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Identification of phospholipids classes and molecular species in different types of egg yolk by using UPLC-Q-TOF-MS

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ABSTRACT

Egg phospholipids (PLs) are currently the products of greatest commercial interest with major area of importance in various fields. Therefore, in this study, duck, hen and quail egg yolk PLs were isolated by solvent extraction with chilled acetone precipitation, and subsequently separated and identified by using ultra-performance liquid chromatography with quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS). Egg PLs were separated on hydrophilic interaction liquid chromatography (HILIC) with ethylene bridged hybrid (BEH) column by gradient elution using acetonitrile/ammonium formate as a mobile phase, and detected by mass spectrometry (MS) under electrospray ionization in positive and negative ion mode. Structural characterizations of 57 molecular species of egg yolk PLs were identified based on MS/MS fragment ion information and elemental composition in MassLynx 4.1 software. The obtained results showed that phosphatidylcholine (16:0–18:1), phosphatidylethanolamine (18:0–20:4), phosphatidylinositol (18:0–18:2), phosphatidylserine (18:0–18:2), sphingomyelin (d18:1/16:0) and lysophosphatidylcholine (16:0) were the predominant species among the different classes of egg yolk phospholipids.

1. Introduction

Egg is considered to be one of the most nutritional foods in human diet, containing many valuable nutrients, such as phospholipids (PLs), essential fatty acids, amino acids, minerals, and vitamins. Egg yolk is probably the richest source of PLs in nature, it approximately contains 10% of phospholipids (on the base of the wet weight), representing 22% of egg yolk total solids. Egg lipids are characterized and distinguished by their high content of PLs, they consisted of 33% PLs, 62% triglycerides, and 5% cholesterol (Anton, 2007).

PLs are an important group of biomolecules (Gunstone, 2008, Chapter 1). Based on the type of alcohol backbone, PLs can be divided into two main groups; glycerophospholipids and sphingolipids. Glycerophospholipids are composed of a glycerol backbone with two fatty acids esterified at the *sn*-1 and *sn*-2 positions, and a phosphorylated alcohol (choline, ethanolamine, inositol, or serine), they involve phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). On the other hand, sphingolipids are composed of the aliphatic amino alcohol sphingosine, fatty acids and sugars or alcohols or phosphoric acid; they include sphingomyelin (SM), gangliosides and cerebrosides (Huang, 2001). Human daily consumption of PLs ranges between 2 and 8 grams/-day, representing 1–10% of the total daily fat intake. Egg PLs contribute a large proportion of the daily intake of PLs (Cohn, Wat, Kamili, & Tandy, 2008). According to the clinical and the biological experiments, egg SM and PC have an effective role in lipid absorption regulation, reducing hepatic lipid levels and inflammation (Blesso, 2015).

Extraction of total lipids and elimination of cholesterol from egg were the main focused points of interest in the beginning. Researchers have investigated many procedures for PLs fractionation, such as extraction with aqueous ethanol and removing the solidified neutral oil with low-temperature crystallization (Sim, 1995), and separation of egg yolk phospholipids by ultrafiltration (Miyata & Matsumoto, 2001). Phospholipids of 47% purity were isolated from egg yolk using high and low concentrations of aqueous ethanol (Palacios & Wang, 2005). The extraction of PLs from

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natural matrices and foodstuffs with high efficiency is needed, because of the availability and the economics of these high-valued lipid products (Wang, Yao, & Wang, 2014). PLs analysis includes several procedures, consisting of lipid extraction, PLs fractions separation from the other lipid classes (neutral lipids and cholesterol), separation and detection of the different isolated fractions of PLs (Donato et al., 2011). Studies on the separation and purification of phospholipids have been undertaken widely, for example, procedures of solvent extraction (Szuhaj, 1989), thin-layer chromatography, high speed countercurrent chromatography (Shibusawa, Takeuchi, Shindo, & Ito, 2005), high performance liquid chromatography (Yoon & Kim, 2002), two dimensional liquid chromatography (Walczak, Bocian, & Buszewski, 2015), and hydrophilic interaction liquid chromatography (HILIC) (Sun, Zhao, & Curtis, 2015; Zhao, Xiong, & Curtis, 2011).

Although mass spectrometry has been extensively used in the analysis of lipids, the large number of PLs species makes the analysis a challenge; because of ion suppression, and the simultaneous occurrence of structural and positional isomers. Also, the difficult detection of the low abundance fractions of PLs when they are analyzed in the existence of the high abundance fractions (Sato, Nakamura, Aoshima, & Oda, 2010). Therefore, chromatographic separation of even phospholipid class before mass analysis ought to increase the detection of the low abundance fractions. Recently, ultra-performance liquid chromatography with quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS) has been used in complicated systems analysis because of its rapid and sensitive separation, and the precision in mass measurement. The current developments and modifications in this technique of analysis resulted in better separations with high resolution, increased sensitivity, and faster analysis. Consequently, the problems linked with complex lipid separation and characterization could be solved (Laaksonen et al., 2006).

This study applies the most recent analytical technique UPLC-Q-TOF-MS for the separation and the identification of phospholipids classes and molecular species in duck, hen and quail egg yolks. Herein, egg yolk PLs were extracted, then HILIC was applied to separate PLs classes based on the polarity of their headgroups using a BEH column, and culminating by the analysis of the fatty acyl residues (fatty acids) of egg yolk phospholipids using gas chromatography.

2. Materials and methods

2.1. Materials

Fresh duck, hen and quail eggs were purchased from local supermarket in Wuxi City, Jiangsu, China. Phospholipids standards; 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmisoyl-*sn*-glycero-3-phosphoinositol (Ammonium Salt) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine 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 chloroform (HPLC grade) were provided by TEDIA (Tedia Company Inc, Ohio, USA).

2.2. Extraction of egg yolk phospholipids

PLs were extracted from duck, hen and quail egg yolks as described by Gładkowski, Chojnacka, Kiełbowicz, Trziszka, and Wawrzeńczyk (2012) with some modification. Briefly, eggshells were carefully washed and broken. Egg yolks were separated from egg whites and stored at 5 °C until use. 40 g of egg yolk were placed in a 200-mL vial with a magnetic stirrer, then 120 mL of ethanol was added and the mixture was stirred for 10 min. The solvent layer was then removed and filtered into a round-bottom flask and the residual was extracted twice with ethanol. The obtained ethanolic solution was subjected to low temperature crystallization at 2–5 °C for 2 h. Afterward, the solvent was evaporated from the solution by a rotary evaporator under reduced pressure and the extracts were collected. The residue was dissolved in 60 mL hexane and transferred to a vial placed in an ice bath (0 °C). Next, 120 mL of cold acetone (−20 °C) was carefully poured into the stirring mixture until PLs precipitation. After the precipitation of PLs, the stirring was stopped and the supernatant was decanted off. The precipitate was carefully washed 5 times with 20-mL portions of cold acetone (−20 °C) and the solvent was removed by decantation each time. Finally, the obtained phospholipids (~3.8–4 g) were dried under a gentle stream of nitrogen and stored at −20 °C for further analysis.

2.3. Analysis of the fatty acids of egg yolk phospholipids

Fatty acids methyl esters of the three types of egg yolk phospholipids were prepared according to the method adapted by Lopez et al. (2008). Briefly, 50 μg of PLs were weighted in screw-capped tubes and 300 μL of 10% BF_3-methanol was added, and the tubes were kept at 100 °C for 90 min. Then, 600 μL of *n*-hexane and 1.5 mL of deionized water were added. The mixtures were centrifuged at 2000 rpm for 5 min, then the upper phase was collected and 1.5 mL of deionized water was added, and the tubes were mixed and centrifuged. The process of washing, mixing and centrifuging was repeated three times. A pinch of anhydrous sodium sulfate was added, and the tubes were kept at 4 °C for 30 min. Finally, the tubes were centrifuged at 2000 rpm for 20 min and the upper layer was collected for gas chromatography analysis.

Gas chromatography (GC) (Agilent 7820A) equipped with an autosampler, a flame ionization detector, and an ionic liquid capillary column (TRACE TR-FAME, 60 m × 0.25 mm × 0.25 μm, Thermo Fisher, USA) was used. The oven temperature was kept at 60 °C, with a running time of 73.5 min. The temperature of the injector and the detector was set at 250 °C. The analysis was achieved using a temperature gradient program from an initial temperature of 60 °C (held for 3 min), increased 5 °C/min to 175 °C (held for 15 min), and then increased 2 °C/min to a final temperature of 220 °C for 10 min. Nitrogen was used as a carrier gas with a flow rate 1.2 mL/min; split ratio 1:100; detector gas 30 mL/min hydrogen; 400 mL/min air and 25 mL/min nitrogen. Identification of GC peaks was finally achieved by comparing their retention times with those of the corresponding standards.

2.4. Ultra-performance liquid chromatography-mass spectrometry

2.4.1. UPLC conditions

The analysis of egg yolk phospholipids was carried on ultra-performance liquid chromatography (UPLC) system (Waters, Milford, Massachusetts, USA) equipped with a BEH-HILIC silica column (100 × 1 mm, 1.7 μm particle size). The mobile phase A was acetonitrile and the mobile phase B was 50 mM ammonium acetate containing 0.1% formic acid (pH = 3.65). Effective separation of egg yolk phospholipids was achieved with a binary gradient started with 5% B for 4 min, then changed 40% B within 10 min and held for 5 min; the total run time was 20 min at a flow rate 300 μL/min. The column and sample room temperatures were 40 °C and 4 °C, respectively. After each analysis, the column was flushed with 5% of the mobile phase B for 5 min before the beginning of the next analysis.

2.4.2. Q-TOF-MS conditions

A quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) instrument (Waters, Milford, Massachusetts, USA) was used for the identification and the quantification of phospholipids molecular species in duck, hen and quail egg yolks. Negative-ion (−ve) mode was used at an optimized condition as follows; the capillary and sampling cone voltage at 3.0 kV and 30 V respectively, the source temperature was 100 °C, desolvation temperature, 400 °C; collision gas, argon; desolvation gas (nitrogen) flow rate, 700 L/h. Data were collected over a mass range between 50 and 1500 Da within 0.2 s scan duration. The MS/MS experiments were completed using different collision energy of 25 and 30 V for each compound. Micromass MassLynx V4.1 system (Waters) software was used for LC/MS data analysis. The identification of each class of egg yolk phospholipids was further confirmed by MS/MS in positive and negative ion mode for the different fractions.

2.5. Statistical analysis

Fatty acids composition of egg yolk phospholipids was analyzed three times, and the data were subjected to the analysis of variance (ANOVA). The results were expressed as mean ± SD. Statistical analysis software (version 6.4, CoStat, Monterey, CA, USA) was used for data treatment. The results were considered statistically significant for P values of less than 0.05.

3. Results and discussion

3.1. Analysis of the fatty acids of egg yolk phospholipids

The major fatty acids associated with egg yolks PLs were C16:0, C18:0, C18:1, C18:2 and C20:4(n-6) (Supplementary section). From the obtained data, there was a significant difference in the content of fatty acids ($P < 0.05$). Palmitic acid was the most abundant fatty acid in the three types of egg yolks, making up 32.68, 30.32 and 24.42%, respectively. C15:0, C20:5(n-3) and C22:5(n-3) were not found in duck egg yolk PLs, while C17:0, C20:2, C21:0 and C20:5 (n-3) were not detected in hen egg yolk. Furthermore, C20:2, C20:3(n-6) and C21:0 were not found in quail egg yolk PLs. Duck egg yolk PLs had a significant lower content of linoleic acid (C18:2) compared to hen and quail egg yolks, making up 8.24, 13.18 and 21.08%, respectively. Arachidonic acid (C20:4) content in duck egg PLs was higher than hen and quail eggs content, making up 15.81, 5.87 and 6.73%, respectively. Similar results were presented in a study on poultry eggs PLs, reported that the content of C20:4 in duck, hen, turkey and quail egg yolk PLs was 11.61, 6.97, 4.79, 5.78% respectively (Couch & Saloma, 1973). The variations in the contents of fatty acids among the different samples were probably a result of the composition of diet which was given to birds. In addition, the differences in analysis methods, species, metabolism, or absorption of fatty acids.

3.2. Separation and identification of phospholipids by UPLC-Q-TOF-MS

Phospholipids have been extracted from different sources and identified using several methods of analysis. Many studies have been reported on the application of high performance liquid chromatography (HPLC) in PLs analysis (Briand, Harold, & Blass, 1981; Chen & Kou, 1982; Hanson, Park, Osborn, & Kiral, 1981; Westenberg et al., 1977). Though, the obtained results were not in agreement, for example, observing different elution orders of the individual classes of phospholipids. Also, for the normal phase liquid chromatography, when it coupled to mass detector, the solvent it used was not convenient. Due to the limitations in these methods of analysis, as they failed to provide inclusive chemical characterization of PLs, in this study the separation method was developed using a BEH column in HILIC by adding 50 mM/L ammonium formate to the mobile phase in order to increase the resolution and the selectivity of separation. In addition, adjusting the mobile phase concentration and pH optimized the separation conditions and resulted in better separation of PLs classes.

The separation analysis of lipids using the HILIC technique was widely reported. It is well known that the HILIC-based separation of PLs depends primarily on the polarity of their polar heads; the less polarity the lipids are, the shorter retention times they are detected. Lipids classes were fractionated using silica-based column in the HILIC analysis method (Lisa, Cifkova, & Holcapek, 2011). Also, phospholipids extracted from different foods have been separated by gradient elution in HILIC using acetonitrile and 10 mM of ammonium acetate as a mobile phase at pH 3.0 (Zhao et al., 2011). Five classes of PLs were analyzed by using isocratic elution containing acetonitrile, methanol and 10 mM ammonium acetate (Schwalbe-Herrmann, Willmann, & Leibfritz, 2010). Different applications of HILIC in lipids analysis have been also reported (Bang, Byeon, & Moon, 2014; Craige Trenerry et al., 2013; Li et al., 2016).

The separation of the different classes of egg yolks PLs was achieved within a runtime of 20 min as shown in Figs. 1and 2 under electrospray ionization (ESI) in both positive and negative ion mode, respectively. The elution of 6 classes of egg yolk PLs studied in this research was as follow, PS > PI > PE > PC > SM > LPC. The identification of PLs classes was carried out by comparing their retention times with those of the reference standard materials. PLs separation was compared to the standards analyzed by Li et al. (2016) who used the same technique and conditions of analysis, and confirmed the precision of this method for the identification of phospholipids. This method was validated for accuracy, linearity, recovery, relative standard deviation (RSD) and limit of quantification (LOQ). Calibration curves presented coefficients of correlation (R) higher than 0.99 with RSDs below 10%. The LOQ of phospholipids was determined at a signal-to-noise ratio of 10. By evaluating this method of analysis, the recoveries of the different classes of PLs were between 94 and 120% as reported in the same literature.

In addition to the individual separated classes of phospholipids and because of the variations of their fatty acyl residues, a partial separation was detected in the various PLs molecular species within each sub-class, with elution order depending on the type of fatty acids on the glycerol backbone; the differences in the retention times for the compounds within one class were less than those from two different classes. Table 1 shows all the identified molecular ions obtained for 6 classes of phospholipids from duck, hen and quail egg yolks, the observed mass, their molecular formulas and relative abundance. The carboxylate anions at the *sn*-1 and *sn*-2 positions were determined from the measured exact mass product ion observed in MS/MS experiments, and the abundances of these anions were associated with the collision energy. The chromatograms of the predominate species of egg yolk phospholipids at a collision energy of 25 or 30 V were presented (Supplementary section). As well, the obtained molecular formulas and fatty acids composition of the detected molecular species separated at different retention times in this research were compared with the literature data (Walczak, Pomastowski, Bocian, & Buszewski, 2016; Wang et al., 2009).

3.3. Molecular species composition of egg yolk phospholipids

Regarding phosphatidylcholine, 20 molecular species of PC were detected (Table 1). PC (16:0–18:1) was the predominant species of PC class in duck, hen and quail egg yolks, making up 18.53, 15.40 and 13.19%, respectively. The mass spectra for the proto-

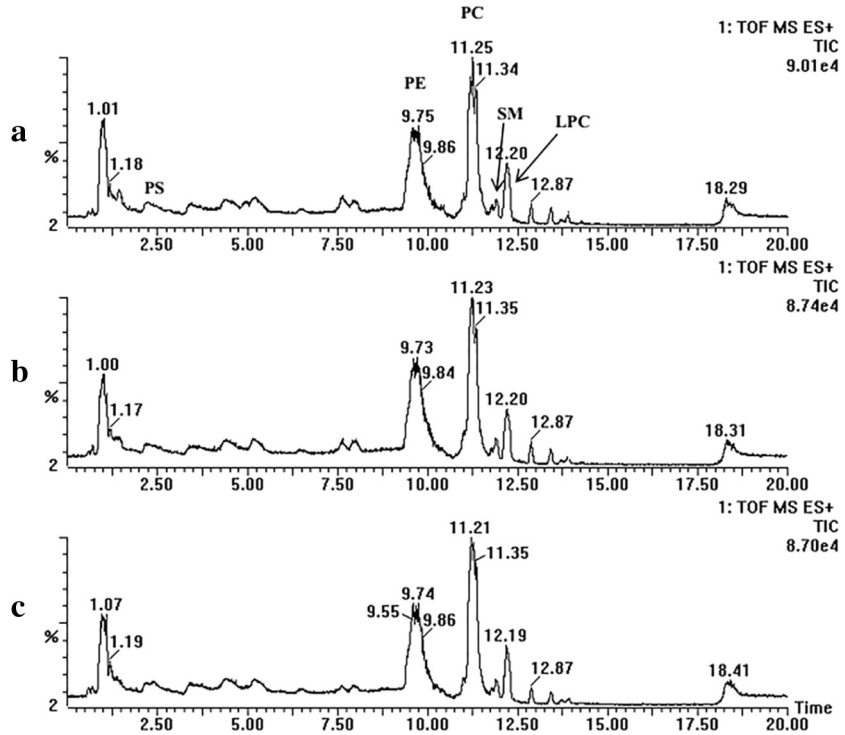

Fig. 1. Total ion chromatogram of phospholipids species in different egg yolks under ESI$^+$ mode within 20 min (a, duck egg yolk; b, hen egg yolk; c, quail egg yolk).

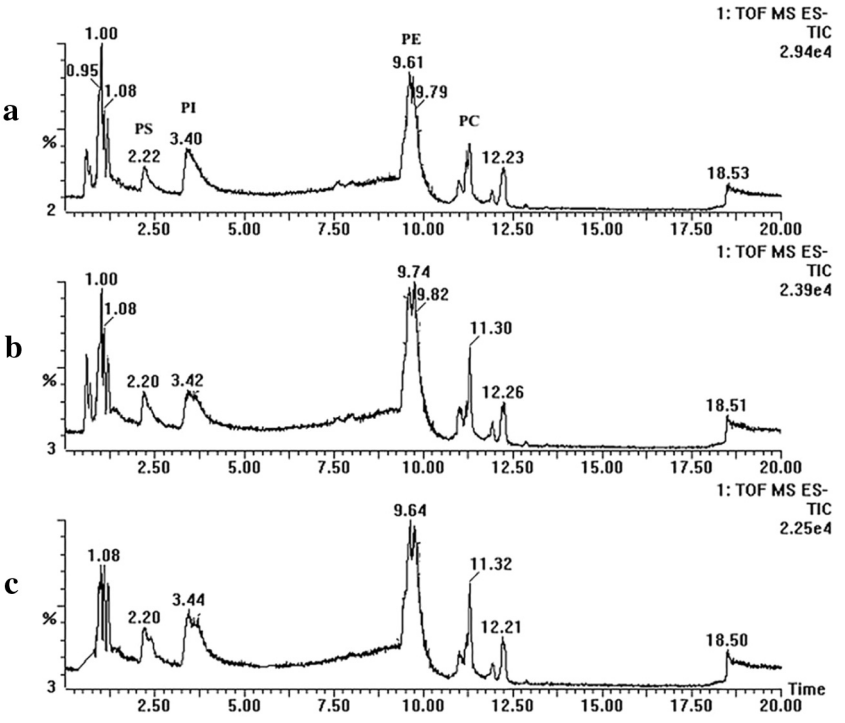

Fig. 2. Total ion chromatogram of phospholipids species in different egg yolks under ESI$^-$ mode within 20 min (a, duck egg yolk; b, hen egg yolk; c, quail egg yolk).

nated form $[M+H]^+$ of PC (16:0–18:1) at m/z 760.5 under the positive ion mode is presented in details in Fig. 3a. From the spectra, significant fragments were detected at m/z 184 correspond to the head of phosphatidylcholine $[C_5H_{14}NPO_4+H]^+$ and m/z 86.1 "dehydrocholine" $[C_5H_{12}N]^+$ (loss of 674.4), the ion mass m/z 184 is a characteristic fingerprint of the PC class (Fuchs, Schober, Richter, Süß, & Schiller, 2007). The other observed fragments such as, the m/z ions 577.5 and 496.3, are corresponding to $[M+H-183]^+$ (loss of phosphocholine) and $[M+H-C_{18}H_{32}O]^+$ (loss of ketene oleic acid). Furthermore, the small m/z ions at 478.3 and 504.3 refer to $[M+H-R_1COOH]^+$ (loss of oleic acid), and the combination of palmitic acid and oleic acid ions, respectively.

PC identification was also confirmed in the negative ion mode $[M+HCOO]^-$, as shown in Fig. 3b for mass spectra of PC (16:0–

Table 1
Phospholipids molecular species in duck, hen and quail egg yolks identified by UPLC-Q-TOF-MS.

Class	*m/z* observed	Molecular formula	Molecular species	Relative abundance (%)		
				Duck egg	Hen egg	Quail egg
PC	706.4265	$C_{38}H_{77}NO_8P+$	C14:0–C16:0	1.01	0.43	0.41
	730.4667	$C_{40}H_{77}NO_8P+$	C14:0–C18:2	1.06	0.80	1.21
	732.4599	$C_{40}H_{79}NO_8P+$	C14:0–C18:1	4.27	4.61	3.15
	734.4458	$C_{40}H_{81}NO_8P+$	C16:0–C16:0	5.50	3.73	3.94
	744.4865	$C_{41}H_{79}NO8P+$	C15:0–C18:2	–	0.49	0.80
	746.4887	$C_{41}H_{81}NO_8P+$	C15:0–C18:1	–	0.74	0.68
	758.4670	$C_{42}H_{81}NO_8P+$	C16:0–C18:2	9.44	15.20	12.53
	760.4573	$C_{42}H_{83}NO_8P+$	C16:0–C18:1	18.53	15.40	13.19
	780.4743	$C_{44}H_{79}NO_8P+$	C14:0–C22:5	1.39	0.66	0.80
	782.4933	$C_{44}H_{81}NO_8P+$	C16:0–C20:4	8.54	6.01	7.48
	784.4738	$C_{44}H_{83}NO_8P+$	C18:1–C18:2	7.50	5.43	6.22
	786.4670	$C_{44}H_{85}NO_8P+$	C18:0–C18:2	5.08	6.28	6.61
	788.4938	$C_{44}H_{87}NO_8P+$	C18:0–C18:1	3.63	5.80	6.77
	806.4861	$C_{46}H_{81}NO_8P+$	C16:0–C22:6	3.94	5.54	6.53
	808.4967	$C_{46}H_{83}NO_8P+$	C16:0–C22:5	7.83	6.75	6.05
	810.5203	$C_{46}H_{85}NO_8P+$	C18:0–C20:4	8.91	7.63	9.58
	812.5357	$C_{46}H_{87}NO_8P+$	C18:0–C20:3	3.68	3.96	–
	832.5099	$C_{48}H_{83}NO_8P+$	C18:2–C22:5	0.79	0.84	1.06
	834.5099	$C_{48}H_{85}NO_8P+$	C18:0–C22:6	3.79	4.49	7.57
	836.5231	$C_{48}H_{87}NO_8P+$	C18:0–C22:5	5.11	5.21	5.42
PE	714.4313	$C_{39}H_{73}NO_8P-$	C16:0–C18:2	5.97	9.47	13.59
	716.4518	$C_{39}H_{75}NO_8P-$	C16:0–C18:1	15.06	8.01	2.81
	728.4761	$C_{40}H_{75}NO_8P-$	C17:0–C18:2	0.23	–	0.38
	738.4459	$C_{41}H_{73}NO_8P-$	C16:0–C20:4	11.88	7.72	4.48
	742.4468	$C_{41}H_{77}NO_8P-$	C18:0–C18:2	8.01	10.47	12.07
	744.4463	$C_{41}H_{79}NO_8P-$	C18:0–C18:1	5.51	7.25	7.29
	748.4935	$C_{42}H_{71}NO_8P-$	C15:0–C22:6	–	4.27	2.05
	762.4708	$C_{43}H_{73}NO_8P-$	C16:0–C22:6	3.52	5.61	9.42
	764.4564	$C_{43}H_{75}NO_8P-$	C18:1–C20:4	11.93	9.12	4.63
	766.4620	$C_{43}H_{77}NO_8P-$	C18:0–C20:4	22.27	20.94	22.25
	768.4760	$C_{43}H_{79}NO_8P-$	C18:0–C20:3	1.14	3.63	–
	776.4850	$C_{44}H_{75}NO_8P-$	C17:0–C22:6	5.11	–	2.96
	790.4611	$C_{45}H_{77}NO_8P-$	C18:0–C22:6	3.58	5.96	13.36
	792.4619	$C_{45}H_{79}NO_8P-$	C18:0–C22:5	5.80	7.54	4.71
PI	833.4466	$C_{43}H_{78}O_{13}P-$	C16:0–C18:2	10.33	16.03	19.47
	835.4684	$C_{43}H_{80}O_{13}P-$	C16:0–C18:1	12.97	13.41	8.00
	857.4497	$C_{45}H_{78}O_{13}P-$	C16:0–C20:4	21.32	16.55	16.40
	861.5123	$C_{45}H_{82}O_{13}P-$	C18:0–C18:2	32.53	30.84	42.00
	863.4988	$C_{45}H_{84}O_{13}P-$	C18:0–C18:1	10.99	11.15	10.53
	883.4642	$C_{47}H_{80}O_{13}P-$	C18:1–C20:4	5.49	3.31	1.60
	885.4581	$C_{47}H_{82}O_{13}P-$	C16:0–C22:4	1.10	0.87	0.27
	911.4812	$C_{49}H_{84}O_{13}P-$	C18:0–C22:5	2.42	4.01	0.93
	913.5063	$C_{49}H_{86}O_{13}P-$	C18:0–C22:4	2.86	3.83	0.80
PS	788.5248	$C_{42}H_{79}NO_{10}P+$	C18:0–C18:2	40.97	32.52	56.94
	814.5222	$C_{44}H_{81}NO_{10}P+$	C18:0–C20:3	11.94	27.18	–
	816.5322	$C_{44}H_{83}NO_{10}P+$	C18:0–C20:2	20.32	–	–
	838.5280	$C_{46}H_{81}NO_{10}P+$	C18:0–C22:5	13.87	22.82	43.06
	862.5252	$C_{48}H_{81}NO_{10}P+$	C20:3–C22:4	12.90	17.48	–
SM	703.4717	$C_{39}H_{80}N_2O_6P+$	d18:1/16:0	80.65	81.69	77.20
	731.5327	$C_{41}H_{84}N_2O_6P+$	d18:1/18:0	11.17	13.04	15.12
	787.5938	$C_{45}H_{92}N2O_6P+$	d18:1/22:0	8.19	5.26	7.67
LPC	494.2747	$C_{24}H_{49}NO_7P+$	C16:1	1.55	3.99	1.91
	496.2592	$C_{24}H_{51}NO_7P+$	C16:0	38.97	44.07	38.39
	522.2939	$C_{26}H_{53}NO_7P+$	C18:1	14.16	19.84	18.79
	524.3002	$C_{26}H_{55}NO_7P+$	C18:0	30.04	19.84	35.18
	544.2903	$C_{28}H_{51}NO_7P+$	C20:4	8.93	5.08	5.73
	546.2953	$C_{28}H_{53}NO_7P+$	C20:3	6.35	7.18	–

UPLC-Q-TOF-MS, ultra-performance liquid chromatography with quadrupole-time-of-flight mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; LPC, Lysophosphatidylcholine; *m/z*, mass to charge.

18:1) at 804.5 *m/z* ion. Tandem MS on $[M-H]^-$ ions did not provide obvious information on the acyl chain. The precursor ion $[M+HCOO]^-$ of PC produced the fragment ion at 744.5 $[M-CH_3]^-$ due to the neutral loss of $C_2H_4O_2$ (60), and also produced the fragment ion $[C_4H_{11}O_4NP]^-$ at *m/z* 168, which was unique to PC (Yan, Li, Xu, & Zhou, 2010). The peaks detected at *m/z* 255.2 and 281.2 are corresponding to palmitic acid and oleic acid, respectively. Also, the signal at *m/z* 480.3 refers to the loss of oleic acid $[M+HCOO-C_{18}H_{33}O_2]^-$.

Referring to phosphatidylethanolamine; based on the nitrogen rule, PE is effectively characterized as $[M-H]^-$, as the molecular ion peak in the negative ion mode and showed signals at even number of *m/z* values. 14 molecular species of PE were detected, and PE (18:0–20:4) was the predominant species in duck, hen

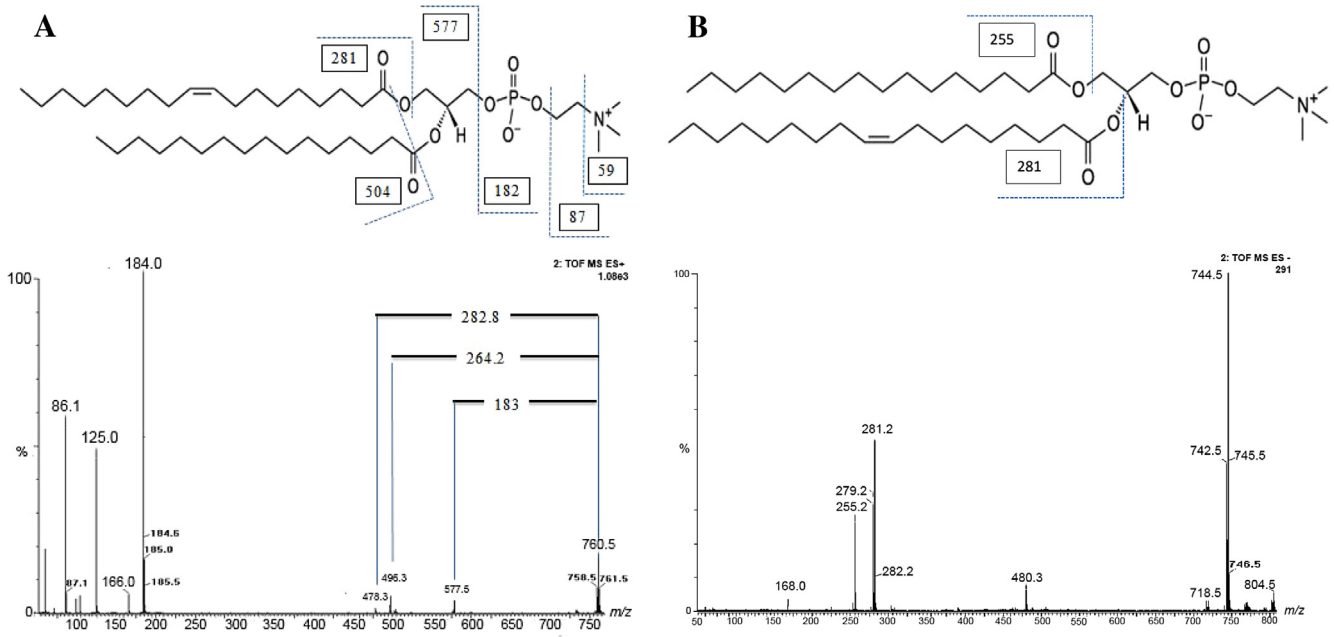

Fig. 3. **(A)** MS/MS fragmentation pathway of PC (16:0–18:1) at *m/z* 760.5 under positive ion mode; **(B)** MS/MS fragmentation pathway of PC (16:0–18:1) at *m/z* 804.5 under negative ion mode.

and quail egg yolks (22.27, 20.94 and 22.25%), respectively (Table 1). Fig. 4a shows the fragmentation spectra of the most abundant species PE (18:0–20:4) at *m/z* ion 766.5 in details. The observed peaks at *m/z* 283.2 and 303.2 correspond to stearic acid and arachidonic acid ions, respectively. PE produced a unique fragment ion at *m/z* 140 refers to PE headgroup $[C_2H_7O_4NP]^-$. The identification of PE fragments was compared with the data reported in literature (Pacetti, Boselli, Hulan, & Frega, 2005) for PE analysis in the negative ion mode. Pulfer and Murphy (2003) identified PE fragments in the positive ion mode.

Phosphatidylinositol identification can be achieved in both positive and negative ion mode (Murphy & Axelsen, 2011). However, the negative ion mode is more abundant and more informative than the positive ion mode because of the substantial negative charge of this fragment headgroup. PI (18:0–18:2) was the major molecular species in duck, hen and quail egg yolks (32.53, 30.84 and 42.00%), respectively. The fragmentation pathway of PI (18:0–18:2) at *m/z* 861.5 $[M-H]^-$ is presented in Fig. 4b. Phosphatidylinositol produced the characteristic fragment ion $[C_6H_{10}O_8P]^-$ at *m/z* 241 due to the condensation of proximate hydroxyl groups on phosphate and inositol. The ion produced at *m/z* 153 $[C_3H_6O_5P]^-$ was also a common fragment generated by PI. The signals observed at *m/z* ions 283.2 and 419.2 correspond to stearic acid ion, and the combination loss of both linoleic acid and PI headgroup (163), respectively. In addition, the detected ion at *m/z* 581.3 results from the loss of linoleic acid.

With regard to phosphatidylserine, 5 molecular species were detected in duck egg yolk, 4 molecular species were detected in hen egg yolk and 2 molecular species were detected in quail egg yolk (Table 1). Although PS could not be confirmed by this method of analysis because this fraction did not produce characteristic ions, the identification of PS was completed and confirmed after the analysis of the other classes of phospholipids. The identification of PS molecular species was confirmed by yielding abundant ion that corresponds to the loss of polar headgroup $[M-184]^+$ in the positive ion mode (Murphy & Harrison, 1994). PS characterization can also be confirmed by identifying the neutral loss of 88 units in MS/MS spectra in the negative ion mode, $[M-H]^-$ ions readily decompose to $[M-88]^-$ ions from the neutral loss of serine headgroup (Murphy & Axelsen, 2011). In negative spraying ionization, the negative precursor ion scan of *m/z* 153 was also suggested to detect PS (Peterson & Cummings, 2006). PS (18:0–22:5) at *m/z* 838.5 spectrum is shown in Fig. 4c, the *m/z* ions 655.5 and 599.4 correspond to the loss of phosphoserine moiety $[M-C_3H_6NPO_6]^+$ and $[M-C_{17}H_{35}]^+$, respectively. The peak observed at *m/z* 313.3 arises from the moiety of docosapentaenoic acid. In addition, the signal observed at *m/z* 300 in low abundance probably comes from stearic acid and ammonia of the phosphatidylserine head ions.

Concerning sphingomyelin, 3 molecular species of SM were detected in egg yolk samples (Table 1). The presence of the quaternary nitrogen atom, with permanent positive charge, dominates the electrospray ionization behavior of this molecule. Therefore, SM produces more abundant ions in the positive ion mode compared to the negative ion mode. SM (d18:1/16:0) was the most abundant molecular species in egg yolk samples. Fig. 5a describes the mass spectra of $[M+H]^+$ ion from SM separated at *m/z* ion 703.5 in details. The fragments at *m/z* 685.5 and 627.5 correspond to the neutral loss of water $[M+H-H_2O]^+$, and the loss of trimethylamine with water $[M+H-C_3H_{11}NO]^+$, respectively. The peak detected at *m/z* 448.2 corresponds to the loss of palmitic acid ion $[M+H-255.3]^+$, and the strong signal observed at *m/z* 184.0 is derived from phosphocholine. The small signals at *m/z* 265.2 and 503.5 probably derived from sphingosine d18:1 chain ion, and the combination loss of palmitic acid and octadecylic sphingoid chain, respectively (Boselli, Pacetti, Curzi, & Frega, 2008; Pulfer & Murphy, 2003).

Lysophosphatidylcholine is a class of phospholipids containing choline in its structure. LPC (C16:0) was the most abundant fraction in the three types of egg yolk (38.97, 44.07 and 38.39%), respectively. Fig. 5b presents the protonated molecular ion $[M+H]^+$ at *m/z* 496.3 in details. The signals observed at *m/z* 478.3, 437.2 and 419.2 can be ascribed to $[M+H-H_2O]^+$, $[M+H-C_3H_9N]^+$ ions and $[M+H-C_3H_{11}NO]^+$, corresponding to the loss of one molecule of water from *sn*-1 position in acyl chain, the neutral loss of trimethylamine, and the combination loss of trimethylamine (59) and water (18), respectively. The fragment detected at *m/z* 313.3

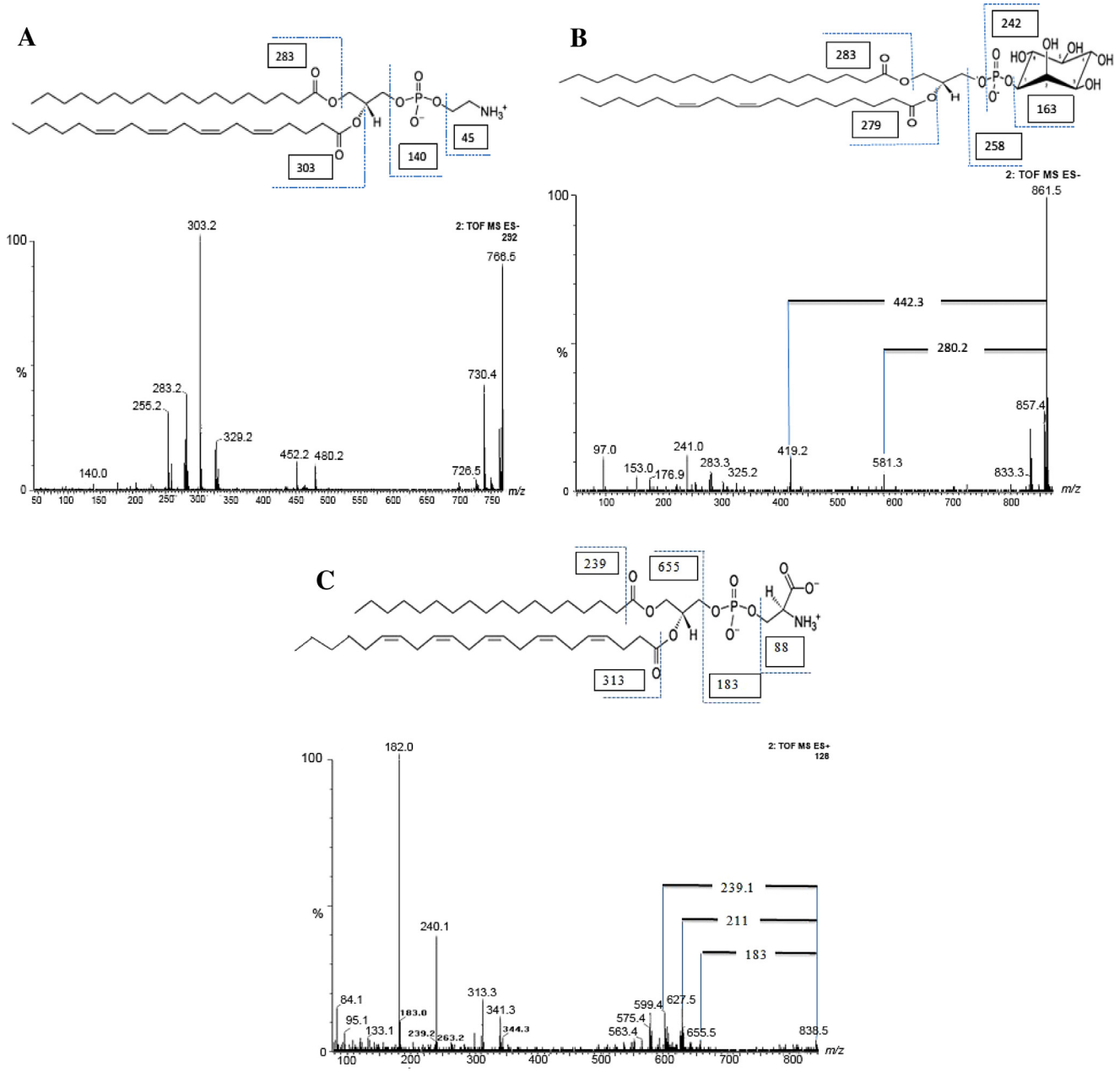

Fig. 4. **(A)** MS/MS fragmentation pathway of PE (18:0–20:4) at *m*/*z* 766.5; **(B)** MS/MS fragmentation pathway of PI (18:0–18:2) at *m*/*z* 861.5; **(C)** MS/MS fragmentation pathway of PS (18:0–22:5) at *m*/*z* 838.5.

corresponds to $[M+H-C_5H_{13}NPO_4]^+$ (loss of phosphocholine), the small signal at *m*/*z* 255 is probably derived from palmitic acid ion, and the strong peaks detected at *m*/*z* 184.0 and 104.2 are derived from phosphocholine and choline, respectively. 6 molecular species of fatty acids (16:0, 16:1, 18:0, 18:1, 20:4 and 20:3) in LPC class were identified in duck and hen egg yolks phospholipids, except LPC (20:3) was not detected in quail egg yolk phospholipids (Table 1).

4. Conclusion

In order to clarify the function properties of PLs, it is really important to conduct analysis methods not only of their classes and sub-classes, but of their molecular species as well. In this study, duck, hen and quail egg yolk PLs were extracted, separated and identified by using UPLC-Q-TOF-MS. 57 molecular species of egg yolk phospholipids were identified simultaneously by analyzing their high-resolution mass spectrometry data obtained by Q-TOF and their characteristic fragmentation ions given by MS/MS data. PC (16:0–18:1), PE (18:0–20:4), PI (18:0–18:2), PS (18:0–18:2), SM (d18:1/16:0) and LPC (16:0) were the predominant species among the different classes of egg yolk PLs. Performing HILIC by 1.7 μm BEH particles column, and the addition of 50 mM/L ammonium formate to the mobile phase provided all the approved advantages of UPLC- faster analysis, with enhanced chromatographic resolution, sensitivity, and reproducibility of separation. The characterization of egg phospholipids by using this method can be suggested as an application to discover new phospholipids compositions associated with their metabolism, functional properties and new applications.

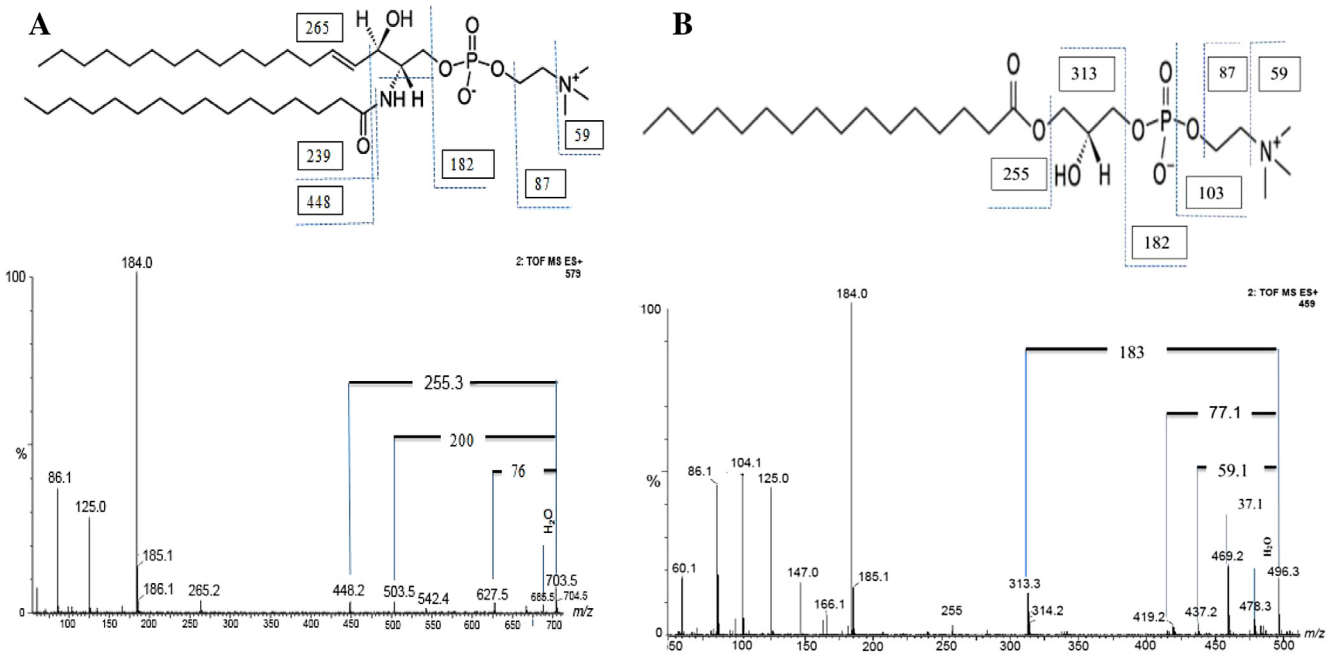

Fig. 5. **(A)** MS/MS fragmentation pathway of SM (d18:1/16:0) at *m/z* 703.5; **(B)** MS/MS fragmentation pathway of LPC (16:0) at *m/z* 496.3.

Conflict of interest

The authors declare that there is no conflict of interests regarding this paper publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.10.043.

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