Determination and Comparison of Phospholipid Profiles in Seven Kinds of Egg Types Using UHPLC-ESI-Q-TOF-MS

Fu Yang, Minghao Zhang, Minjie Zhao, Li Zhou, Jikai Liu, Eric Marchioni

► To cite this version:

Fu Yang, Minghao Zhang, Minjie Zhao, Li Zhou, Jikai Liu, et al.. Determination and Comparison of Phospholipid Profiles in Seven Kinds of Egg Types Using UHPLC-ESI-Q-TOF-MS. Food Chemistry, 2020, 10.1016/j.foodchem.2020.127856 . hal-02917519

HAL Id: hal-02917519

<https://hal.science/hal-02917519>

Submitted on 19 Aug 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Determination and Comparison of Phospholipid Profiles in Seven Kinds of Egg
Types Using UHPLC-ESI-Q-TOF-MS**

Fu Yang,[†] Minghao Zhang,[†] Minjie Zhao,[‡] Li Zhou,^{*,†} Jikai Liu,^{*,†} Eric Marchioni[‡]

[†]The Modernization Engineering Technology Research Center of Ethnic Minority
Medicine of Hubei Province, School of Pharmaceutical Sciences, South-Central
University for Nationalities, Wuhan 430074, P. R. China

[‡]Equipe de Chimie Analytique des Molécules Bioactives et Pharmacognoise, Institut
Pluridisciplinaire Hubert Curien (UMR 7178, CNRS/UDS), 74 route du Rhin, 67400,
Illkirch, France

*To whom correspondence should be addressed, Tel: +86-27-67842267, Email:
zhou2018@scuec.edu.cn; liujikai@mail.scuec.edu.cn

Abstract

Phospholipids from seven egg types were purified (purity > 96%) by solid-phase extraction cartridges, and subsequently identified and quantified by ultra-high-performance liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (UHPLC-ESI-Q-TOF-MS). 10 phospholipid classes were detected and 93 molecular species were characterized. Principal component analysis (PCA) showed that molecular species and concentration of phospholipids in pigeon and hen egg yolks had significant difference with other egg types. Hierarchical cluster analysis further indicated that the phospholipid profiles of pigeon egg yolk were closest to hen egg yolk, following quail, duck, ostrich, emu and goose egg yolks. The quantification of several representative molecular species can be considered to be most responsible for the discrimination among egg yolks phospholipids, for example, SM(d18:1/16:0) in goose egg yolk and PC(16:0/20:4) in duck egg yolk. This study provides a basis for better understanding phospholipid profiles of egg yolks and better evaluating the nutritional value of eggs.

Keywords: Egg yolk, Phospholipids, UHPLC-ESI-Q-TOF-MS, Molecular species, PCA

1. Introduction

Eggs are a favorite food all over the world and a good nutrient source for human health. Egg is inseparable nutrient for most people in daily diet. Egg yolk is a rich source of phospholipids, which accounts for about 33% in lipid composition, other lipids are 62% triglycerides and 5% cholesterol (Anton, 2007; Aro, et al., 2009). Phospholipids are important components of cell membranes which play vital roles in cellular metabolism, signal transduction and membrane transport (Zhang, Xu, Wang, & Xue, 2019; Harrabi, Herchi, Kallel, Mayer, & Boukhchina, 2009; Zhou et al., 2010). Phospholipids can be divided into several classes according to their molecular structures. The common categories include phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS), sphingomyelin (SM), phosphatidylglycerol (PG) and phosphatidic acid (PA), etc. The difference between phospholipid classes was the structure of polar headgroup, for example, PC and PE possess choline group and ethanolamine group, respectively. Each class comprises a diversity of molecular species due to the structures of the fatty acyl group (length of the fatty acid chain and number of double bonds). Hence, the different phospholipid classes possess large structural diversity and complexity.

The purification of egg yolk phospholipids from total lipid is needed when comprehensive analysis of polar lipid classes and their molecular species is aimed. Because high levels of neutral lipids, such as triglycerides, always affect ionization in electrospray ionization (ESI) sources and desolvation of liquid chromatographic effluent droplets, the neutral lipids need to be removed to obtain purified phospholipids. Typically, many methods including solid-phase extraction, thin layer chromatography,

two-dimensional liquid chromatography and supercritical carbon dioxide extraction, were explored for the separation and purification of phospholipids from foodstuffs (Zhou et al., 2010; Walczak, Bocian, & Buszewski, 2015; Boselli, & Caboni, 2000; Graves, Beaulieu, & Drackley, 2007). In general, most of the methods could give good recoveries for most predominant phospholipid classes.

Regarding lipidomics and lipid profiling characterization, mass spectrometry (MS) has been extensively applied in the analysis of lipids. Particularly, ESI-MS/MS is sensitive and results in high specificity for phospholipid identification. Triple quadrupole linear ion trap MS has frequently been used for the identification of molecular species of phospholipids extracted from various food matrices (Gang et al., 2018; Montealegre, Sánchez-Hernández, Crego, & Marina, 2013; Herchi et al., 2011). However, in the last few years, with the development of the lipidomics, great progress has been made in lipid analytical techniques. One of the most useful techniques for the characterization of molecular structure of lipids is high resolution mass spectrometry because of its precise mass accuracy and excellent sensitivity. It exhibits strong power for fragment ion scanning enabling recognition of numerous lipid isomers that were proved difficult to measure by low resolution mass spectrometry (Senyuva, Gökmen, & Sarikaya, 2015). For example, ultra-high performance liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (UHPLC-ESI-Q-TOF-MS) has been applied for the analysis of lipids in milks (Ali, Zou, Huang, Abed, & Tao, 2017), marine shellfish (Zhang et al., 2019) or other biological sources (She et al., 2014; Miao et al., 2015). The information available at present about the detailed molecular species of egg yolk identified by UHPLC-ESI-Q-TOF-MS is limited. To our knowledge, there is

only one literature report that provided the information about molecular species of egg yolks from duck, hen and quail using UPLC-ESI-Q-TOF-MS (Ali et al., 2017). However, there are several other eggs, such as goose egg, pigeon egg, ostrich egg and emu egg. In this study, more kinds of eggs were investigated and the molecular profiles of phospholipid classes contained in egg yolks were characterized. We aimed to evidence lipidomic promising analytical ability to provide the information of the molecular composition of phospholipids in eggs, and to investigate the differences of phospholipid profiles among seven egg types.

Furthermore, although numerous studies have been contributed to characterize phospholipid profiles in different food matrices, these methods reported only the relative abundance of the different molecular species of phospholipid classes (Zhou, Zhao, Ennahar, Bindler, & Marchioni, 2012a; Zhou, Zhao, Ennahar, Bindler, & Marchioni, 2012b; Li et al., 2017; Le Grandois et al., 2009; Wang et al., 2009; Ma et al., 2017). This study presented the first time a detail elucidation and quantification of each molecular species of phospholipid classes in seven kinds of eggs. Results from the present study provided a reference for better evaluation of nutritional value of eggs. In this study, UHPLC-ESI-Q-TOF-MS was thereby applied to identify and quantify phospholipid molecular species from seven egg types including hen egg, duck egg, quail egg, pigeon egg, goose egg, ostrich egg and emu egg. In addition, phospholipid profiles were investigated with software-assisted lipidomic analysis. This is a first investigation regarding a deep comprehensive comparison and determination of phospholipid profiles from various egg types.

2. Materials and methods

2.1. Materials

Fresh hen, duck, goose, quail, pigeon, ostrich and emu eggs were purchased from local supermarket. Seven standards including 1-pentadecanoyl-2-oleoyl(d7)-sn-glycero-3-phosphocholine (15:0-18:1-d7-PC), 1-pentadecanoyl-2-oleoyl(d7)-sn-glycero-3-phosphoethanolamine (15:0-18:1-d7-PE), 1-pentadecanoyl-2-oleoyl(d7)-sn-glycero-3-[phosphor-rac-(1'-glycerol)] (15:0-18:1-d7-PG), 1-pentadecanoyl-2-oleoyl(d7)-sn-glycero-3-phosphoinositol (15:0-18:1-d7-PI), 1-pentadecanoyl-2-oleoyl(d7)-sn-glycero-3-phosphate (15:0-18:1-d7-PA) and sphingomyelin (d18:1/12:0) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All chemicals and solvents used for extraction and analysis were of high purity and analytical grade. Methanol (LC-MS grade), acetonitrile (LC-MS grade) and isopropanol (HPLC grade) were provided by Shanhai Forneeds Biological Technology Co., Ltd. FAME Mix C4-C24 (37 components) was from Supelco.

2.2. Total lipids extraction

Egg yolk powder was obtained by manually removing egg white from fresh eggs, homogenizing and freeze-drying. The extraction of total lipids from egg yolk powder (hen, duck, goose, quail, pigeon, ostrich and emu) was carried out following Folch method (Folch, Lees, & Sloane-Stanley, 1957) with a slight modification. 1.0 g egg yolk powder was placed in 30 mL of a CHCl₃/CH₃OH (2:1, v/v) mixture and shaken at 30°C for 30 min by water-bathing constant temperature shaker (SHA-C, Nanjing, China). The mixture was centrifuged at 4000 g for 15 min. The upper layer was transferred into a new tube and the precipitate was extracted for twice with the same procedure. The combined organic phases were evaporated by a rotary evaporator at 40°C and the residue was dried

under a gentle stream of nitrogen until the obtention of a constant weight. The obtained total lipids were stored at -20°C until use.

2.3. Phospholipid purification

Phospholipids were purified from total lipids according to the procedure of Zhou, Zhao, Ennahar, Bindler, & Marchioni (2012b). Briefly, 50.0 mg of total lipids were dissolved in 1.0 mL CHCl₃, transferred onto a silica gel column (35 cm × 2 cm i. d., 15 g of Si 60 silica gel, particle size 40-63 µm, Geduran, Merck). Firstly, neutral lipids were removed by 200 mL of CHCl₃. Then, the phospholipids were eluted with 150 mL of CHCl₃/1 M formic acid (98:2, v/v). After evaporation of the solvent, 16.4 mg of phospholipids were obtained and stored at -20°C for further use. The purity of phospholipids isolated from different egg yolks was checked by HPLC hyphenated with evaporative light scattering detector (ELSD) according to the method of Zhou et al. (2010).

2.4. Gas chromatographic analysis of fatty acids

Fatty acids methyl esters were obtained after transesterification of purified phospholipids according to our previous study (Zhou et al., 2017). Quantitative analysis was performed on a 3400 Varian GC equipped with a flame ionization detector (FID). Separation was made by a SP 2560 column (0.25 mm × 100 m, 0.20 µm). One microlitre of each sample was injected with a run time of 65 min. Injector was set to 280°C, detector was set to 285°C and the column was set at 140°C and raised to 220°C at a rate of 5°C /min, the final temperature was maintained for 49 min. Peaks were identified by comparison to standards (FAME mix C4-C24). The relative amounts of fatty acids were calculated by the percent area method with external normalization considering the sum of

all areas of the identified fatty acid.

2.5. Preparation of stock solutions

A stable-isotope labelled standard for each molecular species is the preferred option for accurate quantification (Pigini, Cialdella, Faranda, & Tranfo, 2006). They have the same chemical and physical properties as phospholipids to be quantified, but distinguishable in mass spectra. Hence, 6 d7-phospholipid standards and 1 nonendogenous phospholipid standard were used for the quantification, including 15:0-18:1-d7-PC [standard for PC], 15:0-18:1-d7-PE [standard for PE], plasmalylethanolamine (PE-O-alkyl-acyl), lysophosphatidylethanolamine (LPE)], phosphatidylethanol (PEtOH) and oxidized phosphatidylethanolamine (OxPE), 15:0-18:1-d7-PG [standard for phosphatidylglycerol (PG)], 15:0-18:1-d7-PI (standard for PI), 15:0-18:1-d7-PA [standard for phosphatidic acid (PA)] and sphingomyelin (d18:1/12:0) (standard for SM). To equilibrate the analytical platforms and correct the minor variation from different samples, a quality control (QC) sample (cocktail of each sample) were injected regularly every 5 samples.

Stock solutions were prepared in chloroform/methanol (2:1, v/v), and diluted into a series of concentrations (10~5000 ng/mL). The 7 phospholipid standards were added in egg yolk phospholipids and QC samples. The quantification is based on the extracted ion chromatograms (EICs) of individual phospholipids with m/z expansion in ± 5 ppm. Peak areas of the phospholipid standards and egg yolk phospholipids were integrated from EICs. The concentration of each phospholipid molecular species was calculated from this calibration curve constructed from known concentrations of the corresponding standards.

2.6. Identification and quantification of phospholipids by UHPLC-ESI-Q-TOF-MS

2.6.1. UHPLC condition

The identification and quantification of phospholipids were performed by a UHPLC (Shimadzu LC-30A) coupled to a high resolution AB Sciex Q-TOF 6600 mass spectrometry (HRMS) instrument equipped with an electrospray ionization (ESI) source (AB Sciex, Toronto, ON, Canada).

The purified phospholipids were separated on a Phenomenex Kinetex C18 column (100 × 2.1 mm, 2.6 µm). Solvent (A) H₂O: methanol: acetonitrile (1:1:1, v/v/v) with ammonium acetate (5mM) and solvent (B) isopropanol: acetonitrile (5:1, v/v) with ammonium acetate (5mM) were employed as mobile phases. The binary gradients used for the chromatogram were as follows: 80% A for 0.5 min, 80% A to 60% A for 1 min, 60% A to 40% A for 1.5 min, 40% A to 2% A for 5 min, 2% A to 80% A for 2 min. The total run time was 10 min at a flow rate 0.4 mL/min. The column temperature was set at 60 °C. The injection volume was 10 µL.

2.6.2. ESI-Q-TOF-MS conditions

The ESI source conditions were set as follows: spray voltage (5.5 kV), curtain gas (N₂, 99.999%, 35 psi, Domnik Hunter), temperature (600 °C), ion source gas1 (nebulizer gas, 50 psi), ion source gas 2 (turbo gas, 50 psi). Data were collected in both positive and negative ionization mode over a mass range between 100 ~ 1200 *m/z*. The MS/MS experiments were performed using collision energy of 35 eV in negative mode. The mass range was set between 200 ~ 900 *m/z* for fragmentation products.

2.7. Statistical analysis

Phospholipids were identified and quantified using AB SCIEX Analyst 1.6.2 software (Applied Biosystems, Foster City, CA, USA), MS-DIAL and LipidMaps. False

positives were checked manually according to the MS/MS fraction analysis. Discriminatory analysis of molecular composition from various egg yolks were analyzed by Principal Component Analysis (PCA) and hierarchical cluster analysis using MetaboAnalyst 4.0 software.

3. Results and discussion

3.1. Analysis of the fatty acids of egg yolk phospholipids

The major fatty acids associated with seven egg yolk phospholipids were C18:1n9, C16:0, C18:0, C18:2n6 and C20:4n6 (**Supplemental Material Table S1**), which was almost consistent with the conclusion of Ali et al. (2017). Derived from obtained data, the fatty acid profiles of seven egg types were significantly different. Oleic acid was the most abundant fatty acid, occupying 29%, 51%, 30%, 43%, 49%, 51% and 43%, respectively. C18:3n6 and C18:3n3 were detected in hen egg yolk and ostrich egg yolk, while C17:0, C20:1 and C20:2 were not found in emu egg yolk. Furthermore, C17:0 was not detected in duck egg yolk as well. The arachidonic acid (20:4n6) has significant neurocognitive benefits and plays an essential role in the process of treating cardiovascular disease (Brenna, 2016). It was found in all egg samples and the content was higher in hen egg yolk and goose egg yolk than others. Docosahexaenoic acid/eicosapentaenoic acid-phospholipids (DHA/EPA-phospholipids) are one of the major n-3 long-chain polyunsaturated fatty acid dietary forms in our diet, have nutritional functions on antitumor activity, lipid metabolism, and glucose metabolism (Zhang, Xu, Wang, & Xue, 2019). The GC analysis of fatty acid showed that the content of DHA+EPA in pigeon egg yolk (3.77%) was higher than quail egg yolk (2.30%), duck egg yolk (1.62%), ostrich egg yolk (1.45%) and emu egg yolk (1.35%), but it was lower than hen egg yolk (4.27%) and

goose egg yolk (4.81%). As for polyunsaturated fatty acids (PUFA), hen egg yolk, goose egg yolk and pigeon egg yolk showed higher content, making up 29%, 28% and 22%, respectively. The variation in the content of fatty acids in different egg yolks were probably resulted from different species, growth conditions and even diet the birds were fed.

3.2. Purification of phospholipids

The HPLC Chromatogram of purified phospholipids from seven egg types was given in **Supplemental Material Figure S1**. The amount of neutral lipids which usually presented in the first 3 min of the HPLC run, was insignificant. Upscaling to semi-preparative HPLC did not seem to reduce the quality of the separation of the phospholipid classes. This procedure enabled collect adequate amounts of phospholipids fractions. For each egg yolk samples, the purity of phospholipids monitored by HPLC-ELSD were exhibited to be at least 96%.

3.3. Characterization of phospholipids by UHPLC-ESI-Q-TOF-MS

The separation of phospholipid classes of different egg yolks was achieved within a runtime of 10 min under ESI source in negative ion mode (**Figure 1**). **Supplemental material Figure S2** shows an example of EICs of m/z 455.2201, 480.3114, 885.5588, 773.5440, 703.5684, 816.5811 and 766.5458 for hen egg yolk phospholipids. Although the chromatogram under positive ionization mode exhibited higher intensities, either characteristic fragments or fatty acyl identification fragments was not available under ESI⁺ mode for most phospholipids. Only a small part of phospholipids, such as SM was not detected in negative ion mode while can be detected in positive ion mode. Overall, the negative ion mode provided more available information for molecular structure

identification with lower noise and background than positive ion mode. Furthermore, the most abundant product ions derived from collision induced dissociation of $[M-H]^-$ of phospholipids corresponded to the carboxylate anions and loss of a neutral fatty acid or ketene from one or both of the fatty acyl chains (Pulfer, & Murphy, 2003). We detected PE, LPE, OxPE, PE-OH, PG, PI, PA and LPA as $[M-H]^-$ ions, PC as $[M+CH_3COO]^-$ ion, and SM as $[M+H]^+$ ions. The molecular species within one class were classified on the basis of the structures of esterified fatty acid chain (double bonds numbers and alkyl chain length).

The molecular structures of each phospholipid were identified by MS/MS spectrometry. Phospholipids are classified by their different polar headgroups which give rise to the characteristic fragments in the MS/MS, for example, m/z 140.0132 was characteristic of the polar head-group of PE, which can be used to distinguish between different phospholipid classes. Besides, the information of other MS/MS fragments can help to identify the fatty acid chains of phospholipids.

All the characterized phospholipids were quantified based on corresponding standard curves. The limit of detection (LOD) and limit of quantification (LOQ) corresponded to a signal-to-noise ratio of 3 and 10, respectively. As shown in **Table 1**, calibration curves presented linear regression coefficients (R^2) higher than 0.99 and LOD was ≤ 0.8 ng/mL. The spiked recovery method was used to evaluate the method. The recoveries of the seven standards were between 98 and 110%.

3.4. Phospholipid profiles in seven egg types

10 phospholipid classes were detected and 93 phospholipid molecular species were characterized using UHPLC-ESI-Q-TOF-MS. **Table 2** showed the molecular species

identified and quantified of phospholipid classes in seven egg types. 48 species of PE were identified which was the most abundant among phospholipids. It is worth noting that ether phospholipids (PE-O-alkyl/acyl) accounted for a high proportion and other forms of PE such as 1 species of PEtOH, 12 species of LPE and 2 species of OxPE were also found.

The ESI tandem mass spectra of PE $[M-H]^-$ ions yield three fragment categories: (1) a fragment ion (140 Da) was characteristic of the polar head-group $[NH_3(CH_2)_2OPO_3]^-$; (2) two fatty acid $[RCOO]^-$ corresponded to the detached fatty acids; (3) two $[M-H-RCO]^-$ resulted from loss of two acyl groups. **Figure 2a** showed the MS/MS fragmentation spectrum of PE (16:0/18:2) from hen egg yolk at m/z 714.5151. m/z 140.0132 corresponded to the ethanolamine group. The observed peaks at m/z 255.1350 and 279.2347 corresponded to palmitic acid and linoleic acid, respectively. m/z 452.2840 and 476.3142 corresponded to the loss of linoleoyl acyl group and palmitoyl acyl group in the ketene form, respectively. From **Table 2**, we can see that the major PE species from the seven egg types were PE(18:0/20:4), PE(18:0/18:2), PE(16:0/18:2) and PE(16:0/20:4). Many PE(O-alkyl/acyl) were detected. The difference between PE and PE(O-alkyl/acyl) was that one of the two esterified fatty acid was replaced by a saturated ether linkage (-O-alkyl) (Deeley, Thomas, Truscott, Mitchell, & Blanksby, 2009; Park, Suh, Thomson, Ramanujam, & Clandinin, 2006). In general, PE(O-alkyl/acyl) were found in marine organisms or animal tissues (Zhou, Zhao, Ennahar, Bindler, & Marchioni, 2012a; Chapelle, 1987, Mawatari, Hazeyama, & Fujino, 2016). In our previous study, a PE(O-alkyl/acyl) was found in krill oil, but not detected in egg yolk by positive mode in egg yolk by low resolution mass spectrometry (Zhou, Zhao, Ennahar, Bindler, &

Marchioni, 2012a). However, many PE(O-alkyl/acyl) were detected in seven egg types by UHPLC-ESI-Q-TOF-MS in negative ion mode in this study. Apparent disparity to mass spectrometric studies probably attributed to differences in the instrument response function. In addition, a total of 14 egg yolk PEs was detected by UHPLC-Q-TOF-MS in positive ion mode, but no PE(O-alkyl/acyl) was found according to the study of Ali et al. (2017). One major cause of this phenomenon might be related with the UHPLC condition and negative ion mode for more accurate identification. **Figure 2b** showed the fragmentation pathway of PE(O-18:1/18:2) at m/z 726.5488. m/z 279.2321 reflected the deprotonated linoleic acid. m/z 446.2930 and 464.3142 result from the loss of linoleic acid and linoleic acyl group, respectively. In addition, most of the PE(O-alkyl/acyl) contained long chain-polyunsaturated fatty acid. For instance, PE(O-16:1/22:6) (m/z 746.5148) accounted for 83.50 nmol/g in pigeon egg yolk. Furthermore, it is worth noting that the PE species of emu egg yolk was obviously less abundant than in other egg types, only 12 molecular species was detected. **Figure 2c** showed the MS/MS fragmentation spectrum of LPE (18:0) from hen egg (m/z 480.3114). m/z 140.0245 reflected the ethanolamine group. m/z 283.2643 corresponded to stearic acid. m/z 196.0414 corresponded to the neutral loss of stearic acid. As shown in **Table 2**, LPE(16:0) and LPE(18:0) were the most abundant species among the seven egg types. 3 molecular species, such as LPE(O-16:0), PE(16:1) and LPE(20:3) were only identified in hen egg yolks.

The ESI tandem mass spectra of OxPE[M-H]⁻ ions showed more diversified fragmentation patterns, which yields four fragments categories: (1) [RCOO]⁻ and [RCOO+2O]⁻ corresponded to a detached fatty acid and a oxidized fatty acid; (2)

[M-H-RCO-2O]⁻ reflected the loss of oxidized fatty acyl group; (3) A fragment ion (196 Da) corresponded to the residue which was formed by the loss of an fatty acyl group and a fatty acid; (4) [M-H-140-RCO-2O]⁻ corresponded to the simultaneous loss of both the ethanol group (140 Da) and the oxidized fatty acid. A representative MS/MS fragmentation spectrum of OxPE(18:0/22:5+2O) at *m/z* 824.5493 was given in **Figure 2d**. *m/z* 283.2626 and 361.2045 corresponded to the stearic fatty acid (18:0) and oxidized docosapentaenoic acid (22:5+2O), respectively. *m/z* 462.0126 referred to the loss of oxidized docosapentaenoic acid (22:5+2O). *m/z* 323.0717 reflected the simultaneous loss of both the ethanol group (140 Da) and oxidized docosapentaenoic acid (22:5+2O). OxPE(18:0/22:5) was found in almost all the egg yolks except emu egg yolk. OxPE(18:0/18:2+2O) were detected in hen and pigeon egg yolks, accounted for 3.7 nmol/g vs 14.77 nmol/g, respectively. In addition, a special species, PE-OH(16:0/18:2) in pigeon and ostrich egg yolks, accounted for 5.4 nmol/g and 1.68 nmol/g, respectively (**Table 2**). Its MS/MS fragmentation pathway was given in **Figure 2e**. *m/z* 255.2378 and 279.2290 corresponded to palmitoyl acyl group and linoleoyl acyl group, respectively.

PC identification was confirmed in the negative ion mode [M+CH₃COO]⁻. 10 molecular species of PC were detected among the samples (**Table 2**). PC(16:0/18:1) and PC(16:0/18:2) were species with high concentration. A previous study conducted by Campos et al. (2016) developed a HILIC-ESI-ion trap-MS method to determine the molecular species of phospholipids and their changes in eggs from different conditions. 22 molecular species of PC were identified, being PC(16:0/18:1) and PC(16:0/18:2) the most abundant species detected, which was in agreement with our results. In another previous study of Pacetti et al. (2005) with the use of LC-ESI-MS for the identification of

molecular species of phospholipids in eggs from hens fed diets enriched in seal blubber oil, PC(16:0/18:1) and PC(16:0/18:2) were major species, which were also in accordance with our results. **Figure 3a** presented the fragmentation pathway of PC(16:0/18:1) at m/z 818.5958. The fragment ions at m/z 255.2180 and 281.2459 corresponded to deprotonated palmitic acid and oleic acid, respectively. m/z 480.3052 and 506.1038 reflected the loss of oleic acid and palmitic acid, as $[M-CH_3-RCH=C=O]^-$, respectively.

PGs are less abundant than most of the common phospholipid species. Molecular species of PG form abundant negative ions under ESI-MS conditions, as they would be expected for these acidic phospholipids with the neutral polar headgroup. 4 molecular species were detected among the samples. High concentration of PG (16:0/20:1) (52.20 nmol/g) and PG(16:0/18:1) (37.39 nmol/g) were detected in pigeon egg yolk. The fragmentation pathway of PG(16:0/20:1) at m/z 775.5499 were shown in **Figure 3b** as example. The observed peaks at m/z 255.2323 and 309.2855 corresponded to deprotonated palmitic acid and deprotonated eicosanoid acid. m/z 154.0136 corresponded to the phosphate residue of polar headgroup (170 Da). The peaks observed at m/z 519.3211 and 465.2619 corresponded to the neutral loss of palmitic acid and eicosanoid acid, respectively. m/z 245.2249 referred to the combination loss of palmitoyl acyl group and eicosanoid acyl group. m/z 211.2136 derived from the combination loss of palmitic acid and eicosanoid acid.

PI that contains a phosphodiester of the six-carbon sugar inositol as a polar headgroup was found in all samples. 12 molecular species of PI were identified in the negative ion mode. Previous literature reports illustrated that the analysis of acidic phospholipids including PI was typically performed in the negative ion mode (Pacetti,

Boselli, Hulan, & Frega, 2016; Koivusalo, Haimi, Heikinheimo, Kostianen, & Somerharju, 2001). A representative MS/MS fragmentation spectrum of PI(18:0/20:4) at m/z 885.5616 was shown in **Figure 3c**. The observed peaks at m/z 283.0462 and 303.2322 arised from the moieties of stearic acid and arachidonic acid. m/z 418.0431 and 438.6211 corresponded to the simultaneous loss of a fragment of inositol group (163 Da) and arachidonic acid or stearic acid, respectively. As shown in **Table 2**, PI(18:0/20:4) was predominant species among them, the concentration was between 30.95~169.24 nmol/g. PI(18:0/18:2) was second abundant species in most of the samples except for duck, goose and emu egg yolks. Other major species were PI(16:0/20:4), PI(18:0/18:2), PI(16:0/18:2) and PI(16:0/18:1). Our results were similar with the literature report of Ali et al. (2017), but they demonstrated that PI(18:0/18:2) was the most abundant species in duck, hen and quail egg yolks, while PI(18:0/20:4) was not detected in our study. The reason may be related with the diet, feed amount or rearing conditions (Zhou, Zhao, Ennahar, Bindler, & Marchioni, 2012a).

PA has the simplest polar headgroup, and serves as a precursor and metabolite in the biosynthesis and catabolism pathways of phospholipids. Although it is possible to detect PA in both positive ion and negative ion mode, the informative signals in negative ion mode are more accurate. 2 molecular species were detected in negative ion mode. The fragmentation pathway of PA(16:0/18:1) at m/z 673.4848 was shown in **Figure 4a**. m/z 255.2359 and 281.2472 arised from the deprotonation moieties of palmitic and oleic acid, respectively. The observed peak at m/z 152.9936 referred to the combination loss of palmitoyl acyl group and oleic acid. A related molecule is lysophosphatidic acid (LPA), which is produced from lysophospholipids by a plasma enzyme, autotaxin or generated

from phospholipids or diacylglycerol and then deacylated by phospholipase A1 or phospholipase A2 (Aoki, Inoue, & Okudaira, 2008). **Figure 4b** described the fracture mechanism of LPA(16:0) at m/z 409.2340. m/z 152.9859 reflected the loss of palmitic acyl group and a hydroxyl group. m/z 313.2505 corresponded to the loss of phosphate group. m/z 391.2432 corresponds to the loss of H_2O . **Table 2** described that the concentrations of PA and LPA species were relatively high compared to other species. In particular, the content of LPA(16:0) in samples was between 645.17~979.32 nmol/g. The content of PA(16:0-18:1) in hen egg yolk was highest among other sources.

SM is composed of only a single fatty acid esterified to a sphingoid backbone. They possess a large degree of structural diversity based on the variations in the degree of unsaturation, hydroxylation, and methylation of long-chain sphingoid base (sphingosine, sphinganine, etc.) in the nature of the fatty acid, as well as in the nature of the polar headgroup (Zhou, Zhao, Ennahar, Bindler, & Marchioni, 2012b; Fischbeck, Kruger, Blaas, & Humpf, 2009). 1 molecular species, SM(d18:1/16:0), was detected in the different egg yolk samples in positive ion mode. The results were in agreement with Pacetti et al. (2005). The protonated molecule $[M+H]^+$ at m/z 703.5759 in details was shown in **Figure 4c**. m/z 184.0741 corresponded to the phosphocholine group. The signal observed at m/z 264.2862 reflected sphingosine d18:1 chain base, and m/z 685.5613 referred to the loss of one molecular of H_2O from fatty acyl chain.

3.5. Characterization of phospholipid profiles by PCA

PCA involves a mathematical procedure identifying patterns in data set, and aims to highlight the similarities and differences. PCA is a way of identifying patterns in data and expressing the data in such a way as to emphasize their similarities and differences (Shin,

Craft, Pegg, Dixon Phillip, & Eitenmiller, 2010). The discrimination of phospholipid profiles from seven egg types were investigated by PCA. The data matrix (a total of 93 molecular species) was subjected to PCA. The first Principal Component (PC1) expresses the component responsible for the most variance, while PC2 represents the component responsible for the second-most variance. The score plots indicate the difference of phospholipid profiles of various egg yolks. The loading plots reflect the contribution of important variables to the molecular species from different egg yolks.

The first Principal Component (PC1) contributed to 46.8% of the variability in the data set. The second PC (PC2) accounted for 29.7% of the variance in the data was shown in **Supplemental Material Figure S3**. The PC1 and PC2 described 76.5% of the variance and reflect significant variability in the data. The remaining PCs (PC3 to PC5) accounted for 23.5% of the variance and cannot explain significant variability in the data. Thus, only PC1 and PC2 were considered for further study according to Kaiser criterion. (Kaiser, & Rice, 1974). The loading values in PC1 and PC2 represented significant contributions of individual PC molecular species to total variability as presented in **Figure 5a** and **Supplemental Material Table S2**. PC1 loading was highly contributed by many molecular species, for example, PE(18:0/20:3), PE(18:0/18:2), PE(18:1/20:1), PE(18:0/20:1), PE(18:1/20:1), PE(18:0/22:6) and PI(16:0/16:1), and most of them were from PE. Meanwhile, PC2 loading was positively contributed by PC(18:0/18:2), PE(O-20:1/18:2), PE(O-18:1/20:3), PE-OH(16:0/18:2), PE(O-16:1/20:4), PI(18:2/20:5) and PC(16:0/18:2).

Figure 5b presents the score plots of phospholipid profiles in various egg yolks. Goose egg yolk showed negative loadings on PC1 and positive loadings on PC2

indicating goose egg yolk has lower concentration in PC1 and higher concentration in PC2. Meanwhile, the egg yolks from emu, quail, ostrich and duck have negative loadings on PC1 and PC2. Pigeon egg yolk showed positive loadings on PC1 and PC2 with highest score on PC2. Hen egg yolk exhibited positive loading on PC1 and negative loading on PC2. The result indicated phospholipid profiles of pigeon and hen egg yolks had significant difference from other egg yolks.

3.6. Diversity and comparison of different groups by hierarchical cluster analysis

Hierarchical cluster analysis provided a clear clustering tendency of egg yolk samples containing similar profiles of the investigated data. Each colored cell on the map corresponds to a concentration value in the data table. The hierarchical cluster analysis of top 25 (concentration) molecular species of various egg yolks was given in **Figure 5c**. Based on these values, the seven egg samples could be classified correctly into two parts. The pigeon egg yolk and hen egg yolk were arranged in a single portion and other samples were classified as the second part based on the composition similarities. It indicated that the phospholipid profiles of pigeon egg yolk were closest to hen egg yolk, following quail, duck, ostrich, emu and goose egg yolks. The concentrations of molecular species, including PE(18:0/22:6), PE(16:0/18:2), PE(16:0/22:6), LPE(18:0), LPE(16:0), LPE(16:0), PE(16:0/20:4), PE(18:0/20:4) and PE(18:0/22:4), in hen egg yolk, and PE(O-16:1/22:6), OxPE(18:0/18:2+2O), PE(O-18:1/18:2), PC(16:0/18:2), PC(18:0/18:2), PE(O-16:1/20:4), PE(O-18:1/20:3) and PE(O-20:1/18:2), in pigeon egg yolk, SM(d18:1/16:0) and LPA(16:0) in goose egg yolk and PC(16:0/20:4) in duck egg yolk, appeared in significant high concentrations compared to those species in other egg yolks.

4. Conclusion

In summary, we reported a use of UHPLC-ESI-Q-TOF/MS technique to carefully elucidate and quantify molecular species of different phospholipid classes in seven egg types. Purified phospholipids (> 96%) were obtained by adsorption column chromatography. Their molecular structures were further identified by the MS/MS fragmentation pattern of each informative mass ion. Quantification of phospholipid molecular species was achieved using d7-phospholipid standards calibration with high accuracy. The UHPLC-ESI-Q-TOF-MS method was proven to be an effective method for structural identification and quantification of phospholipid. Discriminatory analysis by PCA demonstrated that phospholipid profiles of pigeon and hen egg yolks had significant difference from other egg yolk sources. Hierarchical cluster analysis further indicated that the phospholipid profiles of pigeon egg yolk were closest to hen egg yolk, following ostrich, emu, duck, goose and quail egg yolks. The quantitation of some representative molecular species can be considered to be most responsible for the discrimination among egg yolks phospholipids. Phospholipids might be as used as molecular fingerprints for egg analysis, which could be applied to control origination and tagging, and prevent fraudulent behavior. Furthermore, based on the phospholipid profiles and the content of DHA+EPA and PUFA, hen egg, goose egg and pigeon egg were probably superior to other eggs studied. Herein, hen egg maybe the best choice in consideration of taste and price. Therefore, this study can help scientific research institutions and worker (including food control laboratories, nutritionists, dieticians and so on) better understand the molecular composition of phospholipids in eggs and better select or evaluate the nutritional value of eggs.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (31501521) and the Fundamental Research Funds for the Central Universities (CZY19032). The authors thank the Analytical & Measuring Center, School of Pharmaceutical Sciences, South-Central University for Nationalities for the spectral analyses.

Supporting information

HPLC Chromatogram of purified phospholipids from seven egg types. Extracted individual ion chromatograms of phospholipid molecular species. Pairwise score plot for 5 principal components. Fatty acid profiles of purified phospholipids from seven egg types. Eigen analysis of the correlation matrix loadings of the principal components

References

- Ali, A. H., Zou, X. Q., Huang, J. H., Abed, S. M., & Tao, G. J. (2017). Profiling of phospholipids molecular species from different mammalian milk powders by using ultra-performance liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry. *Journal of Food Composition and Analysis*, 62, 143-154.
- Ali, A. H., Zou, X. Q., Lu, J., Abed, S. M., Yao, Y. P., Tao, G. J., ... Wang, X. G. (2017). Identification of phospholipids classes and molecular species in different types of egg yolk by using UPLC-Q-TOF-MS. *Food Chemistry*, 221, 58-66.
- Anton, M. (2007). Composition and structure of hen egg yolk. In R. Huopalathi, R. Lopez-Fandino, R. Anton, & R. Schade (Eds.), *Bioactive egg compounds* (pp. 1–6). Heidelberg: Springer Verlag Berlin.
- Aoki, J., Inoue, A., & Okudaira, S. (2008). Two pathways for lysophosphatidic acid production. *Biochimica Et Biophysica Acta-molecular and Cell Biology of Lipids*

508 1781(9), 513-518.

509 Aro, H., Järvenpää, E. P., Könkö, K., Sihvonen, M., Hietaniemi, V., & Huopalahti, R.
510 (2009). Isolation and purification of egg yolk phospholipids using liquid extraction and
511 pilot-scale supercritical fluid techniques. *European Food Research and Technology*,
512 228(6), 857-863.

513 Brenna, J. T. (2016). Arachidonic acid needed in infant formula when docosahexaenoic
514 acid is present. *Nutrition Reviews*, 74(5), 329-336.

515 Boselli, E., & Caboni, M. F. (2000). Supercritical carbon dioxide extraction of
516 phospholipids from dried egg yolk without organic modifier. *The Journal of*
517 *Supercritical Fluids*, 19(1), 45-50.

518 Campos, A. M., Ricardo, F., Alves, E., Reis, A., Couto, D., Domingues, P., ...
519 Domingues, M. (2016). Lipidomic investigation of egg' yolk: changes in lipid profiles
520 of eggs from different conditions. *Food Research International*, 89(1), 177-185.

521 Chapelle, S. (1987). Plasmalogens and O-alkylglycerophospholipids in aquatic animals.
522 *Comparative Biochemistry and Physiology. B: Biochemistry and Molecular Biology*,
523 88, 1-6.

524 Deeley, J. M., Thomas, M. C., Truscott, R. J. W., Mitchell, T. W., & Blanksby, S. J.
525 (2009). Identification of abundant alkyl ether glycerophospholipids in the human lens
526 by tandem mass spectrometry techniques. *Analytical Chemistry*, 81, 1920-1930.

527 Fischbeck, A., Kruger, M., Blaas, N., & Humpf, H. U. (2009). Analysis of sphingomyelin
528 in meat based on hydrophilic interaction liquid chromatography coupled to
529 electrospray ionization-tandem mass spectrometry. *Journal of Agricultural and Food*
530 *Chemistry*, 57, 9469-9474.

531 Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957). A simple method for the isolation
 532 and purification of total lipids from animal tissues. *The Journal of Biological*
 533 *Chemistry*, 226, 497–509.

534 Gang, K. Q., Zhou, D. Y., Lu, T., Liu, Z. Y., Zhao, Q., Xie, H. K., ... Shahidi, F. (2018).
 535 Direct infusion mass spectrometric identification of molecular species of
 536 glycerophospholipid in three species of edible whelk from Yellow Sea. *Food*
 537 *Chemistry*, 245, 53–60.

538 Graves, E., Beaulieu, A., & Drackley, J. (2007). Factors affecting the concentration of
 539 sphingomyelin in bovine milk. *Journal of Dairy Science*, 90 (2), 706–715.

540 Harrabi, S., Herchi, W., Kallel, H., Mayer, R. M., & Boukhchina, S. (2009). Liquid
 541 chromatographic-mass spectrometric analysis of glycerophospholipids in corn oil.
 542 *Food Chemistry*, 114, 712-716.

543 Herchi, W., Sakouhi, F., Khaled, S., Xiong, Y. P., Boukhchina, S., Kallel, H., & Curtis, J.
 544 M. (2011). Characterisation of the glycerophospholipid fraction in flaxseed oil using
 545 liquid chromatography-mass spectrometry. *Food Chemistry*, 129(2), 437-442.

546 Kaiser, H. F., & Rice, J. (1974). Little jiffy, mark Iv. *Educational and Psychological*
 547 *Measurement*, 34, 111-117.

548 Koivusalo, M., Haimi, P., Heikinheimo L, Kostainen R, & Somerharju P. (2001).
 549 Quantitative determination of phospholipid compositions by ESI-MS: Effects of acyl
 550 chain length, unsaturation, and lipid concentration on instrument response. *Journal of*
 551 *Lipid Research*, 42, 663-672.

552 Le Grandois, J., Marchioni, E., Zhao, M. J., Giuffrida, F., Ennahar, S., & Bindler, F.
 553 (2009). Investigation of natural phosphatidylcholine sources: separation and

554 identification by liquid chromatography-electrospray ionization-tandem mass
 555 spectrometry (LC-ESI-MS²) of molecular species. *Journal of Agricultural and Food*
 556 *Chemistry*, 57, 6014-6020.

557 Li, Q. Q., Zhao, Y., Zhu, D., Pang, X. M., Liu, Y., Frew, R., & Chen, G. (2017)
 558 Lipidomics profilings of goat milk, soymilk and bovine milk by UPLC-Q-Exactive
 559 Orbitrap Mass Spectrometry. *Food Chemistry*, 224, 302-309.

560 Ma, L., MacGibbon, A. K. H., Jan Mohamed, H. J. B., Loy, S., Rowan, A., McJarow, P.,
 561 & Fong, B. Y. (2017). Determination of phospholipid concentrations in breast milk and
 562 serum using a high-performance liquid chromatography-mass spectrometry-multiple
 563 reaction monitoring method. *International Dairy Journal*, 71, 50-59.

564 Mawatari, S., Hazeyama, S., & Fujino, T. (2016). Measurement of ether phospholipids in
 565 human plasma with HPLC-ELSD and LC/ESI-MS after hydrolysis of plasma with
 566 phospholipase A1. *Lipids*, 51(8), 997-1006.

567 Miao, H., Chen, H., Pei, S. W., Bai, X., Vaziri, N. D., & Zhao, Y. Y. (2015). Plasma
 568 lipidomics reveal profound perturbation of glycerophospholipids, fatty acids, and
 569 sphingolipids in diet-induced hyperlipidemia. *Chemico-Biological Interactions*,
 570 228(25), 79-87.

571 Montealegre, C., Sánchez-Hernández, L., Crego, A. L., & Marina, M. L. (2013).
 572 Determination and characterization of glycerophospholipids in olive fruit and oil by
 573 nonaqueous capillary electrophoresis with electrospray-mass spectrometric detection.
 574 *Journal of Agricultural and Food Chemistry*, 61, 1823-1832.

575 Pacetti, D., Boselli, E., Hulan, H. W., & Frega, N. G. (2005). High performance liquid
 576 chromatography-tandem mass spectrometry of phospholipid molecular species in eggs

577 from hens fed diets enriched in seal blubber oil. *Journal of Chromatography A*, 1097,
578 66-73.

579 Park, E. J., Suh, M., Thomson, A. B. R., Ramanujam, K. S., & Clandinin, M. T. (2006).
580 Dietary gangliosides increase the content and molecular percentage of ether
581 phospholipids containing 20:4n-6 and 22:6n-3 in weanling rat intestine. *Journal of*
582 *Nutritional Biochemistry*, 17, 337-344.

583 PIGINI, D., CIALDELLA, A. M., FARANDA, P., & TRANFO, G. (2006). Comparison between
584 external and internal standard calibration in the validation of an analytical method for
585 1-hydroxypyrene in human urine by high-performance liquid chromatography/tandem
586 mass spectrometry. *Rapid Communications in Mass Spectrometry*, 20, 1013-1018.

587 Pulfer, M., & Murphy, R. C. (2003). Electrospray mass spectrometry of phospholipids.
588 *Mass Spectrometry Reviews*, 22(5), 332-364.

589 Senyuva, H. Z., Gökmen, V., & Sarikaya, E. A. (2015). Future perspectives in
590 OrbitrapTM-high-resolution mass spectrometry in food analysis: A review. *Food*
591 *Additives and Contaminants - Part A Chemistry*, 32, 1568-1606.

592 She, Y. Q., Song, J. N., Zhao, L. L., Zhong, Y. M., Rui, W., Feng, Y. F., & Wu, X.
593 (2014). Rapid identification of glycerophospholipids from RAW264.7 cells by
594 UPLC/ESI-QTOF-MS. *Biomedical Chromatography*, 28, 1744-1755.

595 Shin, E. C., Craft, B. D., Pegg, R. B., Dixon Phillip, R., & Eitenmiller, R. R. (2010).
596 Chemometric approach to fatty acid profiles in Runner-type peanut cultivars by
597 principal components analysis. *Food Chemistry*, 119(3), 1262-1270.

598 Walczak, J., Bocian, S., & Buszewski, B. (2015). Two-dimensional high-performance
599 liquid chromatography-mass spectrometry for phosphatidylcholine analysis in egg yolk.

600 *Food Analytical Methods*, 8(3), 661–667.

601 Wang, D. Y., Xu, W. M., Xu, X. L., Zhou, G. H., Zhu, Y. Z., Li, C. B., & Yang, M. (2009).

602 M. Determination of intramuscular phospholipid classes and molecular species in

603 Gaoyou duck. *Food Chemistry*, 112, 150-155.

604 Zhang, T. T., Xu, J., Wang, Y. M., & Xue, C. H. (2019). Health benefits of dietary marine

605 DHA/EPA-enriched glycerophospholipids. *Progress in Lipid Research*, 75, 100997.

606 Zhang, Y. Y., Liu, Y. X., Zhou, Z., Zhou, D. Y., Du, M., Zhu, B. W., & Qin, L. (2019).

607 Improving lipidomic coverage using UHPLC-ESI-Q-TOF-MS for marine shellfish by

608 optimizing the mobile phase and resuspension solvents. *Journal of Agricultural and*

609 *Food Chemistry*, 67, 8677-8688.

610 Zhou, L., Grandois, J. L., Marchioni, E., Zhao, M. J., Ennahar, S., & Bindler, F. (2010).

611 Improvement of total lipid and glycerophospholipid recoveries from various food

612 matrices using pressurized liquid extraction. *Journal of Agricultural and Food*

613 *Chemistry*, 58, 9912-9917.

614 Zhou, L., Wang, Y., Wang, X. L., Liang, Y., Huang, Z., & Zeng, X. X. (2017).

615 MALDI-TOF/TOF mass spectrometric determination and antioxidant activity of

616 purified phosphatidylcholine fractions from shrimp species. *Journal of Agricultural*

617 *and Food Chemistry*, 2017, 65, 1229-1238.

618 Zhou, L., Zhao, M. J., Ennahar, S., Bindler, F., & Marchioni, E. (2012a). Determination

619 of phosphatidylethanolamine molecular species in various food matrices by liquid

620 chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS²).

621 *Analytical and Bioanalytical Chemistry*, 403, 291-300.

622 Zhou, L., Zhao, M. J., Ennahar, S., Bindler, F., & Marchioni, E. (2012b) Liquid

623 chromatography-tandem mass spectrometry for the determination of sphingomyelin
624 species from calf brain, ox liver, egg yolk and krill oil. *Journal of Agricultural and*
625 *Food Chemistry*, 60, 293-298.

Figure captions

Figure 1. Total ion chromatogram of molecular species from phospholipids under negative ion mode. (a) hen egg yolk; (b) duck egg yolk; (c) goose egg yolk; (d) pigeon egg yolk; (e) quail egg yolk; (f) ostrich egg yolk; (g) emu egg yolk.

Figure 2. MS/MS fragmentation pathway of PE(16:0/18:2) (m/z 714.5151) (a), PE(O-18:1/18:2) (m/z 726.5488) (b), LPE (18:0) (m/z 480.3089) (c), OxPE(18:0/22:5) (m/z 824.5493) (d), PE-OH(16:0/18:2) (699.4998) (e) under negative ion mode.

Figure 3. (a) MS/MS fragmentation pathway of PC((16:0/18:1) (m/z 818.5958), (b) PG (16:0/20:1) (m/z 775.5499) and (c) PI(18:0/20:4) (m/z 885.5569) under negative ion mode.

Figure 4. MS/MS fragmentation pathway of PA(16:0/18:1) (m/z 673.4848) (a) and LPA(16:0) (m/z 409.2340) (b) under negative ion mode, and SM(d18:1/16:0) (m/z 703.5759) (c) under positive ion mode.

Figure 5. (a) Loading plot of PC1 and PC2 for phospholipid in seven egg types. (b) Score plots for phospholipids in seven egg types. (c) Hierarchical cluster analysis based on top 25 molecular species in seven egg types. Colors represent different concentrations indicated by the color bar.

Figure 1

Figure 2

Figure 3

Figure 4

(a)

(b)

(c)

Table 1. Calibration curves, retention time (RT), linear regression coefficients (R^2), limit of detection (LOD) and limit of quantification (LOQ) of seven phospholipid standards

Standards	Formula	RT (min)	m/z	Equations	R^2	LOD (ng/mL)	LOQ (ng/mL)
15:0-18:1-d7-PE	$C_{38}H_{67}D_7NO_8P$	4.06	709.5519	$y = 2.0415x + 0.1129$	0.9982	0.5	1.7
15:0-18:1-d7-PI	$C_{42}H_{72}D_7O_{13}P$	2.93	828.5625	$y = 3.3967x - 0.1603$	0.9993	0.3	1.0
15:0-18:1-d7-PC	$C_{41}H_{73}D_7NO_8P$	4.74	811.6189	$y = 1.5318x - 0.1809$	0.9988	0.6	2.0
15:0-18:1-d7-PA	$C_{36}H_{62}D_7O_8P$	3.07	666.5097	$y = 2.9346x - 1.2760$	0.9961	0.4	1.3
15:0-18:1-d7-PS	$C_{39}H_{67}D_7NO_{10}P$	3.08	753.5417	$y = 1.4189x - 0.5214$	0.9942	0.8	2.6
15:0-18:1-d7-PG	$C_{39}H_{68}D_7O_{10}P$	3.01	740.5464	$y = 4.2594x - 0.1083$	0.9981	0.2	0.7
d18:1/12:0-SM	$C_{35}H_{71}N_2O_6P$	3.34	647.5123	$y = 1.9735x - 0.0236$	0.9921	0.5	1.7

plasmamylethanolamine (PE-O-alkyl-acyl), lysophosphatidylethanolamine (LPE)], phosphatidylethanol (PEtOH) and oxidized phosphatidylethanolamine (OxPE),

Table 2. Molecular species profiles of purified phospholipids from seven egg types

Precursors	<i>m/z</i>	Error (ppm)	RT (min)	Species	Concentrations (nmol/g)						
					Hen	Duck	Goose	Pigeon	Quail	Ostrich	Emu
PE											
[M-H] ⁻	688.4958	+5.08	5.27	16:0/16:1	30.76	5.53	7.78	3.97	10.66	24.99	nd
[M-H] ⁻	698.5128	-0.29	5.69	O-16:1/18:2	14.20	nd	2.48	52.06	3.98	18.95	nd
[M-H] ⁻	700.5293	+0.86	5.76	O-16:0/18:2	16.91	1.19	nd	14.92	3.80	1.41	nd
[M-H] ⁻	700.5326	+5.57	6.10	O-16:1/18:1	41.79	9.20	12.55	105.39	16.04	59.09	1.21
[M-H] ⁻	702.5483	+5.70	6.18	O-16:0/18:1	30.98	5.53	9.33	24.77	14.95	15.88	4.58
[M-H] ⁻	712.4940	+2.38	5.01	16:1/18:2	8.36	nd	nd	nd	nd	1.33	nd
[M-H] ⁻	712.4955	+4.49	5.15	16:0/18:3	1.14	nd	nd	nd	nd	4.75	nd
[M-H] ⁻	714.5151	+4.06	5.41	16:0/18:2	499.73	48.02	55.06	121.55	52.98	61.26	10.65
[M-H] ⁻	716.5284	+6.70	5.79	16:0/18:1	2.27	nd	nd	nd	nd	nd	nd
[M-H] ⁻	718.5428	+5.01	6.24	16:0/18:0	4.65	nd	nd	nd	nd	nd	nd
[M-H] ⁻	722.5172	+5.81	5.65	O-16:1/20:4	6.57	nd	2.05	79.77	nd	11.25	nd
[M-H] ⁻	724.5321	+4.69	5.79	O-18:2/18:2	11.43	nd	3.99	56.36	nd	4.10	nd
[M-H] ⁻	726.5463	+2.75	6.19	O-18:2/18:1	9.74	6.23	14.37	25.45	nd	9.41	nd
[M-H] ⁻	726.5488	+6.19	6.25	O-18:1/18:2	40.56	nd	3.17	135.40	3.97	9.39	nd
[M-H] ⁻	728.5261	+3.43	5.68	17:0/18:2	7.54	nd	nd	nd	nd	2.04	nd
[M-H] ⁻	728.5618	+2.61	6.68	O-18:1/18:1	28.17	3.70	8.69	54.80	3.82	5.63	nd
[M-H] ⁻	730.5413	+2.87	6.07	O-17:0/18:1	8.70	nd	3.08	2.95	2.43	2.79	nd
[M-H] ⁻	730.5777	+2.87	6.76	O-18:0/18:1	32.86	nd	6.46	21.03	15.51	1.96	3.23
[M-H] ⁻	736.4948	+3.39	4.96	16:1/20:4	7.58	nd	nd	nd	nd	2.65	nd
[M-H] ⁻	738.5129	+6.63	5.37	16:0/20:4	402.30	43.25	113.19	35.23	26.00	56.59	2.85
[M-H] ⁻	740.5275	+5.27	5.50	18:1/18:2	6.79	nd	4.66	nd	nd	1.11	nd
[M-H] ⁻	740.5568	-4.19	4.40	O-22:3/15:0	10.08	9.90	7.57	13.34	9.52	7.30	7.59
[M-H] ⁻	742.5430	+5.11	5.95	18:0/18:2	582.06	22.78	126.25	474.04	110.66	92.91	23.22
[M-H] ⁻	742.5443	+6.89	4.38	18:1/18:1	97.05	85.55	41.25	46.73	22.61	23.18	5.11
[M-H] ⁻	744.5580	+4.80	6.35	18:0/18:1	1.95	nd	nd	nd	nd	nd	nd
[M-H] ⁻	746.5148	+2.41	5.55	O-16:1/22:6	32.44	4.78	9.19	83.50	7.17	26.37	nd
[M-H] ⁻	748.5314	+4.68	5.88	O-16:1/22:5	27.31	4.22	13.39	18.14	2.94	18.24	nd
[M-H] ⁻	748.5345	+7.75	5.63	O-16:0/22:6	25.74	nd	3.78	nd	16.41	nd	nd
[M-H] ⁻	750.5505	+8.26	5.79	O-16:0/22:5	25.86	nd	2.96	nd	4.68	nd	nd
[M-H] ⁻	750.5478	+4.46	6.04	O-16:1/22:4	5.44	nd	7.68	15.31	nd	6.64	nd
[M-H] ⁻	752.5655	+7.44	6.48	O-18:1/20:3	nd	nd	nd	15.77	nd	nd	nd
[M-H] ⁻	754.5787	+4.11	6.82	O-20:1/18:2	nd	nd	nd	6.53	nd	nd	nd

[M-H] ⁻	762.5111	+4.20	5.27	16:0/22:6	208.01	43.29	33.29	24.02	26.26	10.23	5.40
[M-H] ⁻	764.5240	+0.52	5.45	18:1/20:4	117.98	20.61	43.10	11.96	17.86	26.99	2.66
[M-H] ⁻	766.5423	+4.04	5.91	18:0/20:4	956.93	127.67	282.99	254.84	118.45	146.41	14.66
[M-H] ⁻	768.5568	+2.47	6.14	18:0/20:3	36.01	3.27	10.15	24.88	2.25	3.25	nd
[M-H] ⁻	770.5718	+1.56	6.46	18:1/20:1	12.20	0.00	3.80	10.20	nd	nd	nd
[M-H] ⁻	772.5327	+5.18	5.63	O-18:2/22:6	7.22	2.08	4.15	31.74	nd	nd	nd
[M-H] ⁻	772.5873	+1.42	6.91	18:0/20:1	6.38	nd	2.25	5.56	nd	nd	nd
[M-H] ⁻	774.5493	+6.46	6.09	O-18:1/22:6	6.13	nd	2.76	nd	3.67	nd	nd
[M-H] ⁻	776.5636	+4.76	6.18	O-18:0/22:6	19.90	2.93	3.08	6.26	22.58	nd	nd
[M-H] ⁻	778.5762	+0.77	6.60	O-18:1/22:4	6.70	nd	7.05	16.78	nd	nd	nd
[M-H] ⁻	778.5785	+3.72	6.54	O-18:0/22:5	35.28	nd	1.14	nd	5.56	nd	nd
[M-H] ⁻	780.5947	+4.48	6.72	O-18:0/22:4	21.03	nd	2.34	3.99	3.00	nd	nd
[M-H] ⁻	788.5248	+1.52	5.36	18:1/22:6	9.75	4.18	2.91	3.14	4.59	nd	nd
[M-H] ⁻	790.5425	+4.17	5.80	18:0/22:6	95.77	23.70	17.25	43.30	26.54	8.38	4.21
[M-H] ⁻	792.5581	+4.03	6.15	18:0/22:5	141.64	17.45	43.96	12.72	8.99	11.59	nd
[M-H] ⁻	794.5729	+2.89	6.32	18:0/22:4	26.37	3.68	6.47	6.75	1.98	nd	nd
PE-OH											
[M-H] ⁻	699.4998	+4.00	5.72	16:0/18:2	nd	nd	nd	5.4	nd	1.68	nd
LPE											
[M-H] ⁻	438.2630	+0.91	2.50	15:0	4.79	5.49	4.17	6.18	5.27	3.97	4.12
[M-H] ⁻	438.2986	-0.91	2.99	O-16:0	7.04	nd	nd	nd	nd	nd	nd
[M-H] ⁻	450.2639	+2.89	2.38	16:1	3.61	nd	nd	nd	nd	nd	nd
[M-H] ⁻	452.2783	0	2.76	16:0	239.84	14.03	24.63	13.09	10.25	4.79	nd
[M-H] ⁻	464.3143	-0.65	3.09	O-18:1	7.42	nd	2.35	nd	2.69	nd	nd
[M-H] ⁻	466.3311	+1.72	3.47	O-18:0	11.15	nd	nd	nd	2.75	nd	nd
[M-H] ⁻	476.2781	-0.42	2.55	18:2	34.61	3.81	7.25	61.77	9.59	4.72	nd
[M-H] ⁻	480.3089	+4.16	3.23	18:0	419.46	22.31	33.33	49.84	18.06	7.36	2.52
[M-H] ⁻	500.2780	-0.60	2.54	20:4	39.82	nd	10.26	42.02	7.56	3.90	nd
[M-H] ⁻	502.2967	+5.57	2.76	20:3	3.10	nd	nd	nd	nd	nd	nd
[M-H] ⁻	524.2783	0	2.50	22:6	21.54	3.64	7.46	24.25	11.83	3.16	nd
[M-H] ⁻	526.2944	+0.95	2.77	22:5	41.48	2.93	10.52	5.30	3.72	2.17	nd
OxPE											
[M-H] ⁻	774.5359	+8.78	4.47	18:0/18:2+2O	3.70	nd	nd	14.77	nd	nd	nd
[M-H] ⁻	824.5493	+5.58	5.95	18:0/22:5+2O	10.14	2.64	3.09	9.79	3.12	2.31	nd
PC											
[M+CH ₃ COO] ⁻	790.5649	+5.69	5.22	16:0/16:1	87.14	111.74	211.36	120.25	42.61	178.46	41.26
[M+CH ₃ COO] ⁻	814.5631	+3.31	4.92	16:1/18:2	129.27	34.94	63.52	131.89	30.17	35.93	17.24

[M+CH ₃ COO] ⁻	816.5810	+6.12	5.34	16:0/18:2	1156.53	642.03	1241.58	4255.32	395.23	387.96	278.62
[M+CH ₃ COO] ⁻	818.5958	+5.01	5.73	16:0/18:1	1783.42	1367.30	2098.15	3614.85	480.53	686.58	475.86
[M+CH ₃ COO] ⁻	840.5803	+5.08	5.30	16:0/20:4	245.91	312.73	275.86	320.26	143.21	128.87	63.00
[M+CH ₃ COO] ⁻	842.5933	+1.90	5.44	18:1/18:2	137.63	118.35	187.84	620.90	83.28	96.86	61.12
[M+CH ₃ COO] ⁻	844.6126	+6.28	5.88	18:0/18:2	302.96	311.90	625.30	1892.91	224.69	230.50	175.12
[M+CH ₃ COO] ⁻	846.6256	+3.07	6.31	18:0/18:1	274.89	223.34	480.44	628.00	127.77	151.38	121.00
[M+CH ₃ COO] ⁻	864.5805	+5.20	5.22	16:0/22:6	88.49	43.08	68.67	80.78	65.93	nd	28.69
[M+CH ₃ COO] ⁻	866.5956	+4.50	5.53	16:0/22:5	103.78	66.94	67.55	nd	59.20	nd	nd
PG											
[M-H] ⁻	747.5183	+0.13	4.58	16:0/18:1	12.61	3.12	5.46	37.39	3.79	14.64	nd
[M-H] ⁻	771.5226	+5.70	4.42	16:0/20:3	1.99	nd	nd	nd	nd	nd	nd
[M-H] ⁻	773.5392	+6.98	4.65	16:0/20:2	3.98	1.62	2.51	16.02	1.34	1.34	nd
[M-H] ⁻	775.5499	+0.52	4.94	16:0/20:1	9.00	2.39	5.97	52.20	4.22	1.51	nd
PI											
[M-H] ⁻	807.5096	+8.30	4.15	16:0/16:1	0.25	0.03	0.07	0.09	0.05	0.04	0.03
[M-H] ⁻	833.5223	+4.44	4.25	16:0/18:2	12.82	4.40	3.00	11.11	17.74	5.40	2.58
[M-H] ⁻	835.5376	+4.07	4.47	16:0/18:1	8.96	4.26	6.28	4.92	32.20	11.99	2.53
[M-H] ⁻	855.5109	+9.35	4.05	16:0/20:5	0.07	0.30	0.02	0.21	0.20	0.16	0.05
[M-H] ⁻	857.5223	+4.31	4.24	16:0/20:4	14.67	20.73	8.27	2.79	22.88	8.88	6.07
[M-H] ⁻	861.5520	+2.44	4.59	18:0/18:2	18.50	8.21	8.05	33.33	49.90	15.29	4.98
[M-H] ⁻	863.5724	+8.00	4.87	18:0/18:1	4.35	2.83	3.40	6.81	20.33	7.95	2.05
[M-H] ⁻	879.5092	+7.16	3.90	18:2/20:5	0.00	0.04	nd	0.07	0.00	0.03	0.00
[M-H] ⁻	881.5224	+4.31	4.08	18:1/20:5	0.18	0.22	0.03	0.11	0.27	0.05	0.09
[M-H] ⁻	883.5401	+6.68	4.35	18:0/20:5	1.44	1.43	0.76	0.33	6.21	1.10	1.96
[M-H] ⁻	885.5569	+7.90	4.58	18:0/20:4	65.05	105.89	60.00	125.99	169.24	43.19	30.95
[M-H] ⁻	887.5735	+9.01	4.73	18:0/20:3	4.62	3.36	8.13	4.63	7.17	3.57	0.57
PA											
[M-H] ⁻	671.4678	+2.98	5.33	16:0/18:2	220.96	132.61	277.57	330.15	141.54	203.85	202.19
[M-H] ⁻	673.4848	+5.05	5.70	16:0/18:1	351.72	208.09	304.66	288.87	241.89	231.15	257.72
LPA											
[M-H] ⁻	409.2340	-5.13	2.72	16:0	775.87	597.56	930.25	645.17	979.32	701.30	765.50
SM											
[M+H] ⁺	703.5759	-9.10	5.03	d18:1/16:0	31672	5912	30542	27027	3502	6097	2323

nd means not detected; RT means retention time.

Graphic Abstract