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2D2D HILIC-ELSD/UPLC-Q-TOF-MS Method for Acquiring Phospholipid Profiles and the Application in *Caenorhabditis elegans*

Xiaotian Sun, Tao Zhang, Pinzhen Zhao, Guanjun Tao, Ruijie Liu, Ming Chang,* and Xingguo Wang

Phospholipids are the main constituent of cellular membranes and have recently been identified to have diagnostic value as biomarkers for many diseases. Accordingly, much emphasis is now laid on developing optimal analytical techniques for the phospholipid profiles of various biological samples. In the present study, different classes of phospholipids are first separated by optimized hydrophilic interaction chromatography with evaporative light scattering detector (HILIC-ELSD). The phospholipids in each class are then identified by ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). Validation results confirm that this approach meets the requirements of quantitative analysis. Finally, the approach is adopted to analyze the phospholipid profiles in *Caenorhabditis elegans*. A total of 111 phospholipid species is identified according to the mass fragments. Major fatty acyl chains in phospholipids are found to be formed by oleic acid (C18:1), arachidonic acid (C20:4), and eicosapentaenoic acid (C20:5). Overall, this study improves current knowledge on analytical techniques of the phospholipid composition in *C. elegans* and provides a basis for future lipidomics research.

Practical applications: Phospholipids reportedly play a crucial role in the development of many diseases. Until now, only a small portion of phospholipids in *Caenorhabditis elegans* has been reported by using one-dimensional analysis strategy. The offline 2D2D liquid chromatography method developed in this study identifies 111 phospholipid species in *Caenorhabditis elegans*. The obtained phospholipid profiles complement the lipid database of *Caenorhabditis elegans*. The study also provides the basis for the future development of a 2D online approach.

1. Introduction

Phospholipids are widely acknowledged as the main constituents of biofilm. Several classes of phospholipids have been reported, including phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylcholine (PC), as well as their hydrolytic degradation products, such as lysophosphatidylcholine (LPC).^[1] Each class of phospholipids contains different head groups with various fatty acyl chains. Interestingly, an environmental change can reportedly affect the phospholipid composition and structure, thus regulating the biological membrane function.^[2] Phospholipids can be separated by reverse-phase high-performance liquid chromatography (RPLC),^[3] normal-phase high-performance liquid chromatography (NPLC),^[4] and hydrophilic interaction liquid chromatography (HILIC),^[5] and subsequently analyzed by ultraviolet absorption detector (UV), evaporative light scattering detector (ELSD), or mass spectrometry (MS).^[6] Phospholipids are eluted in order of increasing polarity, and solvents with lower polarity are often utilized as the mobile phase during NPLC. NPLC-ELSD is nowadays widely used to separate, identify, and quantify different classes of

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phospholipids. However, studies have shown that this method failed to discriminate phospholipids with different fatty acyl chains.^[7] Another disadvantage of using NPLC-ELSD is that the retention times are not reproducible over a long time due to the inevitable accumulation of polar compounds on the silica gel, which decreases its adsorption ability.^[8] HILIC uses a mobile phase system similar to RPLC, giving the same elution order as NPLC with high reproducibility and less retention time drift.^[9,10] HILIC provides similar benefits to NPLC, which uses simple mobile phases for efficient separation. An increasing body of evidence suggests that HILIC coupled with MS is suitable for analyzing polar compounds.^[11,12] However, the drawbacks of HILIC and NPLC are similar. HILIC can only separate different classes of phospholipids and is unable to separate phospholipids from the same class. In contrast, RPLC can separate other molecules from the same phospholipid class but exhibits poor ability to separate phospholipids of different classes.^[13,14] Importantly, differences in the molecule hydrophobicity determined by fatty acyl chains allow phospholipids to be separated by RPLC.^[15] However, the overlap of different phospholipids in the RPLC chromatogram negatively affects the quantification process. Therefore, HILIC or RPLC alone allows the separation of phospholipids with limited resolution.

NPLC,^[16] HILIC,^[17] and silver-ion chromatography (for non-polar lipids)^[18] have previously been coupled with online or offline RPLC. Determination of online and offline modes was based on whether the two-step procedure was automated or required manual handling. Complex phospholipid samples can be separated with the head groups in one dimension and fatty acyl chains with hydrophobic properties in the other dimension. Online 2D techniques allow a fully automated process with low sample loss and degradation risks. However, this method is compromised by the difficulties in synchronizing the two dimensions and the requirement of advanced instruments. Offline 2D techniques are more laborious but allow the conditions for each dimension to be fully optimized to yield the phospholipids of interest.

Over the past decades, *Caenorhabditis elegans* has been widely utilized in life science studies, especially as an animal model for aging and disease-related *in vivo* studies.^[19,20] Phospholipids are the main constituent of the biological membrane and can assist in the diagnosis of many diseases. For instance, PE deficiency has been found to disrupt α -synuclein homeostasis in *C. elegans* models of Parkinson's disease.^[21] Moreover, PC and sphingomyelin (SM) have been associated with aging,^[22] while PC-34:1 and PC-34:3 were reportedly positively associated with longevity but negatively associated with diabetes.^[23] Accordingly, documentation of the phospholipid metabolite profiles of different diseases would improve current knowledge on the metabolic pathways that are altered during disease development. In this respect, many studies have sought to identify phospholipid profiles in *C. elegans* in recent years. For example, in a study where gas chromatography (GC-MS) was applied to analyze phospholipids in *C. elegans*, PC and PE were mainly identified due to their high abundance.^[24] Only a small portion of phospholipids in *C. elegans* have been reported using a one-dimensional chromatography analysis approach. Accordingly, the purpose of this study was to develop an offline 2D method (HILIC-ELSD/UPLCQ-TOF-MS) to identify phospholipids in *C. elegans*. In the first dimension, phospholipids

were separated into different classes using a HILIC column. The separated phospholipids in each class were collected and then identified using a C18 column in the second dimension. Importantly, the phospholipid profiles obtained in this study was helpful for establishing a phospholipid database and contributed to future lipidomic studies in *C. elegans*.

2. Results and Discussion

2.1. Comparison of RPLC and HILIC

It is widely acknowledged that different classes of phospholipids can be separated on a HILIC column based on the heterogeneity of their head group composition. RPLC can also separate different phospholipids due to hydrophobic interactions with the stationary phase and is also influenced by the carbon chain length and the number of double bonds in the acyl chains. As shown in **Figure 1**, RPLC and HILIC were adopted to analyze five classes of phospholipids with the same saturation degree and chain length. The C18 column was used for RPLC separation and the HILIC column for HILIC separation. The results indicated that an individual peak was observed for each phospholipid class separated. Phospholipids showed a closer retention time to each other by using RPLC, which were between 8.18 and 10.47 min. The HILIC approach showed a superior peak resolution (**Figure 1B**) while the retention time of different phospholipids did not overlap, ranging from 8.06 to 17.29 min. Various phospholipids classes were effectively separated using HILIC-ELSD. A large sample loop was used for HILIC separation, which facilitated the collection of more samples for subsequent identification.

2.2. First Dimension Separation by HILIC-ELSD

As shown in **Figure 2**, LPC, PA, PC, PE, PG, PS, and SM were effectively separated. Acetonitrile with ammonium acetate (10 mmol L⁻¹) was chosen as gradient elution to get better resolution. The efficiency of the HILIC column was compromised at temperatures lower than 30 °C or higher than 50 °C. Thus, the column temperature was kept lower than 50 °C to avoid the degradation of phospholipids during analysis. Analyses performed at 30, 35, and 40 °C are shown in **Figure 2A–C**. The optimal column temperature was at 35 °C. Additionally, the ELSD drift tube temperature significantly affected the response intensity. Optimizations were also performed at 45, 50, and 55 °C (**Figure 2D–F**). As shown in **Figure 2E**, acceptable resolution and optimal repeatability were observed at 50 °C. Overall, different classes of phospholipids were effectively separated using the optimized conditions. The retention time indicated that phospholipids with lower polarity were first eluted. For instance, LPC was eluted before PC due to its relatively lower polarity. In the present study, the retention time of various phospholipids increased in the following order: PG < PA < PS < PE < LPC < PC < SM.

The operating conditions, including the column type, mobile phase, column temperature, and drift tube temperature, were optimized to obtain good separation by HILIC-ELSD. As for detector selection, ELSD allowed the detection of nonvolatile substances with good sensitivity compatible with gradient elution.

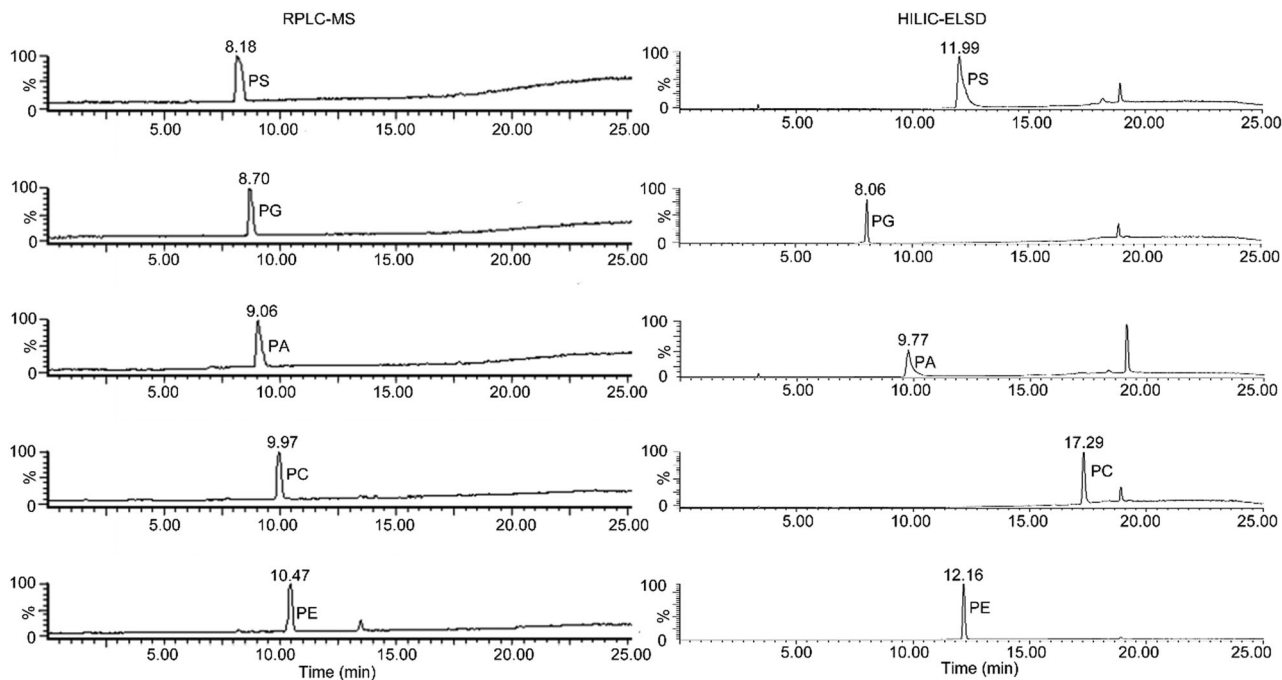


Figure 1. Chromatograms of five classes of phospholipids (with C14:0 fatty acyl chains) obtained by A) RPLC and B) HILIC. The chromatograms performed with the hilic column temperatures at C) 30 °C, D) 35 °C, E) 40 °C and drift tube temperatures at F) 45 °C, G) 50 °C, H) 55 °C.

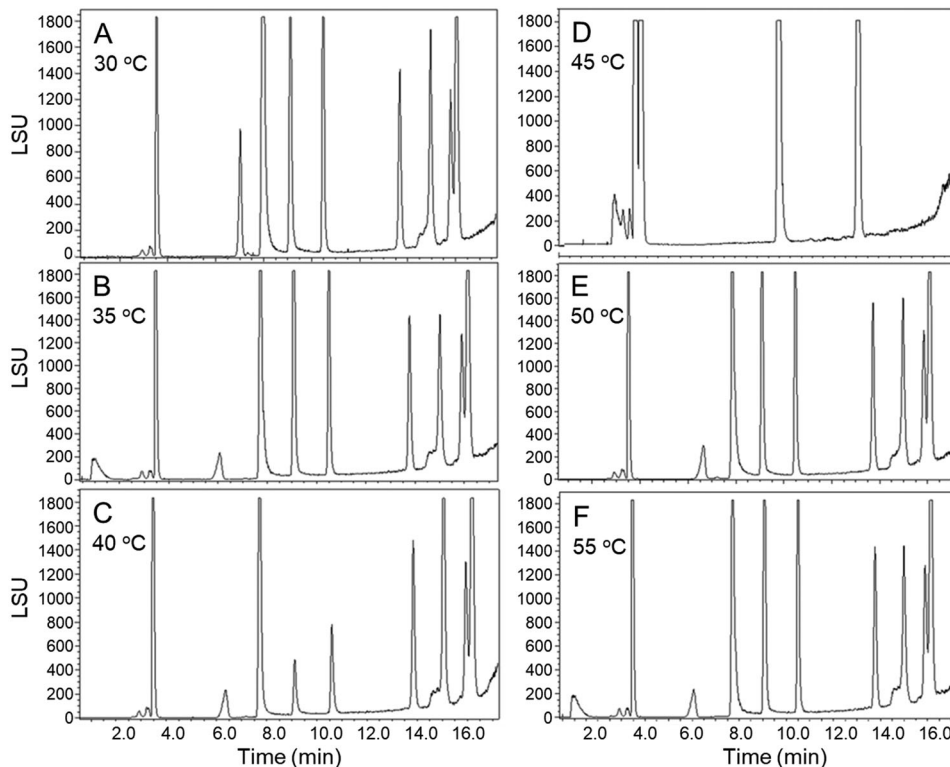


Figure 2. The chromatograms performed with the column temperatures at A) 30 °C, B) 35 °C, C) 40 °C and drift tube temperatures at D) 45 °C, E) 50 °C, F) 55 °C.

Table 1. Linear calibration curves, limits of detection (LOD), limits of quantification (LOQ), and recovery for different classes of phospholipid.

Compounds	Calibration curves	R^2	LOD [$\mu\text{g mL}^{-1}$]	LOQ [$\mu\text{g mL}^{-1}$]	Recovery [%]	R.S.D. [%] of retention time [min]	R.S.D. [%] of peak area
PG	$y = 120.93x - 354.88$	0.9905	1.5625	6.00	93.1	0.17	3.03
PA	$y = 46.454x - 709.55$	0.9950	6.25	12.50	114.7	0.07	5.13
PS	$y = 14.618x - 94.958$	0.9913	6.25	18.75	93.8	0.29	4.05
PE	$y = 247.93x - 906.58$	0.9892	3.125	12.50	101.3	0.15	1.79
PC	$y = 174.13x - 812.63$	0.9982	0.75	1.50	102.0	0.04	1.69
SM	$y = 61.472x + 16.845$	0.9998	3.125	6.25	96.0	0.73	4.96
LPC	$y = 176.75x - 1170.7$	0.9924	3.125	6.25	87.2	0.06	5.25

The potential of ELSD in the analysis of different lipid classes has been established.^[25] Even though Refractive index detectors (RID) are compatible with HPLC, they are limited by the presence of only one single mobile phase, leading to poor ability to separate complex samples effectively. Moreover, the main disadvantage of UV-based detectors is their limited quantification ability. In addition, UV detection results can reportedly be biased by low absorption and uncharacteristic spectrum.^[26] Therefore, ELSD optimization was performed in our study. Notwithstanding that phospholipids have been characterized by HILIC-ELSD in previous studies,^[27,28] better peak shapes were obtained in the present study using the HILIC column compared to other NPLC columns such as the silica gel column. The elution gradient for HILIC-ELSD was subsequently optimized. Hexane and chloroform were unsuitable since the eluent used in HILIC usually possesses higher polarity than hexane or chloroform. Hexane and chloroform as low-polarity mobile phases which are suitable for silica gel columns. In addition, phospholipids have poor solubility in hexane.

2.3. Separation Performance and Method Validation

Seven classes of phospholipids in a mixed sample were identified and quantified using the optimized HILIC-ELSD. For quantitative analysis of the phospholipids, the method was first validated in terms of linearity, recoveries, detection limits, and quantitation. As shown in **Table 1**, the correlation coefficients (R^2) were higher than 0.99 except for PE (0.9892). The precision of the peak area ranged from 2.07% to 8.77%. The recoveries of the seven phospholipid classes ranged from 87.2% to 114.7%, indicating that the method performed was acceptable. The relative standard deviations of retention time were lower than 0.29% except for SM (0.73%). All the relative standard deviations of peak area were lower than 5.25%. Overall, the results indicated our method was reliable for separating and quantifying different classes of phospholipids.

2.4. Second-Dimension Analysis by UPLC-Q-TOF-MS

Before the second-dimension analysis by UPLC-Q-TOF-MS, phospholipid fractions separated by HILIC-ELSD in the first dimension were separately collected based on the retention time of each phospholipid class. The total ion chromatograms of dif-

ferent phospholipids collected from *C. elegans* are shown in **Figure 3A–G**. The retention time of PG, PA, PS, PE, and PC ranged from 5 to 15 min. SM was eluted from 10 to 15 min, while LPC was eluted between 3.0 and 10 min. Though the elution pattern of different species overlapped, a chromatograph was generated from independent injections. Accordingly, we could identify various phospholipid profiles in each collected fraction.

The MS/MS spectra of different phospholipid classes are shown in **Figure 3H–N**. The mass fragments could help identify the structure of phospholipids, including PG, PA, PS, PE, PC, SM, and LPC. From the mass spectrum results, it could be inferred that these phospholipids had three different fragmentation patterns. PG, PA, and PS are anionic lipids that give $[M-H]^-$ parent ions in negative ionization mode. These three phospholipids have been reported to be susceptible to neutral loss due to their polar heads.^[29] Fatty acyl chains at the sn-1 and sn-2 positions can generate $[RCOO]^-$ fragment ions during the collision. The above characteristics allow the confirmation of the acyl chains in the molecule. For example, PA-18:0/20:5 was identified by the parent ion m/z 721.50, and the corresponding MS/MS spectrum is shown in **Figure 3M**. The main peaks were m/z 721.56, 438.33, 419.30, 301.25, 283.30, and 153.01. In the negative ionization mode, the ester bond in PA was prone to fragmentation. Thus, the peaks at m/z 301.25 and m/z 283.30 were fragment ions produced by the terminal fatty acyl chains of C18:0 and C20:5, respectively. The m/z 438.33 and 419.30 were fragment ions of LPA-18:0 and LPA-20:5 generated after fatty acid loss. The peak at m/z 153.01 was the typical fragment ion of glycerol phosphate skeletons, and the phospholipid at m/z 721.50 was identified as PA-18:0/20:5. Other anionic phospholipids such as PE, PG, and PS had similar fragmentation patterns to PA. Interestingly, it has been reported that cleavage of the ester bond is related to various factors, including the type of phospholipids and the collision energy.^[30] Therefore, the position of fatty acid (sn-1/sn-2) could not be confirmed. According to a previous study, most phospholipids in *C. elegans* contained polyunsaturated fatty acids, generally at the sn-2 position.^[31]

LPC, SM, and PC are neutral lipids that easily form $[M+H]^+$ ions and other daughter ions in positive ionization mode. These phospholipids have a polar head with the same structure, thus giving the characteristic ion at m/z 184. The MS/MS spectrum of m/z 522.33 LPC is shown in **Figure 3H**. The main peaks were found at m/z 522.33, 504.31, 184.06, and 86.09. Removal of the head group (m/z 184.06) and the glycerol skeleton (m/z 56) from LPC (m/z 522.33) indicated that the fatty acyl chain consisted of

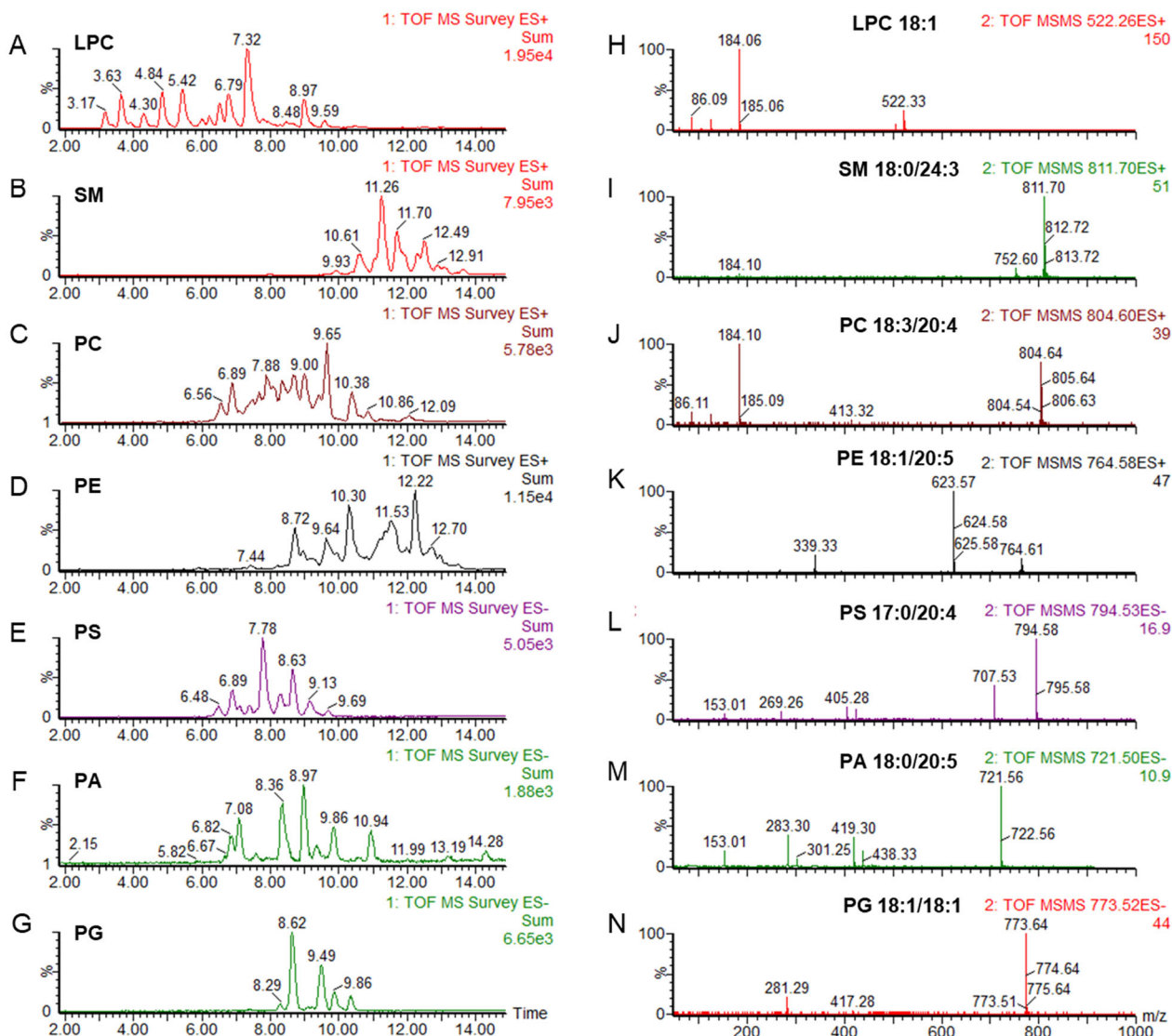


Figure 3. Total ion chromatograms of different phospholipid classes and the MS/MS spectrum of corresponding phospholipid species A) LPC, B) SM, C) PC, D) PE, E) PS, F) PA, G) PG; and MS/MS spectrum of H) LPC-18:1, I) SM-d18:0/24:3, J) PC-18:3/20:4, K) PE-18:1/20:5, L) PS-17:0/20:4, M) (B) PA-18:0/20:5, N) PG-18:1/18:1.

C18:1. PE is a weak anionic phospholipid that can be ionized in either positive or negative ionization mode. It was found that PE showed a higher response in positive ionization mode than in negative ionization mode. As shown in Figure 3K, the parent ion produced in positive ionization mode was $[M+H]^+$ at m/z 764.58. It has been reported that $[M+H-141]^+$ (m/z 623.57) was the characteristic ion of PE in the positive ionization mode.^[32]

The traces of precursor ions of different PA species (PA-17:0/18:1, PA-18:2/19:0, PA 18:1/18:1, and PA-18:0/20:5) are shown in the extract ion chromatogram (EIC) (Figure 4A–D). The corresponding retention times in the negative ion mode were 10.94, 9.86, 8.97, and 8.36 min. The elution order was based on the equivalent carbon number (ECN). The ECN was calculated based on the total carbon number and the total number of double bonds. The above four PAs had the same polar phospholipid head groups but different ECNs (33, 33, 32, and 29, respectively).

As shown in Figure 4A, the retention time increased with an increase of ECN and the corresponding MS/MS spectra is presented in Figure 4E–H.

2.5. Phospholipids Profile in *C. Elegans*

Overall, 111 phospholipid species were identified in *C. elegans* using the 2D untargeted analysis by HILIC-ELSD/UPLC-Q-TOF-MS (Table S1, Supporting Information). The quantitative results of different phospholipid classes are shown in Figure 5. PC was the dominant phospholipids, representing 37.23% of the total phospholipid content (Figure 5A), while PE was the second most abundant phospholipids in *C. elegans* (18.1%). Our results were consistent with previous reports that the major phospholipids in *C. elegans* were PC and PE.^[33] Furthermore, we found that

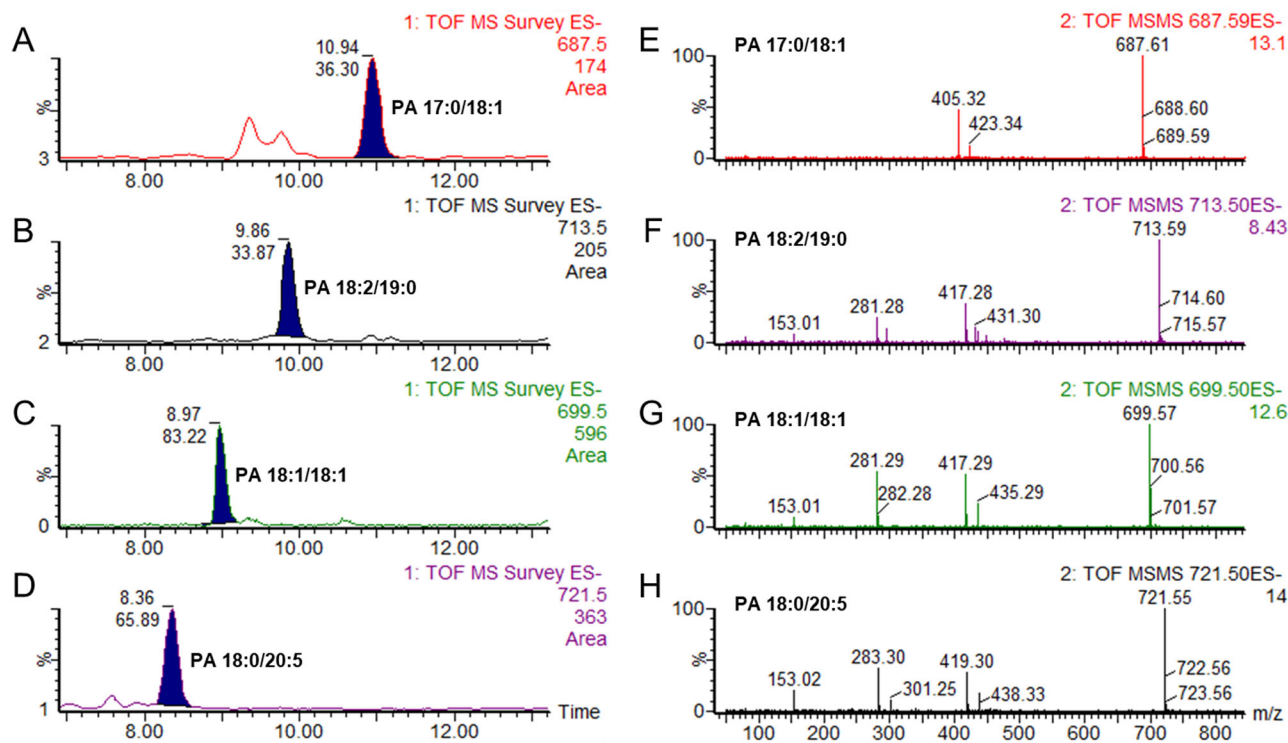


Figure 4. A–D) Extract ion chromatograms and E–H) the corresponding MS/MS spectrum of typical PA species. A,E) PA-17:0/18:1, B,F) PA-18:2/19:0, C,G) PA 18:1/18:1, and D,H) PA-18:0/20:5.

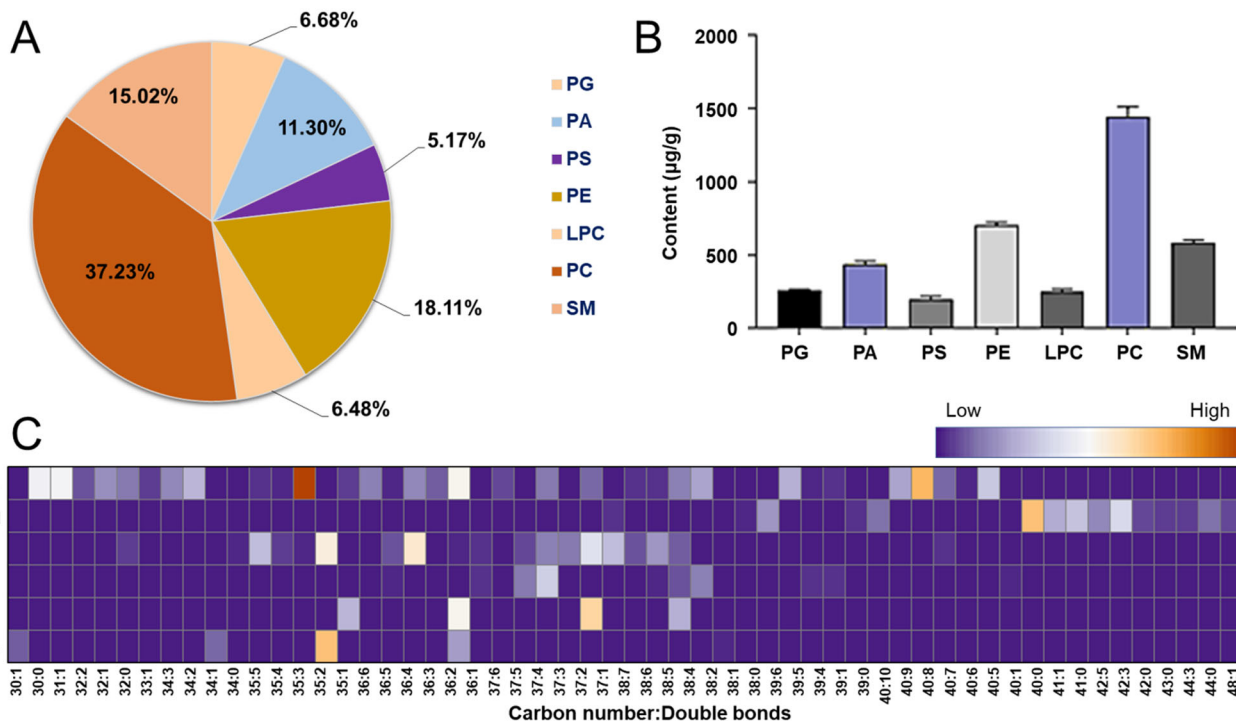


Figure 5. Quantitative analysis and heat map analysis of phospholipid profiles obtained from the 2-D HILIC-ELSD/UPLC-Q-TOF-MS method. A) Percentage pie chart, B) content column chart, and C) heat map analysis.

most phospholipids consisted of oleic acids (C18:1), such as PG, PA, and LPC, which was reasonable since C18:1 was the major fatty acid in *C. elegans*.^[34] The eicosapentaenoic acid (C20:5) is an important polyunsaturated fatty acid, mainly in PC and SM. In addition, the main PC species found were PC-20:5/20:5, PC-20:4/20:5, PC-18:1/20:5, PC-18:1/18:1, and PC-18:1/20:4. As seen in Figure 5C, a heatmap of phospholipids identified showed an abundant distribution of various PC. Notably, a higher carbon number with a lower degree of unsaturation was found for SM compared to PE, PS, PA, or PG. The SM metabolism regulates development in *C. elegans*. Consistently, a previous study indicated that SM in *C. elegans* mainly consisted of mono-unsaturated long-chain fatty acids.^[35] The fatty acid composition in SM in *C. elegans* has been reported to consist mainly of C18:0, C18:1, and other long-chain fatty acids, with low proportions of polyunsaturated fatty acids.^[36] Only three species of SM were found with polyunsaturated fatty acyl chains in the present study.

Phospholipids containing polyunsaturated fatty acids in *C. elegans* have been shown to play an important role in maintaining growth and adapting to environmental changes.^[37] For instance, the membrane phospholipids in Dauer larva are reportedly enriched in polyunsaturated fatty acids.^[38] Furthermore, PC can act as a lipid messenger for developing a stable matrix for intracellular membranes. Moreover, many signaling pathways are involved during the hydrolysis of PC to LPC with free fatty acids.^[39] PA and SM contributed 11.3% and 15.0% of the total amount of phospholipids, respectively. PA is an important precursor for the synthesis of many phospholipids. PA metabolites affect membrane dynamics involved in cell fusion and fission.^[40] Different phospholipids possess different fatty acyl chains with heterogeneous degrees of unsaturation and lengths and exhibit distinct properties. It has been established that the membrane lipid composition can affect its physical properties and hence the physiological functions of tissues and organs. In a previous study, PS with abundant ω -3 fatty acid acyl chains improved brain function.^[41] The PS in *C. elegans* predominantly consisted of fatty acyl chains of arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5).

The obtained phospholipid profiles of *C. elegans* were compared with the data reported in the literature. In contrast with most studies, more species of phospholipids were identified in our research. PC and PE were first analyzed mainly due to their higher abundance.^[24] Phospholipids in *C. elegans* consisted of 55% PE, 32% PC, and 8% SM; other phospholipids, such as PI and LPC, accounted for the remaining 5%.^[20] However, PI was not detected in most studies due to its low abundance or limitations in the methods applied. The reported procedures, which included TLC-GC and HPLC-MS, were performed during a relatively short analysis time, and the fatty acyl chains of the phospholipids were ignored.^[42–45] Hameed et al. only analyzed a small part of the phospholipids of *C. elegans*.^[31] It is worth mentioning that PI was identified by Triebel et al., who reported a relatively rich phospholipid profile, including 179 species from seven different classes.^[46] Many species of PE and PC were identified while PA was not identified, and phospholipid acyl chains were not investigated. Interestingly, the acyl chain in phospholipids, especially polyunsaturated fatty acyl chains, has been reported to act like a contortionist to facilitate fast protein movements and membrane bending.^[47] Accordingly, it is important to elucidate the role of the fatty acyl chain in phospholipids. It should be borne in mind

that Q-TOF-MS was utilized in this study while orbitrap-MS was adopted by Triebel et al. In recent years, Q-TOF-MS has gained considerable attention given its high sensitivity and specificity in compound detection.^[48] Overall, the 2D HILIC-ELSD/UPLC-Q-TOF-MS strategy provided a practical method to identify the phospholipid profile in *C. elegans*.

3. Conclusion

An offline 2D HILIC-ELSD/UPLC-Q-TOF-MS method was developed to identify phospholipid profiles in *C. elegans*. In the first dimension, phospholipids were separated into seven classes using a HILIC column. The phospholipids were then identified in the second dimension using a C18 column. Finally, 111 phospholipid species in *C. elegans* were identified. The dominant phospholipids were PC and PE, which accounted for 37.2% and 18.1% of the total phospholipids, respectively. Interestingly, oleic acid, arachidonic acid, and eicosapentaenoic acid were the most abundant phospholipids. Overall, these results complemented the existing literature on the phospholipid content of *C. elegans* and provided an important reference for the future development of a 2D online instrument.

4. Experimental Section

Chemicals and Materials: Yeast extract, peptone, agar, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, calcium chloride, magnesium sulfate, and ammonium acetate were purchased from Sinopharm Chemical Reagent Co., Ltd. Ammonium formate, isopropanol (IPA) and formic acid were acquired from Anaqua Chemicals Supply Co., Ltd. Methanol, acetonitrile (ACN), and chloroform (LC-MS grade) were provided by TEDIA Company Inc. Standards of L- α -lysophosphatidylcholine- γ -oacyl (LPC, >99%) and SM (\geq 98%) were purchased from Avanti Polar Lipids (USA). PS (>99%), PG (>99%), PC (>99%), PE (>99%), and PA (>99%) were obtained from Sigma Aldrich. Phospholipid solutions were prepared by dissolving the appropriate amount of each in chloroform.

C. Elegans Sample Preparation: Wild type *C. elegans* strain Bristol (N₂) was provided by the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, USA). *C. elegans* was cultivated in nematode growth medium (NGM) seeded with dead bacteria (*Escherichia coli*, strain OP50) as a food source as we previously described.^[34] After bleaching, the L1 worms were incubated in an NGM plate for 48 h at 20 °C. The collected L4 worms were frozen immediately in liquid nitrogen until lipid extraction.

Extraction of Phospholipids: Phospholipids were extracted in triplicate from 100–200 mg lyophilized powder of *C. elegans* under ice bath conditions using a method previously described with slight modifications.^[49] Briefly, a mixture of methanol (1.5 mL), chloroform (3 mL), and distilled water (1.2 mL) was added to samples. After the mixtures were vortexed for 5 min and then sonicated with a JY92-IIN Scientz Ultrasonic Homogenizer (Ningbo, Zhejiang, China) for 10 s with 5 s interval, and 75% power for 15 min. Chloroform (0.75 mL) and water (0.75 mL) were added and then shaken for 20 min. The mixture was centrifuged at 8000 rpm for 10 min. The lower organic phase was evaporated under nitrogen. The dried residue was dissolved in chloroform/methanol (2:1, v/v) before analysis.

HILIC-ELSD Separation for the First Dimension: Hydrophilic-mode separation of phospholipids was performed on a HILIC column (250 mm \times 4.6 mm, 5 μ m, Waters). The high-performance liquid chromatography (HPLC) system consists of a Waters 2695 liquid chromatography and a Waters 2414 Infinity ELSD. The optimized gradient started at 95% A for 2 min and was decreased stepwise to 90% A over 1 min, to 80% A in 9 min and finally to 70% A for 3 min (A: ACN; B: ultrapure water containing 10 mmol L⁻¹ ammonium acetate). The flow rate was 0.3 mL min⁻¹

with an injection volume of 10 μL . The column temperature was kept at 35 $^{\circ}\text{C}$. For ELSD, the nebulizer and evaporator temperature were both set at 50 $^{\circ}\text{C}$. Nitrogen was used as the nebulizing gas, and the pressure was set at 40 psi. Different phospholipids fractions were collected using the Waters Fraction Collector (Palo Alto, CA, USA). For the second-dimensional analysis, the collected sample was dried under a nitrogen stream and dissolved in 100 μL of chloroform/methanol (2:1, v/v).

UPLC-Q-TOF-MS Identification for the Second Dimension: The different phospholipid fractions were analyzed using the ACQUITY Ultra-higher Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA, USA), according to a previously described method.^[5] The fractions were separated using a BEH C18 column (50 mm \times 2.1 mm \times 1.7 μm , Waters). The column temperature was 40 $^{\circ}\text{C}$. Binary gradient elution was performed with different ratios of eluents A (IPA/ACN, 90:10, v/v) and B (ACN/water, 40:60, v/v). Both eluents contained 10 mmol L^{-1} ammonium acetate and 0.1% formic acid. The gradient was as follows: 0–20 min, 45–90% A; 20–22 min, 90–100% A; 22–25 min, 45% A. The flow rate was 0.3 mL min^{-1} , and the injection volume was 5 μL .

Mass spectrometry was performed using the Xevo C2-S Q-TOF-MS spectrometer (Waters, Milford, MA, USA) with an electrospray ionization (ESI) source.^[50] The capillary voltage and the cone voltage were maintained at 3.5 kV and 30 eV, respectively. The ion source temperature and the desolvation temperature were held at 100 and 400 $^{\circ}\text{C}$. The flow rates of cone gas and desolvation gas were 700 and 50 L h^{-1} , respectively. The data-dependent analysis (DDA) scanning modes parameters included the interval scan, MS scan times, and MS/MS scan, which were set at 0.02, 1, and 0.2 s, respectively. The low collision energy was set as 6 eV for MS. The high collision energy ranged from 15 to 25 eV for MS/MS. The scan range was 50–1000 m/z . Instrument control was performed with MassLynx 4.1 software.

Data Analysis: Phospholipid classes with different concentrations were detected using our optimized method. Eight concentrations were analyzed to construct the calibration curves for the quantification of phospholipids in *C. elegans*. Mass Spectrometry data analysis was processed using the MassLynx software (version 4.1). All statistical analyses were conducted from at least three independent experiments with the data were expressed as means \pm standard deviations (SD) and presented using GraphPad Prism8.0 software (GraphPad Software, San Diego, CA, USA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

X.S.: Conceptualization; Writing – original draft. T.Z.: Supervision; Writing – original draft; Writing – review & editing. G.T.: Formal analysis. R.L.: Supervision. M.C.: Supervision; Writing – review & editing. X.W.: Project administration.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

2D, *Caenorhabditis elegans*, fatty acids, HILIC-ELSD, phospholipids, Q-TOF-MS

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