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Targeted lipidomics profiling of marine phospholipids from different resources by UPLC-Q-Exactive Orbitrap/MS approach

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ABSTRACT
Marine phospholipids (MPLs) are valuable components that can be applied within diverse areas like nutrition, pharmacy, and medicine as well as basic scientific research, particularly due to their high concentration of long chain polyunsaturated fatty acids. In this work, lipidomics approach by UPLC-Q-Exactive Orbitrap/MS was used for the identification, quantification, comparison, and characterization of phospholipids (PLs) from three different marine resources (shrimp head, codfish roe, and squid gonad). In total, 310 PL molecular species containing 34 different structures of fatty acid chains were identified simultaneously by Lipidsearch software. Significant differences between three groups in the PL classes, compositions and contents were revealed. A list of characteristic PL species that represent significant differences among groups was determined by lipidomics analysis. Until now, the information about the deep comprehensive description of PL from marine sources is limited. Thus, this study will give a very potential starting point to develop MPL products and establish the quality standards for different marine raw materials.

1. Introduction

Phospholipids (PLs) are structural components of biological membranes and play a crucial role in cellular and tissue physiology, such as being signaling and regulatory molecules [1−3]. Additionally, PL signaling events are related to various diseases [4,5]. The importance of PL signaling and its potential therapeutic targeting have been discussed by researchers from different groups [5]. According to their structural features, PLs could be divided into several classes: phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), phosphatidylinositol

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(PI), phosphatidylglycerol (PG), phosphatidic acid (PA), and sphingomyelin (SM), etc.. The structures of the fatty acyl groups determine the individual PL molecular species [6,7].

Marine phospholipids (MPLs) contain high levels of physiologically important long chain polyunsaturated fatty acids (PUFAs), which attracted more and more attention [8]. The marine resources for obtaining MPLs mainly include krill, fish eggs, fish meal and fish processing by-products. PC is the most abundant MPLs and the most dominant PL molecular species derived from marine resources are C16:0-C20:5 PC and C16:0-C22:6 PC. Therefore, the MPLs are rich in the eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) [9]. Moreover, MPLs provide a better bioavailability of EPA and DHA [10], a much wider range of health benefits for both animals and humans [11], and a better resistance to oxidation [12]. The various health benefits have been proved by many clinical studies, such as the effect for premenstrual syndrome [13], cardiovascular disease [14], inflammation [15], and so on. MPLs can also affect the physical condition of cancer patients [16,17]. Additionally, the uptake and utilization of PUFAs bound to MPLs are more efficient than those bound to triglycerides (TGs) [9]. MPLs are considered to be a new alternative and of major nutritional impact for supplement PUFAs [18]. The marine resources in the earth are very rich, and there is a broad development prospects for MPLs. To clarify the composition characterization of various PLs from different marine resources is one of the basic problems for the application of MPLs.

Due to a wide concentration range with varying polarities, the analysis of PL constituents becomes a huge challenge. Researchers strive to improve methods at different stages of analysis process, including novel preparation (extraction and fractionation/isolation) methods or an improvement in chromatographic conditions. Studies on the extraction of PLs have been undertaken widely, such as extraction with different concentrations of aqueous ethanol [19], supercritical fluid extraction [20], low-temperature crystallization [21], and ultrafiltration [22]. Fractionation steps of PLs can be carried out prior to analysis by open column chromatography [23], thin-layer chromatography (TLC) [24], or solid phase extraction (SPE) columns [25]. Furthermore, high-performance liquid chromatography (HPLC) methods using the evaporative light scattering detector (ELSD) [26], charged aerosol detector (CAD) [27] or mass spectrometry (MS) [28] detector have been established as reliable solutions that can bypass the tedious steps of isolation and purification. Besides, gas chromatography (GC) method could analyze the variation of derivatized fatty acid chains of PLs, while HPLC can be used to analyze the whole PL molecules especially when coupled to MS/MS [29]. The presence of PUFA side chains greatly enriches the variety of MPLs. Therefore, a comprehensive and overall analysis is the key to reveal the characteristics of MPLs.

Lipidomics is an important field in metabolomics, and one of its objectives is to determine the structures of lipid molecules from MS data and analyze the changes in these molecules under specific circumstances [30]. The identification of lipids is challenging and has the risk of misidentification, because of the presence of a large

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number of structural isomers and the abundant ions with various adducts [31]. The correct identification of lipids requires three basic factors: accurate mass value of molecular weight-related ions, characteristic fragment ions, and good separation and reproducible retention time in liquid chromatography (LC) [32]. In lipidomics, the innovation of soft ionization, such as electrospray ionization (ESI), and tandem mass spectrometry in combinatiion with LC enables us to fully detect lipids in excess of 100 species [30]. The employment of a high-resolution mass spectrometer that could achieve the high mass accuracy in both full scan and MS/MS stages is very informative for structural characterization [29]. Besides, there are still many other approaches for lipidomics being reported [33−40].

In this article, we report a UPLC-Q-Exactive Orbitrap/MS study of the PLs from three different kinds of marine sources (shrimp head, codfish roe, and squid gonad). These MPLs were identified, quantified and characterized. Finally, their differences in PLs molecular species were compared by software-assisted lipidomic analysis. This is the first investigation regarding a deep comprehensive comparison and characterization of MPLs compositions from these three marine sources.

2. Materials and methods

2.1. Chemicals and materials

Frozen shrimp head, codfish roe, and squid gonad were provided by Penglai Marine (Shandong) Co., Ltd (Penglai, China). All chemicals and solvents used for extraction and sample preparation were of high purity and analytical grade. HPLC-grade solvents, acetonitrile (ACN), ammonium acetate and isopropanol (IPA) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Extraction of marine lipids

Lipids were extracted from shrimp head, codfish roe, and squid gonad following a method mentioned by Bligh and Dyer [41,42]. Briefly, each sample (10 g) was cut into small pieces, then 300 mL of methanol (MeOH), 150 mL of chloroform and 120 mL of distilled water were added. After stirring for 3 min and ultrasonication for 30 min, 150 mL of chloroform and 150 mL of distilled water were further added to the mixture. After ultrasonication for another 30 min, the mixture was filtrated and then placed in a separatory funnel. The lipid phase in the chloroform layer was isolated and evaporated under reduced pressure to give the lipid extracts. Finally, the obtained lipids (~400-600 mg) were dried under a gentle stream of nitrogen (N_2) and stored at −20 °C.

2.3. Sample preparations for UPLC-Q-Exactive Orbitrap/MS analysis

After the addition of H_2O (200 μL) and homogenization for three cycles (each cycle: 5500 rpm for 20 s) with cooled N_2 gas flow from liquid N_2, lipid extracts (50

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mg) were extracted using 400 μL of methyl tert-butyl ether (MTBE), 80 μL of MeOH and 200 μL of H_2O. The mixture was treated by vortexing for 30 s and ultrasonication for 10 min, and then was centrifuged for 15 min at 3000 rpm to separate phases. The upper MTBE layer was decanted and evaporated to dryness. Finally, the reconstituted extract was re-dissolved in 100 μL of dichloromethane/MeOH (1:1, v/v) prior to analysis. Because the instrument itself is relatively stable with a small amount of samples, we did not use the pooled QC sample in this analysis.

2.4. Ultra-performance liquid chromatography-mass spectrometry

2.4.1. UPLC conditions

The analysis of MPLs was carried on an UltiMate^{TM} 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an XSelect CSH C18 column (Waters, Milford, Massachusetts, USA; 2.1 × 100 mm, 2.5 μm). The mobile phase A was ACN/H_2O (6:4) containing ammonium acetate (0.77 g/L), and the mobile phase B was IPA/ACN (9:1). The binary gradients used for the chromatogram were as follows: 63% A for 1.5 min, 63% A to 55% A for 2.5 min, 55% A to 48% A for 1 min, 48% A to 42% A for 3 min, 42% A to 34% A for 3 min, 34% A to 30% A for 3 min, 30% A to 25% A for 4 min, and 25% A to 2% A for 2 min. The total run time was 20 min at a flow rate 250 μL/min and the column temperature was 45 °C. After each analysis, the column was flushed with 63% A for 5 min before the next analysis.

2.4.2. Q-Exactive Orbitrap/MS conditions

A Q-Exactive Orbitrap mass spectrometry instrument (Thermo Fisher Scientific, Waltham, MA, USA) was used for the identification and the quantification of PLs molecular species from three kinds of marine sources. ESI source conditions were set as follows: spray voltage (2.8 kv), source temperature (320 °C), sheath gas flow rate (35 Arb), auxiliary gas flow rate (10 Arb). The data were collected in both negative and positive ionization mode with dependent MS/MS acquisition at ranges of *m/z* 200–2000 and *m/z* 240–2000, respectively. The full scan and fragment spectra were collected at a resolution of 70,000 and 17,500, respectively. The Q-Exactive Orbitrap mass spectrometer was used for its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software continuously evaluates the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. In each cycle (1.2 s), top 10 precursor ions were chosen for fragmentation at collision energy (CE) of 15, 25 and 35 V.

2.5. Statistical analysis

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Lipids were identified and quantified using Lipidsearch softwave version 4.1.16 (Thermo Fisher Scientific, Waltham, MA, USA) according to accurate mass and fragment matching. PLs, glycolipids, sphingolipids and neutral lipids are included in the database. False positives are checked manually.

The LC-MS data was exported into Microsoft Excel and was normalized before multivariate analyses. According to the ‘‘80 % rule’’ [43,44], peaks present in more than 80 % samples of either group were kept for further analysis. The resulting three-dimensional matrix involving peak index (RT-*m/z* pair), sample names (observations), and normalized peak area percentage were submitted to the SIMCA-P 14.1 softwave (Umetrics AB, Umeå, Sweden). Because a large amount of information can be found in each analysis, a number of different multivariate modeling techniques can be applied. A principal component analysis (PCA) modeling was firstly used to evaluate the ability to predict the different groups. Next, we relied on this statistical analysis to establish an appropriate classification model by using the data obtained from samples and to identify key factors that lead to the sample differences. The partial least squares-discriminate analysis (PLS-DA) explored the differences between PLs from different groups by incorporating the known classification and indicated the variable importance of PLs with a loading plot (indicating the responsible variable for deviations from normal operation) according to variable importance plot (VIP). The statistically significant PLs responsible for discrimination of three groups were obtained from VIP (VIP > 1.0) in the projection Score from PLS-DA model and p-value (significance at $p \leq 0.05$) based on Student’s t test.

3. Results and discussion

3.1. PLs profiling using UPLC-Q-Exactive Orbitrap/MS

UPLC-Q-Exactive Orbitrap/MS was applied for the non-targeted LC-MS lipidomic profiling of the three different kinds of seafood processing byproducts (shrimp head, codfish roe, and squid gonad). Six PL classes were mainly detected in the negative mode search. They included PC, PE, PI, PA, SM and PS with all fatty acyl chains ranging from 14 to 24 carbons and 0 to 6 double bonds per chain, allowing lysophospholipids as well. No PL class was detected in the positive ionization mode. A total of 310 distinct PL species with 34 kinds of fatty acyl chain structures were searched and identified by Lipidsearch softwave, which mainly involved 83 PC, 50 PE, 33 LPC, 24 PI, 17 LPE, 7 PA, 21 SM, and 22 PS. The total ion chromatograms (TICs) of three samples were shown in Fig. 1. 296 PLs in shrimp head, 309 PLs in codfish roe and 293 PLs in squid gonad were identified at sufficient levels for quantification. Table S1 shows all the molecular ions obtained for 8 classes of PLs from three groups, the observed mass, molecular formulas, RT and the normalized peak area percentage. The relative quantifications of individual PL molecular species were calculated by comparing their total peak areas. From the obtained data, there was a significant difference in the content of PL classes. By

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comparison (Fig. 2), PC was the most dominant type of PL class in all three MPL extracts, making up 53.62, 51.83 and 49.24%, respectively. Shrimp head PLs contained a significant larger amount of lysophosphatidylcholine (LPC) (30.25%) and PI (4.48%), and a smaller amount of PE (5.06%) and SM (0.73%) compared to PLs in codfish roe and squid gonad. PS content in codfish roe PLs was higher than those in shrimp head and squid gonad PLs, making up 2.83, 0.24 and 0.52%, respectively. In addition, there is no significant difference for the contents of PUFA PLs contained in the total PLs of shrimp head, codfish roe and squid gonad, with 64.78, 57.71 and 64.12%, respectively. As shown in Fig. 3, the most predominant PUFA PL in the shrimp head and squid gonad was PC, making up 43.50 and 32.74%, respectively, while the largest amount of PUFA PL in the codfish roe was PE (25.64%).

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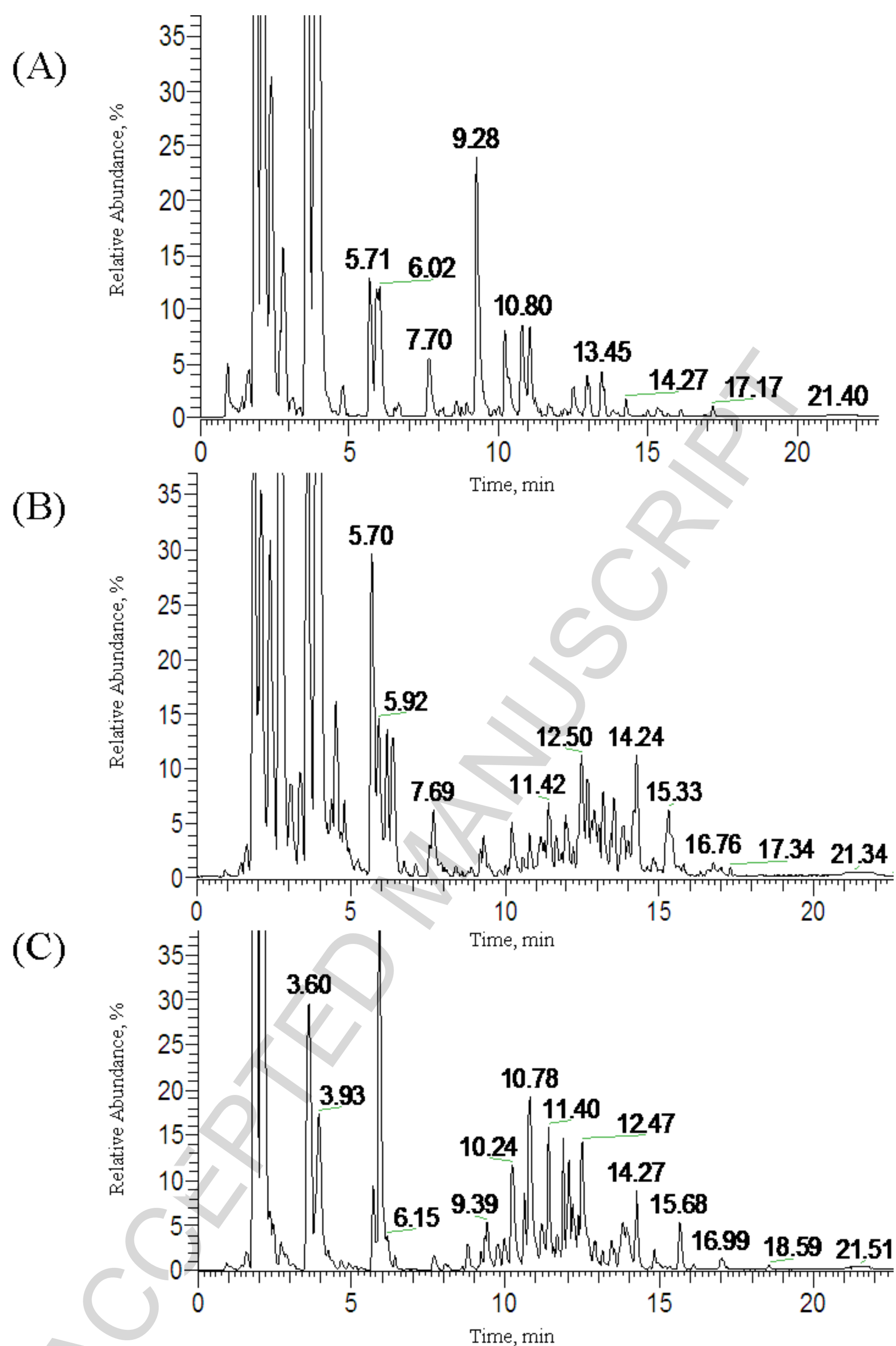

Fig. 1. Total ion chromatograms (TICs) of PLs extracts from (A) shrimp head, (B) codfish roe, and (C) squid gonad by UPLC-Q-Exactive Orbitrap/MS analysis.

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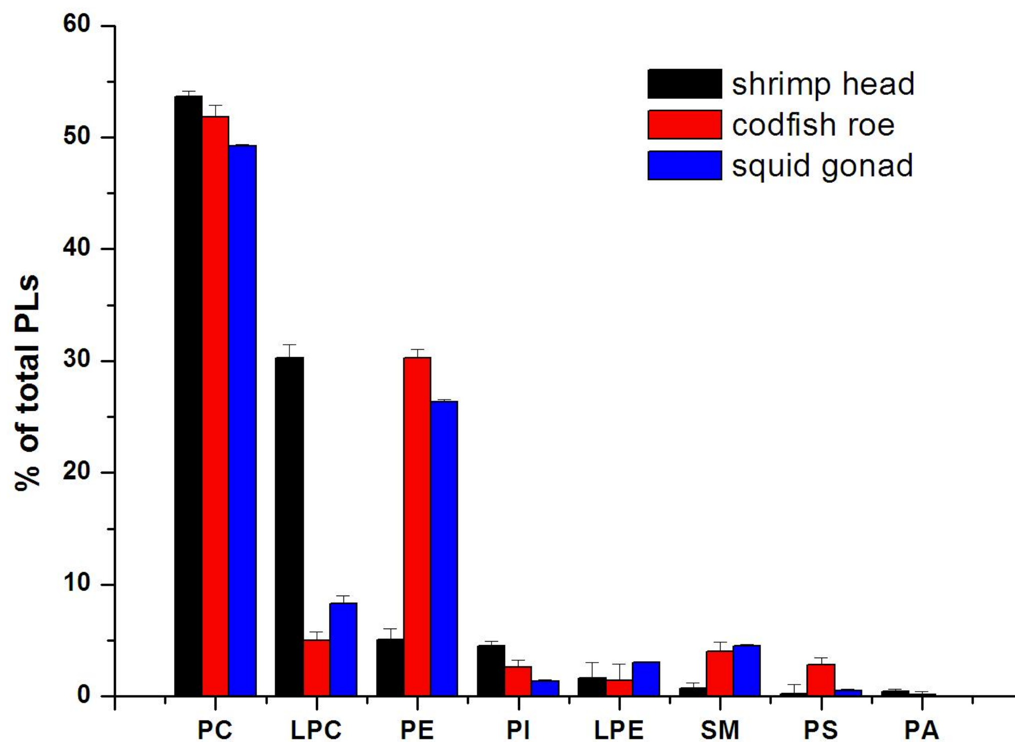

Fig. 2. PL profile (percent of total PLs) in extracts of shrimp head, codfish roe and squid gonad (n = 3).

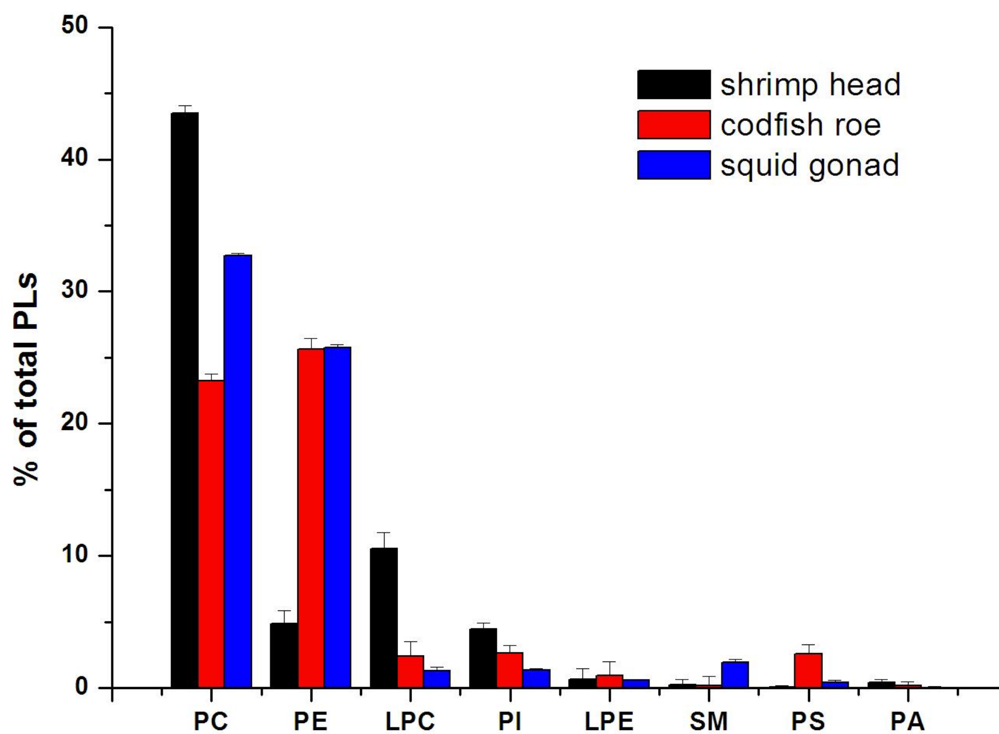

Fig. 3. PUFA PL profile (percent of total PLs) in extracts of shrimp head, codfish roe and squid gonad (n = 3).

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3.2. Identification results of molecular species in main PL classes

In negative ion mode, PL precursors can be divided into two groups: $[M - H]^-$ ion for PC, SM and LPC, and $[M + CH_3COO]^-$ ion for PE, PI, PA, PS and LPE. Among these identified PLs (Table S1), PUFA PLs accounts for a significant proportion. For example, the area ratios of PUFA PCs within the total PCs of shrimp head, codfish roe and squid gonad were 81.23, 44.87 and 66.43%, respectively, while the area ratios of PUFA PEs within the total PEs were 96.38 (shrimp head), 84.65 (codfish roe) and 97.94% (squid gonad). In detail, PC (16:0/20:5), PC (16:0/22:6), PC (18:1/20:5) and PC (18:1/22:6) were major components of shrimp head PCs (Table S1). Conversely, saturated and monounsaturated fatty acyl chains containing PCs such as PC (16:0/18:1), PC (16:0/20:1), PC (18:1/18:1) and PC (16:0/20:5) were major components in codfish roe. Moreover, the most abundant PCs in squid gonad were PC (16:0/22:6), PC (16:0/18:1), PC (16:0/20:5), PC (16:0/20:1) and PC (18:0/22:6). For PE class, EPA and DHA-containing PEs, such as PE (18:1/22:6), PE (16:0/22:6), PE (18:1/20:5) and PE (18:1p/20:5) were very rich in the shrimp head, making up 52.47% of the total PEs. Correspondingly, PE (18:0p/20:5), PE (18:0/20:5), PE (18:0p/22:6) and PE (16:0e/20:4) in the codfish roe and PE (20:1/20:5), PE (16:0/20:5), PE (18:0/20:5) and PE (20:1/22:6) in the squid gonad were also abundant according to their area ratios (27.40 and 43.87%) in the corresponding total PE.

3.3. Multivariate analysis

Characterization and discrimination of marine PLs from three sources were interpreted using multivariate statistical analysis by SIMCA-P 14.1 software. The PLS-DA analysis model (Fig. 4A) was able to clarify the segregation between PLs of shrimp head, codfish roe and squid gonad. To check the reliability of the model, the permutation test was carried out and indicated a higher predictive ability of the model by the analysis of the R2 ($R2 < 0.5$) and Q2 ($Q2 < 0$) values (Fig. 4B). Hierarchical cluster analysis (HCA) showed that the differences between groups were greater than those among within group, revealing the distinct clustering of three groups (Fig. 4C). Additionally, the 89 PLs were selected with VIP values > 1.0 from PLS-DA model, which were considered to be most responsible for the discrimination as shown in Fig. 4D. Furthermore, the univariate analysis ($p < 0.05$) by using Student's *t* test was performed to determine the final statistical significant PLs (Table 1).

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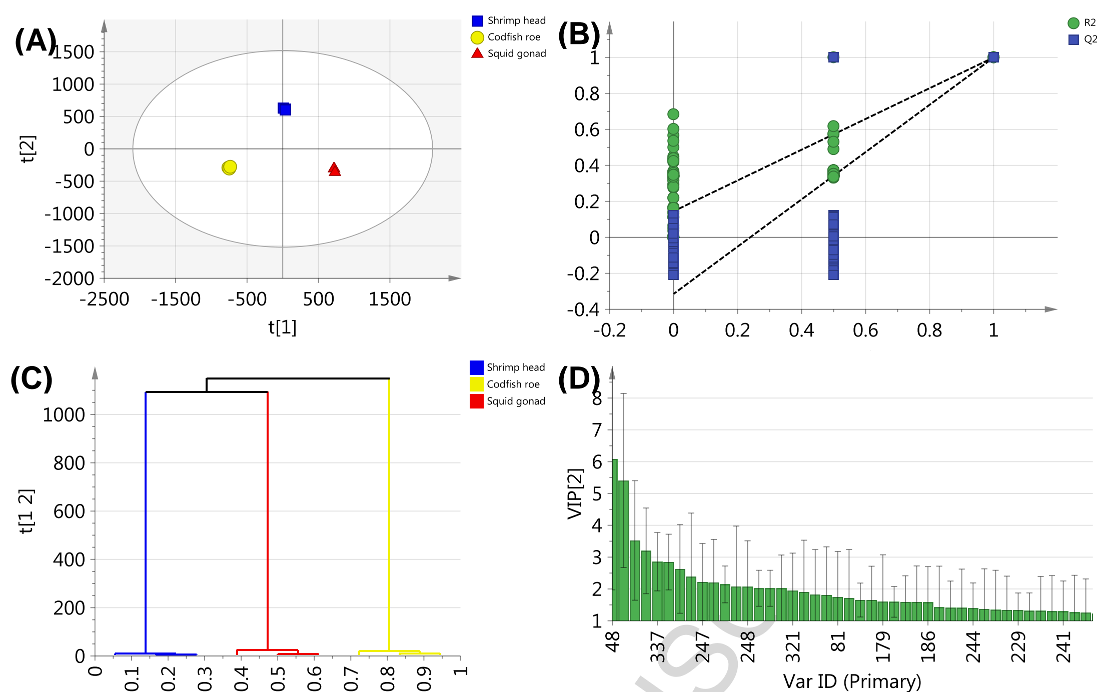

Fig. 4. Multivariate analysis of PLs. (A) PLS-DA score plot for shrimp head group (n = 3, blue), codfish roe group (n = 3, yellow) and squid gonad group (n = 3, red), (B) plot obtained after performing a random permutation test on PLS-DA model, R2 is the explained variance, and Q2 is the predictive ability of the model. Low value of Q2-intercept depicts the high predictability of the model, (C) hierarchical clustering analysis showing clustering of shrimp head (blue), codfish roe (yellow) and squid gonad (red) groups, (D) VIP score plot depleting VIP values (VIP > 1.4) that were responsible for the discrimination in the score plot.

Table 1

(A) Top 5 PLs showing significant differences between shrimp head and codfish roe groups; (B) Top 5 PLs showing significant differences between shrimp head and squid gonad groups; (C) Top 5 PLs showing significant differences between codfish roe and squid gonad groups.

Sr. no.	PL species	VIP score	*p* value
(A) Shrimp head versus codfish roe			
1	PC(17:1/18:1)	1.17	1.99E−10
2	PE(18:0p/22:6)	1.83	9.40E−10
3	PE(18:0p/20:5)	2.65	1.14E−09
4	PE(16:0p/20:5)	1.60	4.34E−09
5	PC(16:0/20:1)	2.59	6.08E−09

Sr. no.	PL species	VIP score	*p* value
(B) Shrimp head versus squid gonad			
1	PE(16:0p/20:5)	1.32	5.51E−10
2	PE(18:0p/22:5)	1.31	2.62E−09
3	PE(18:0p/20:5)	1.62	1.13E−08
4	PE(18:0/20:5)	2.31	2.61E−08

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Sr. no.	PL species	VIP score	p value
5	LPC(20:5)	1.39	2.77E–07
(C) Codfish roe versus squid gonad			
1	PE(18:0p/20:5)	2.59	5.87E–09
2	PC(16:1/22:6)	1.26	8.65E–09
3	PE(16:0p/20:5)	1.31	7.37E–08
4	PE(18:0p/16:0)	1.51	1.15E–07
5	PC(17:0/18:1)	1.98	1.30E–07

3.4. Comparative analysis of PLs in three different marine sources

Comparative analysis (*t*-test) of three marine sources (shrimp head, codfish roe, and squid gonad) revealed many variances in PL species. From the list of 89 PLs selected by PLS-DA model (VIP > 1.0), 55 PLs showed significant differences (*t*-test $p < 0.05$) between shrimp head and codfish roe groups. Among them, two PC (PC (17:1/18:1) and PC (16:0/20:1)) and three PE (PE (18:0p/22:6), PE (18:0p/20:5) and PE (16:0p/20:5)) represent the most prominent differences (Table 1A). 30 PLs showed significant differences ($p < 0.05$) between shrimp head and squid gonad groups. The top 5 most discriminating PLs of them were confirmed to be PE (16:0p/20:5), PE (18:0p/22:5), PE (18:0p/20:5), PE (18:0/20:5) and LPC (20:5) (Table 1B). Besides, 39 PL species showed significant differences between codfish roe and squid gonad groups. Among them, three PE and two PC, including PE (18:0p/20:5), PE (16:0p/20:5), PE (18:0p/16:0), PC (16:1/22:6) and PC (17:0/18:1), were top 5 differentially expressed PL species between these two groups (Table 1C).

3.5. Method validation

This method was validated in terms of accuracy, linearity, limit of determination (LOD), limit of quantitation (LOQ) and the relevant analytical parameters for analysis of the lipids standards (Table S2). In addition, the same UPLC-Q-Exactive Orbitrap/MS approach has been used to characterize lipid extracts from Bee pollen [45] which proved the validity and reliability of the method.

4. Conclusion

The aim of this study was to identify, compare and characterize PLs in three byproducts from different seafood processing (shrimp head, codfish roe and squid gonad) by using high-resolution UPLC-Q-Exactive Orbitrap/MS lipidomics as an aid to develop the different MPL products. In contrast to studies of general PL classes and fatty acid compositions, the analysis of lipidomics of seafood processing byproducts are rarely described in the literatures [8,46,47]. Lipidomics is a very effective research strategy to reveal the overall characteristics of MPLs composition and the degree of unsaturation of their side chains. In addition, Q-Exactive Orbitrap mass spectrometry, with extremely high resolution, sensitivity, and mass accuracy, is

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a powerful technique for fragment ion scanning [48]. It has been successfully used for lipidomics profiling in complex matrices. For example, a practical workflow for high-throughput and exhaustive lipid profiling was developed with the help of Q-Exactive Orbitrap mass spectrometry coupled with LC, and over 400 lipid compounds in mouse plasma was identified with below 2 % relative standard deviation (RSD) of relative retention time and capable of detecting low-abundance lipid compounds [49]. In this experiment, total 310 PL molecular species containing 34 different kinds of fatty acid chains and involving 8 PL classes (PC, PE, LPC, PI, LPE, PA, SM and PS) were identified simultaneously by Q-Exactive Orbitrap mass spectrometry with Lipidsearch software. As far as we know, this is the first report focusing on high-resolution UPLC-Q-Exactive Orbitrap/MS to the lipidomics profiling of these three different marine resources with optimization of sample preparation, chromatographic conditions and mass parameters.

MPLs are valuable ingredients that can be used in diverse areas of nutrition, pharmacy, medicine and basic research because of the PUFA side chains [8]. The compositions of MPLs are known to be different considerably from different sources. In this study, many visible differences in the PL compositions of three kinds of marine resources were verified by the relative quantifications. Meanwhile, PUFA PLs were further confirmed to be very rich in the seafood processing byproducts. The results of the UPLC-Q-Exactive Orbitrap/MS on the basis of lipidomic profiling study revealed the significantly different PLs between three groups. All of the differences shown above will provide opportunities to broaden the biological and commercial applicability of MPLs. Furthermore, the differential markers found through the lipidomics can be the basis for future exploration of MPL products, and establishing the quality standards for different marine raw materials.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online

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version, at https://www.journals.elsevier.com/journal-of-chromatography-b.

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Highlights

1. Phospholipids in different marine sources were identified and compared overall.

2. Characteristic phospholipids representing significant differences were determined.

3. Lipidomics approach by UPLC-Q-Exactive Orbitrap/MS was used for identification and analysis.

4. The deep comprehensive description of marine phospholipids was carried out.

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