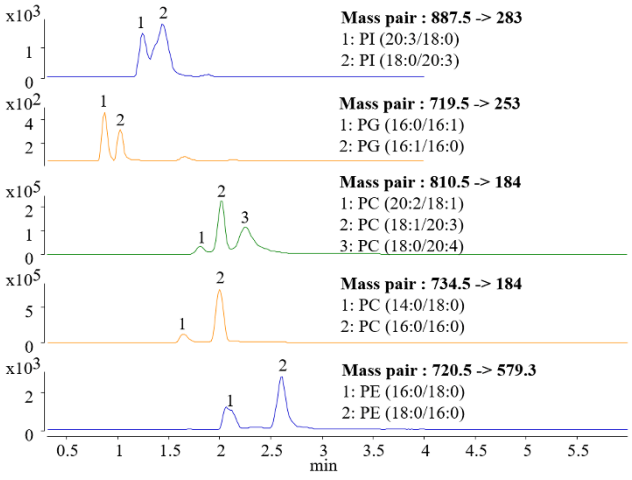

Fig. 2. The separation of isomers in mixed phospholipid standards. PG and PI were analyzed in negative ion mode, and PC, PE were analyzed in positive ion mode with an Agilent ZORBAX Eclipse plus C18 (2.1×100mm, 1.8μm) column.

Identification of phospholipids

A two-step procedure was developed in our method for the identification of phospholipids. The first step was to identify numbers of phospholipids using MRM mode employing a pooled-sample and the second step was to identify structure of phospholipid species in the pooled sample, which was achieved by performing MS/MS spectra. To employ MRM detection, all the possible transitions of phospholipids was firstly established. It is well established that glycerophospholipids are constituted by two non-polar acyl chains (sn-1 and sn-2 positions) and a phosphoric acid polar head, which can be PC, PE, PG, PI, PS and PA when proton of the phosphoric acid replaced by choline, ethanolamine, etc. (Fig. S-2). Lyso-glycerophospholipids (LPA, LPC, LPE) can be formed by hydrolyzing one of the esterified

nonpolar acyl chains (sn-1 or sn-2 positions) of glycerophospholipids (Fig. S-2). The basic unit of sphingomyelins (SM) is ceramide or dihydroceramide, and SM is formed when the hydroxyl group of ceramides or dihydroceramide is replaced by phosphorylcholine (Fig. S-2). There are total of 21 commonly known fatty acids in mammalian cells.[29] Therefore, the combination of 21 fatty acids and with above mentioned 10 classes of phospholipid could generate a total of 2751 possible phospholipid species (2646 glycerophospholipids, 63 lyso-glycerophospholipids, 42 SMs). Finally, a total of 2751 possible phospholipid species could be selected for detection.

The choice of ions and fragments for detecting of these phospholipids has previously been reported in many studies.[19, 30-32] The most abundant ions are protonated molecules $[M+H]^+$ (PC, PE, SM, LPC and LPE), neutral losses of water $[M+H-H_2O]^+$ (SM, LPC and LPE) in positive ion and deprotonated molecules $[M-H]^-$ (PG, PI, PS, PA and LPA) in negative ion mode. The MS/MS spectra of phospholipids provided the well-known characteristic fragment ions and neutral losses (Table 1). The fragment ion of *m/z* = 184 is observed in PC, LPC and SM which contain phosphocholine, *m/z* = 153 is observed in PA, LPA and PG while *m/z* = 241 is observed in PI. The neutral loss of $\triangle m/z$ = 141 is observed for PE and LPE which contain phosphoethanolamine, and $\triangle m/z$ = 87 for PS. In addition, the acyl chains of glycerophospholipids are lost to form ketene ($[M+H-RCHCO]^+$ or $[M-H-RCHCO]^-$) and a carboxylic acid ($[M+H-RCOOH]^+$ or $[M-H-RCOOH]^-$) in MS/MS spectra, which can help to identify the position and composition of acyl chains. However, the carboxylate anions $[RCOO]^-$ from the acyl chain is the main fragment in negative ion mode. Combined with the above information, we calculated all the fragment ions for the possible 2751 phospholipid species (Fig. S-2). For example, 16:0 and 18:1 fatty acid can generate 4 PCs including PC(16:0/16:0), PC(16:0/18:1), PC(18:1/16:0) and PC(18:1/18:1), and it can also form 4 PEs, 4 PAs, 4 PGs, 4 PIs, 4 PSs, 2 LPCs, 2 LPEs, 2 LPAs and 4 SMs, the detailed structure and fragment ions are shown in Table S-1. We then verified the calculated fragment ions with 10 classes of phospholipid standards (Fig. S-3), and the results showed that the fragments of each class of phospholipid standard is consistent with the calculated information.

The first MRM measurement was established with the most abundant ions as the precursor ions, and the characteristic fragment ions or neutral losses for the identification of phospholipid species as the daughter ions (Table 1). Since isomers have the same precursor ions and daughter ions (Fig. 2), the 2751 possible phospholipid species can produce 584 mass pairs for MRM analysis. For a given batch of sample, we pooled small aliquot of each sample and used a pre-scan by the first MRM measurement. If there presented a precursor ion without chromatographic peak (e.g. m/z = 872.5, Fig. 3), then this suggested that the phospholipid was not present in the sample, hence was filtered out in the subsequent MS/MS spectra. If the precursor ions with chromatographic peaks (e.g. m/z = 760.5, Fig. 3) were observed, then further MS/MS spectra were performed with different gradient collision energy through the product ion scan (Fig. 3). At this first step, we were able to identify the number of phospholipid species per class presented in given sample set.

Table 1. The MRM parameters for qualitative and quantitative analysis of phospholipids

Phospholipids	Precursor ion	Daughter ion (qualitative analysis)		Daughter ion (quantitative analysis)		Fragmentor (V)	Collision energy (V)
		Positive ion mode	Negative ion mode	Positive ion mode	Negative ion mode		
PC	$[M+H]^+$	184		184		200	30
PE	$[M+H]^+$	$[M+H-141]^+$		$[M+H-141]^+$		160	15
PA	$[M-H]^-$		153		$[RCOO]^-$	210	25
PG	$[M-H]^-$		153		$[RCOO]^-$	270	45
PI	$[M-H]^-$		241		$[RCOO]^-$	290	45
PS	$[M-H]^-$		$[M-H-87]^-$		$[RCOO]^-$	200	20
SM	$[M+H]^+$	184		184		160	25
LPC	$[M+H]^+$	184		184		160	25
LPE	$[M+H]^+$	$[M+H-141]^+$		$[M+H-141]^+$		160	15
LPA	$[M-H]^-$		153		153	130	25

Table 2. The calculation equation of the relative response

GP	Equation	Range (nmol/mL)	R^2	Slope (RSE, %)	Intercept (RSE, %)	Compounds (n)
PC	y = -0.050 x + 2.510	0.01-1	0.966	8.00	6.33	6
PE	y = -0.043 x + 2.314	0.01-1	0.981	6.98	4.02	6
PA	y = -0.064 x + 2.995	0.02-1	0.982	6.25	5.98	4
PG	y = -0.076 x + 3.371	0.02-1	0.825	26.32	21.95	5
PI	y = -0.082 x + 3.550	0.02-1	0.999	0.24	0.30	3
PS	y = -0.056 x + 2.741	0.02-1	0.996	3.64	3.47	3

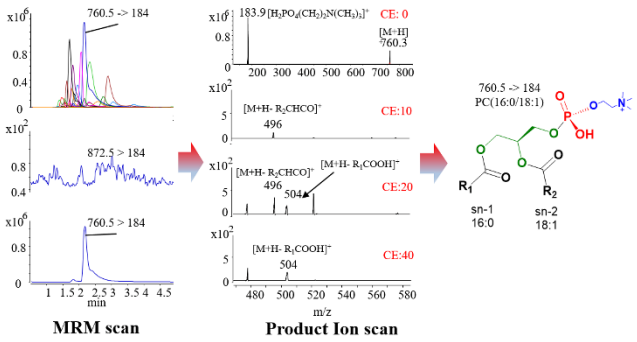

Fig. 3. Process for structure identification of phospholipids [e.g. PC (16:0/18:1)]. Phospholipid species were screened on pooled samples by MRM mode, and the precursors without chromatographic peaks (e.g. 872.5 -> 184) were filtered out, while the precursors with chromatographic peaks (e.g. 760.5 -> 184) were further fragmented with the MS/MS spectra from Product ion scan. The MS/MS spectra of precursor ion according MRM with gradient collision energy, such as 760.5 -> 184. This data used to identify the position and composition of acyl chains of glycerophospholipids based on the rule that the sn-2 (m/z = 496, $[M+H-R_2CHCO]^+$) is more likely to lose with lower collision energy than sn-1 (m/z = 504, $[M+H-R_1COOH]^+$).

The second step of the method was to identify the phospholipid structure with product ion scan. Since phospholipid standards are not available for all species, the identification of acyl chains is necessary, this was achieved by performing MS/MS spectra, which is a widely accepted approach for analysis of lipids.[11, 13] The product ion scan were repeated with identical chromatographic conditions of step one based on the determined precursor ions and retention time. The MS/MS spectra were used to identify the fatty acid compositions and their positions based on the rule that the fatty acid in sn-2 is more likely to lose with lower collision energy than sn-1.[33] For example, the MS/MS spectra of m/z 760.5 with gradient collision energy (Fig. 3), the sn-2 (m/z = 496, $[M+H-R_2CHCO]^+$) is more likely to lose with lower collision energy than sn-1 (m/z = 504, $[M+H-R_1COOH]^+$), and the structure is PC (16:0/18:1) combined with the structural and fragment ion of PC. Based on the first step of identify phospholipid species, in the second step of identification of acyl chain step, we only need to conduct verification for the existing phospholipids. Phospholipid identification was performed by manual MS/MS spectra interpretation and compared with all the fragment ions of 2751 possible phospholipid species that we previously calculated.

Quantitative analysis of phospholipids

Once we identified phospholipids presented in the pooled samples, quantification of phospholipids in individual samples can be carried out by MRM mode. We used $[M+H]^+$ or $[M-H]^-$ as the precursor ions, the characteristic fragment ions as the product ions of PC, LPC, LPA, SM, the neutral losses as the product ions of PE, LPE and $[RCOO]^-$ as the product ions of PA, PG, PI, PS (Table 1). The final detection method only runs for 7 minutes in positive ion mode and 4 minutes in negative ion mode for a sample. Hence, our qualitative analysis method is high throughput and suitable for analyzing large number of samples.

Odd number fatty acyl of phospholipid was selected as the internal standard for each phospholipid class as suggested by previous publications.[19, 34, 35] However, internal standards are not available for all species of phospholipids, which will lead to inaccuracy for quantification because the MS response varies for different acyl chain lengths. [30] In order to overcome this problem, we performed dilution experiments on 6 PC standards having different acyl chain lengths (Fig. 4A). We found that there presented a good linear relationship between the carbon numbers and the relative response for all dilutions within 0.01-1 nmol/mL (Fig. 4B). Then we performed multiple linear regression using results obtained from all the different dilutions (0.01, 0.02, 0.05, 0.1, 0.5, 1 nmol/mL) and generated a new linear equation,

which could be used to correct differences in MS response for different acyl chain length (Fig. 4C). Similarly, relative response for PE, PA, PI, PS, PG were generated in a similar way (Table 2, Fig. S-4). The relative standard error (RSE) of intercept and slope between experimental values and those obtained from new linear equation is less than 10% with exception of PG (Table 2). The relative response for each class of phospholipids containing different carbon numbers were listed in Table S-2, which could be used for accurately quantification of phospholipids in biological samples with one internal standard.

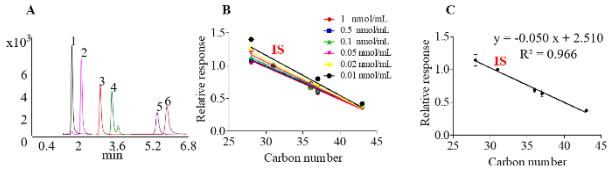

Fig. 4. The curve of the relative responses. **A:** The MRM of 6 PCs used to calculate the relative response. 1: PC (14:0/14:0) 2: PC (17:0/14:1) 3: PC (17:0/20:4) 4: PC (18:1/18:1) 5: PC (21:0/22:6) 6: PC (18:0/18:0); **B:** The curves of the relative response of PC at 6 different concentrations. The horizontal axis is the carbon number of two acyl chains of phospholipids, and the vertical axis is the relative response. C: The new linear curve of the relative response of PC obtained by multiple linear regression.

Validation of the method

We further evaluated the limit of detection (LOD) and the limit of quantitation (LOQ) of our method. This was performed based on the analysis of 10 internal standards with a serious of dilutions. The LOD and LOQ were determined from values of signal-to-noise ratios (*S/N*). The LOD and LOQ were set when *S/N* equals 3 and 10, respectively. The results showed that the LOD was 0.04-33 pmol/mL and LOQ was 0.1-110 pmol/mL (Table 3), the LOD improved by 3-500 times comparing to the method previously published [19, 34], suggesting better sensitivity of our method.

We also evaluated the precision and accuracy of the method developed currently. Precision was conducted by assessing the reproducibility of peak area and retention time from 5 consecutive measurements of mixed phospholipid standards for low (0.01 nmol/mL), middle (0.1 nmol/mL) and high (1 nmol/mL) concentrations. The intraday (morning, noon and evening) and inter-day (two consecutive days) was evaluated and expressed as the relative standard deviation (RSD) of the peak area and retention time. The results showed that 5.1 ± 2.7 % for average RSD in peak area and 4.8 ± 2.3 % for average RSD in retention time of mixed phospholipid standards (Table S-3), indicating that the methodology has better precision. Accuracy was represented by the relative error between the experimental value and the theoretical value after quantitative correction by the relative response of the phospholipid standards at different concentrations (Table S-4). The results showed that the relative error of all glycerophospholipids except PG was almost between ± 15%, PI and PS were even between ± 10% (Table S-4), indicating that the methodology has better accuracy.

Phospholipid analysis of biological samples

Finally, the developed method was applied to a range of common biological samples of plasma (human plasma, rat plasma), cells (A549, 16HBE and adipocyte) and organ tissue (rat liver) to test the applicability. We identified a total of 308 phospholipid species across 10 phospholipid classes in the plasma, cells and liver tissue and these included 86 PCs, 53 PEs, 12 PAs, 22 PSs, 31 PGs, 26 PIs, 16 LPAs, 18 LPCs, 17 LPEs, 27 SMs (Table S-5). Among these, 221 phospholipid species were quantified using relative response and internal standards, and 74

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Table 3. The LOD and LOQ of phospholipids

Phospholipids	LOD (pmol/mL)	LOQ (pmol/mL)	Phospholipids	LOD (pmol/mL)	LOQ (pmol/mL)
PC (17:0/14:1)	0.04	0.10	PI (17:0/20:4)	0.71	2.35
PE (17:0/20:4)	0.09	0.21	SM (d18:1/17:0)	0.04	0.10
PA (17:0/14:1)	1.11	3.69	LPA (17:1)	33.14	110.45
PG (17:0/14:1)	0.80	2.64	LPC (17:1)	0.11	0.37
PS (17:0/14:1)	0.78	2.60	LPE (17:1)	1.21	4.01

The LOD and LOQ were determined from signal-to-noise ratios (S/N) 3 and 10, respectively. LOD: The limit of detection. LOQ: The limit of quantitative.

Table 4. The total numbers of phospholipid species per class in different biological samples

Phospholipids	Serum			Cells				Tissue	
	Human serum		Rat serum	A549 cell		16HBE cell	Adipocyte	Rat liver	
	Our method	Reference		Our method	Reference			Our method	Reference
PC	59	41	43	42	29	42	29	43	21
PE	28	18	15	31	25	31	15	25	14
PA	3	ND	1	10	ND	10	1	2	ND
PG	10	4	7	11	8	11	8	18	14
PI	16	17	15	16	22	16	8	22	5
PS	5	7		11	21	11	8	16	7
SM	14	19	12	15	13	15	14	16	11
LPC	14	21	17	11	14	11	10	17	12
LPE	16	6	10	10	8	10	9	15	8
LPA	13	ND	11	11	ND	11	3	10	ND
Total	178	133	131	168	140	168	105	184	92

phospholipid species (LPC, LPE, LPA and SM) were quantified using internal standards for relative quantification (Table S-5). The rest of 13 phospholipid species were not quantified as the concentration was lower than the LOQ (Table S-5). The results showed that the total number of phospholipid species was different for different biological samples (Table 4). The total number of PC and PE was the most abundant phospholipids in all the biological samples investigated here, which was anticipated as PC and PE are the main phospholipids in membrane.[1] The PCA results showed that different biological samples had different phospholipid composition, and the distribution of different biological samples showed an obvious separation trend (Fig 5). These results showed that the number and the concentration of phospholipid species were very different for different biological samples.

In total, we identified 178 phospholipid species in human plasma (Table S-5), which is comparable to those observed from human plasma [35] (Table 4). However, since we used targeted analysis, which is more quantitative. We also identified a total of 168 phospholipid species in A549 cells and a total of 184 phospholipid species in rat liver (Table S-5), which is also comparable to those obtained by others (Table 4). [34] These results showed that our method is able to better quantify phospholipid species. We also noted that there were 41 common phospholipid species (11 PCs, 1 PE, 5 PIs, 1 PG, 2 LPA, 8 LPCs, 6 LPEs, 7 SMs) in the samples studied (Fig. S-5). These 41 phospholipids are probably the most abundant in lipidomic extracts, but the levels of these phospholipids were variable among these different biological samples.

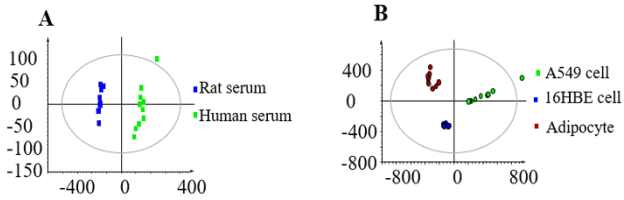

Fig. 5. The principle component analysis of phospholipids in plasma (R^2X=0.957, Q^2=0.886) and cells (R^2X=0.967, Q^2=0.907) that is generated from data displayed in Table S-5, n = 10.

Conclusions

In the current research, we developed a UHPLC-MS method that is capable of quantifying 10 classes of phospholipids with higher sensitivity and analysis efficiency. The method can be performed in three steps: the first step is to identify phospholipid class using MRM on mixed aliquots of all samples and the second step is to identify the structure of phospholipids in the mixture using MS/MS spectra performed on only selected precursor ions that presented in samples. The final step is to quantify the phospholipids on samples. In our method, more

comprehensive phospholipid species is covered, hence could identify more phospholipid species compared to previous published method. Our method was able to quantify 10 classes of phospholipids in 11 min with a high throughput: LOD of 0.04-33 pmol/mL and LOQ of 0.1-110 pmol/mL, which is more sensitive and more efficient (especially the large sample size) compared to previously published methods. In addition, we developed relative response for better quantification of PC, PE, PA, PG, PI and PS by taking different MS response of acyl chain length into consideration. Furthermore, applicability of this method was performed on different biological samples, including plasma, cells and organ tissues, demonstrating that the method is widely applicable.

Acknowledgments

This work was supported by grants from the National Key R&D Program of China (2017YFC0906800) and National Science Foundation of China (21675169)

Conflicts of interest

There are no conflicts to declare.

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Graphical abstract