Shotgun Lipidomics Strategy for Fast Analysis of Phospholipids in Fisheries Waste and Its Potential in Species Differentiation

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Ⓢ *Supporting Information*

ABSTRACT: An efficient shotgun lipidomics strategy was established and optimized for fast phospholipid profiling of viscera from three fish species: *Lateolabrax japonicas*, *Ctenopharyngodon idellus*, and *Carassius auratus*. This strategy relies on direct infusion of total lipid extracts into a tandem mass spectrometer without additional separation of the individual molecular species. Four classes of phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), were analyzed, and at least 81 molecular species of phospholipids were identified, including 34 species of PC, 24 species of PE, 12 species of PS, and 11 species of PI, in both positive- and negative-ion electrospray ionization mode. The results show that fish viscera, which are traditionally discarded as fisheries wastes, are nutritional in phospholipids with total contents of the four detected phospholipid classes ranging from 1.52 to 3.29 mg/g in the three tested fish species. Regardless of the tested fish species, PC and PE are the dominant phospholipid classes, followed by PI and PS. Furthermore, principal component analysis (PCA) was applied to normalize the relative amounts of the identified phospholipid species. The results demonstrate that PS 18:0/22:6, PI 18:0/20:4, and PI 18:0/20:5 were the main contributors of cumulative value and could be used as an indicator for fish species differentiation. This shotgun lipidomics method was >10 times faster than traditional methods, because no chromatographic separation was needed. The successful application of this strategy paves the way for full utilization of traditionally discarded fisheries wastes and provides an alternative means for fish species differentiation.

KEYWORDS: phospholipids, shotgun lipidomics, fish viscera, multidimensional mass spectrometry, principal component analysis

■ INTRODUCTION

Lateolabrax japonicas, *Ctenopharyngodon idellus*, and *Carassius auratus* areeconomically important fish species in China aquaculture production.[1] They are generally processed to wine-marinated fillets for domestic consumption and also exported to Europe and the United States. During fish processing, solid wastes, including heads and viscera, are generated and constitute as much as about 30% of the original material. Traditionally, they are considered as waste and have been utilized only to a minor extent, representing an important commercial loss and posing an environmental problem to the fishing industry. Recent studies showed that fisheries waste constitutes an important source of nutritional compounds.[2−4] There is obviously an increased need to utilize our sea resources with more intelligence and foresight.[5,6]

Lipid content of fish samples is an important feature used in biochemical, physiological, and nutritional studies. Especially phospholipids, which are major components of cellular membranes, play important roles in cell signaling and proliferation.[7,8] Phospholipids have complicated molecular types due to a glycerol backbone with one headgroup (choline, ethanolamine, serine, inositol, glycerol, or hydrogen attached to a phosphate group) at the sn-3 position and a fatty acid substituent at the sn-1 and/or sn-2 position.[9] In addition, the diversity of molecular species can be attributed to the number of carbon and double bonds in the fatty acid substituent and the substituents' location on the glycerol backbone. There are

some papers about phospholipids profiling of fish viscera, but most of them were devoted to analyzing the total content of phospholipids as well as the individual phospholipid fractions.[10,11] However, only a few studies focused on phospholipid species of viscera and other byproduct characterization.[12] Therefore, efficient and robust methods are required for fast phospholipid evaluation before conversion of these byproducts into more profitable and marketable products.

Lipidomics is a research field that studies cellular lipidomes on a large scale and at the intact-molecule level.[13] The emergence of this new discipline is built on the foundation established by numerous pioneering studies with multiplexed catalysts, including advances in instrumentation, interest in metabolism of lipids, and increased recognition of the roles of lipids in diseases.[14] One of the major analytical platforms in current lipidomics practice is a multidimensional mass spectrometry (MDMS)-based shotgun method.[15,16] MS-based lipidomics profiling ideally yields comprehensive, quantitative, and reproducible analytical data in an efficient and robust manner from any given biological sample, without deliberate bias or optimization for a specific lipid class or species. Such shotgun lipidomics techniques have been successfully applied
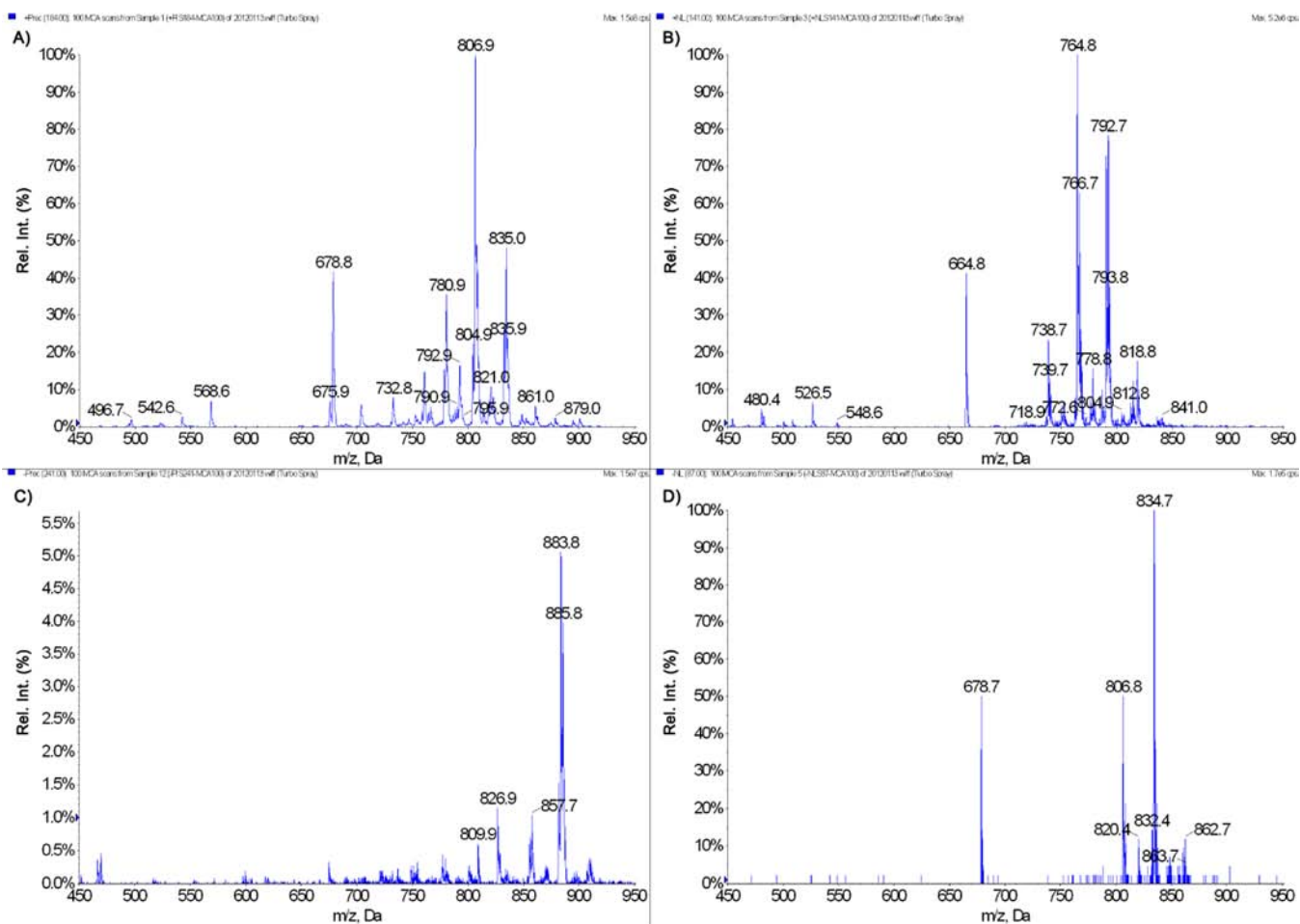
Figure 1. PIS and NLS mass spectra of phospholipids from fish viscera of *L. japonicas*: (a) PIS of *m/z* 184 Da in the positive-ion mode, identifying PC species; (b) NLS of 141 Da in the positive-ion mode, identifying PE species; (c) PIS of *m/z* 241 Da in the negative-ion mode, identifying PI species; (d) NLS of 87 Da in the negative-ion mode, identifying PS species. The IS peaks *m/z* 678.8 (PC 14:0/14:0), *m/z* 664.9 (PE 15:0/15:0), *m/z* 809.7 (PI 16:0/16:0), and *m/z* 678.7 (PS 14:0/14:0) shown are used to quantify PC, PE, PI, and PS, respectively.

for the characterization of glycerophospholipids,[17] ceramides,[18] glycerolipids,[19] and sterol lipids.[20]

In this study, we aimed to establish an efficient strategy for the fast analysis of phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), in viscera from *L. japonicas*, *C. idellus*, and *C. auratus* by using shotgun lipidomics with direct infusion electrospray ionization tandem mass spectrometry (ESI-MS/MS). This study could be useful to evaluate the phospholipid profile of fish processing byproducts, paving the way for enhancing utilization of fisheries wastes at an industrial scale.

■ MATERIALS AND METHODS

Materials and Reagents. The fish samples (*L. japonicas*, *C. idellus*, and *C. auratus*) with weight of ∼0.4 kg/fish were kindly given by Yuanyang Fisheries Ltd. (China) and authenticated by Zhejiang Research Institute of Marine Fisheries (Zhejiang, China). After capture, the fish were immediately transported to the Institute of Aquatic Products Processing, Zhejiang Gongshang University, within 2 h.

High-performance liquid chromatography (HPLC) grade methanol (MeOH) and chloroform (CHCl$_3$) were purchased from Merck (Darmstadt, Germany). Internal standards, PC (14:0/14:0), PE (15:0/15:0), PI (16:0/16:0), and PS (14:0/14:0), were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and prepared in MeOH/

CHCl$_3$ (1:1, v/v) to a final concentration of 1 μg/mL. These internal standards were selected because they represent ≤1% of endogenous cellular lipid molecular species present as demonstrated by ESI-MS lipid analysis without the addition of these internal standards. High-purity water with a resistivity of 18.2 MΩ/cm was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All other chemicals and solvents used in this study were of analytical grade and purchased from Huadong Chemicals Co., Ltd. (Hangzhou, Zhejiang, China).

Lipid Extraction. Upon arrival, fish samples with no evident signs of parasites as described by Mazorra-Manzano et al.[21] were collected and killed by ice-shocking. The fish viscera were separated from muscles, rinsed with cold distilled water, and then homogenized at 1500 rpm using an Ultra Turrax (T25, IKA-Werka, Germany). Lipids were extracted from fish viscera samples according to a modified version of the Bligh and Dyer method.[22] Briefly, 0.1 g of viscera sample was accurately weighed, placed in a 5 mL polytetrafluoroethylene tube, and mixed with 3 mL of CHCl$_3$/MeOH (2:1, v/v) solution. After extraction by an ultrasonic cleaning device for 15 min, a potion of 1 mL of water was added to separate the phase. After centrifugation at 8000g for 10 min, the lower organic phase was recovered and transferred to a new glass tube by pipet. The aqueous phase was re-extracted with 2 mL of CHCl$_3$ for another two times and then handled as described before. The collected organic phase were combined and evaporated under nitrogen flow. Dried lipid extracts were dissolved in 1 mL of CHCl$_3$/MeOH (2:1, v/v) and stored in the dark at −80 °C for electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis. Internal standards (ISs) were added to each viscera sample

prior to extraction of lipids. It is worth mentioning that to minimize the risk of oxidation of the polyunsaturated fatty acids or lipid hydrolysis during the process of isolation, it is always recommended that the extraction process of lipids should be completed at low temperature (4 °C) as soon as possible after collection of the viscera from living fish body.

Mass Spectrometry. Shotgun lipidomics analysis was performed on a 3200 triple-quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with a TurboIon-Spray interface (AB Sciex, Concord, Canada). Instrument control, data acquisition, and processing were performed using the associated Analyst 1.5.1 software (AB Sciex). Prepared samples were taken directly into the ion source by a sample pump (Harvard 11 Plus Syringe Pump, Harvard Apparatus, Inc., Holliston, MA, USA).

The TurboIon-Spray interface was operated in both positive- and negative-ion modes. To obtain maximum sensitivity for the identification and detection of phospholipids, ion spray voltage (IS) was always set at 5.5 and 4.5 kV capillary voltage in positive- and negative-ion modes, respectively. The ion source temperature (TEM) was set at 450 °C. Ion source gas 1 (GS1) and ion source gas 2 (GS2) were used as the drying and nebulizer gases at back pressures of 30 and 35 psi, respectively. Curtain gas (CUR) was 20 psi. The MS/MS experiments combining precursor ion scan (PIS) and neutral loss scan (NLS) were performed to monitor the different phospholipid classes. The declustering potential (DP) and collision energy (CE) were optimized using ISs and set to DP of 100 V and CE of 40 V (+PIS 184), DP of 90 V and CE of 35 V (+NLS 141), DP of 110 and CE of 30 V (−PIS 241), and DP of 100 V and CE of 40 V (−NLS 87). Each scan was acquired in multiple-channel acquisition (MCA) mode, which improves the ion statistics by summing the number of scans. The injection rate was set at 5 μL/min, and the MS scan range was from m/z 350 to 1150.

Identification and Quantitation of Phospholipids. Peak identification and isotope effects correction of acquired data were performed using LipidView software, version 1.1 (AB Sciex). Only the peaks that comprise >0.5% intensity and a signal-to-noise ratio (SN) > 3 were identified and quantified. The present method does not directly identify the acyl residues or their positions in the lipid molecules, but rather provides the total number of acyl chain carbons and double bonds. The major omega-3 molecular species of PC and PE can be identified by −PIS 327 and 301. Because abundant $[M − 15]^-$ peaks of PC species can be also generated by the negative-ion ESI/MS/MS PIS 327 and 301, overlapping with $[M − H]^-$ ions of PE species in the mass range of 700−850 Da when crude lipids are directly infused into the mass spectrometer without LC separation of lipid classes, we assigned the fatty acid chain constitutes for minor both PC and PE molecular species by consulting with other results published previously.[23−29] For PS and PI, information on the acyl chain composition can be obtained by multidimensional PIS to monitor the formation of specific m/z product ions corresponding to deprotonated FA ions in the negative-ion mode. For example, −PIS 255, −PIS 283, −PIS 311, −PIS 329, and −PIS 327 were taken to monitor FA 16:0, FA 18:0, FA 20:0, FA 20:5, and FA 22:6, respectively. The quantification of a class of lipid molecular species in lipid extracts was made by normalization of the individual molecular ion peak intensity to selected internal standard after correction for ^{13}C isotope effects.

Statistical Analysis. Statistical analysis and calculation of the mean, standard deviation, and level of significance were performed by using Microsoft Excel 2007. Principal component analysis (PCA) was applied to determine the main sources of variability present in the data sets and to establish the relationship between samples (objects) and phospholipids (variables). ESI-MS raw data were directly imported into the statistical software package MarkerView, version 1.2.1 (AB Sciex), and the software would look for the difference in the mass fragments (m/z), the retention time, and the abundance.

Table 1. Total Contents (mg/g) of Phospholipids and Each Phospholipid Class from Fish Viscera of *L. japonicas*, *C. idellus*, and *C. auratus*[a]

lipid class	*L. japonicas*		*C. idellus*		*C. auratus*	
	content	SD	content	SD	content	SD
LPC	0.48	0.04	0.24	0.02	0.18	0.01
LPE	0.46	0.03	0.21	0.02	0.09	0.01
LPI	nd		0.01	0.00	0.01	0.00
LPS	nd		nd		0.03	0.00
PC	15.87	1.48	7.13	0.68	7.29	0.42
PE	13.60	1.26	5.65	0.52	4.22	0.30
PI	1.45	0.12	0.86	0.07	0.78	0.06
PS	3.86	0.36	2.37	0.21	2.98	0.74
Total	35.72	3.29	16.47	1.52	15.54	1.54

[a]The concentration data of phospholipids are shown as means and SD ($n = 6$). nd, not detected.

RESULTS AND DISCUSSION

Extraction Efficiency. One of the most critical factors obviously affecting the quantitative analysis of lipids in fish viscera samples is the isolation step of phospholipids from tissues by extraction with organic solvents and removal of the nonlipid component from the extracts. The target phospholipids studied in this experiment belong to polar lipids due to their polar headgroups. To analyze polar phospholipids from one lipid extract, several methods were designed to establish a protocol for the exhaustive extraction of phospholipids from fish viscera samples. Figure S-1 of the Supporting Information shows the performance of several lipid extraction methods, including the modified Bligh and Dyer method,[22] the Folch method, which uses chloroform/methanol in a ratio of 2:1 and large volumes of water for washing out the nonlipid components,[30] and the Nichols method, which uses an initial isopropanol extraction for enzyme inactivation following a "Folch"-like procedure.[31] The extraction efficiency was determined in fish viscera homogenate by adding a phospholipid IS mixture before and after extraction. The results indicate that the recovery for the Folch method is the lowest among the three utilized methods, about 60% (average value of the tested phospholipids). This may be due to the inherit drawback of the Folch method, emulsions formation, which makes the extraction process difficult to control. Mean recovery for the Bligh and Dyer method was about 78% and did not vary with concentration of ISs added. For the Nichol method, the mean recovery was unstable and the values varied greatly, with a standard deviation of >10%, although the mean recovery was the highest (about 82%). This is partially caused by the loss and contamination of target phospholipids during tedious extraction procedures, including solvent evaporation, reconstitution, and washing. Therefore, the Bligh and Dyer method was chosen for phospholipid extraction from fish viscera samples, and it was shown to be efficient, reliable, and reproducible.

To further improve the mean recovery, the efficiencies of the Bligh and Dyer method by using vortex dispersion and ultrasonic assisted extraction were also tested. The mean recovery of the former is about 8−16% lower than that of the latter, which indicates that ultrasonic assisted extraction is a critical step in releasing polar lipids from viscera cells and exhaustively extracting polar lipids from fish viscera sample due to the efficiency of ultrasonic power in cell-wall breaking. Three

Table 2. Molecular Species and Abundance (%) of Lysophospholipids from Fish Viscera of *L. japonicas*, *C. idellus*, and *C. auratus*[a]

LPL[b]	m/z	*L. japonicas*		*C. idellus*		*C. auratus*	
		abundance	SD	abundance	SD	abundance	SD
LPC 16:1	494.6	0.17	0.02	0.16	0.02	0.19	0.02
LPC 16:0	496.6	0.41	0.04	2.06	0.59	0.96	0.79
LPC 18:2	520.6	nd		0.25	0.03	0.20	0.01
LPC 18:1	522.6	0.23	0.02	0.77	0.10	0.74	0.06
LPC 20:5	542.6	0.59	0.06	0.07	0.02	0.13	0.01
LPC 20:4	544.6	nd		0.53	0.07	0.39	0.26
LPC 20:3	546.6	nd		0.13	0.02	nd	
LPC 22:6	568.6	1.52	0.10	0.85	0.10	0.73	0.05
LPC 22:5	570.6	nd		0.30	0.02	0.14	0.02
LPE 16:0	454.6	0.43	0.03	1.23	0.23	nd	
LPE 18:1	480.4	0.96	0.12	1.06	0.22	0.80	0.06
LPE 18:0	482.6	0.57	0.03	0.45	0.03	0.31	0.02
LPE 20:4	502.6	nd		0.32	0.03	0.29	0.03
LPE 20:1	508.6	0.25	0.03	0.20	0.02	nd	
LPE 22:6	526.6	1.27	0.16	0.40	0.05	0.49	0.06
LPE 22:5	528.6	0.06	0.02	0.50	0.09	0.19	0.01
LPI 26:6	699.8	nd		0.85	0.38	1.02	0.09
LPS 16:0	496.3	nd		nd		1.22	0.19
LPS 18:0	524.4	nd		nd		1.47	0.27

[a]The concentration data of lysophospholipids are shown as means and SD ($3n = 6$). nd, not detected. [b]The LPL molecular species are shown in the format *X Y:Z*, where *X* represents the phospholipid classes, *Y* represents the number of total carbon atoms of the fatty acid chain, and *Z* represents the number of total double bonds of the fatty acid chain.

ultrasonic assisted extractions is considered to be nearly complete because there were no detectable ISs and polar lipid components in extracts from the fourth extraction.

Moreover, appropriate amounts of HCl and NH_4OH (final concentration = 0.01 M) were added to improve the extraction efficiency in both positively and negatively charged phospholipids. However, there is a concern that the acidic or alkaline conditions would induce hydrolysis of endogenous lipids, resulting in artificial generation of lysophospholipids and free fatty acids.

Finally, we utilized the Bligh and Dyer method combining an ultrasonic assisted method for phospholipids extraction from fish viscera samples.

Identification and Quantization of Phospholipids. Headgroup fragmentation scanning by employing selective PIS and NLS mode analysis strategies was utilized for rapid and sensitive monitoring of the molecular compositions and abundances of individual phospholipid species in unfractionated lipid extracts. The use of multiple scans for phospholipid identification enabled the compositions of the overlapping ions present at a particular m/z values in either the positive- or negative-ion mode mass spectra to be more fully elucidated and allowed the list of spectral features to be significantly expanded. In this study, each phospholipid class showed fragmentation during CID at the phosphodiester linkage, resulting in headgroup fragmentation.[32] All peaks in the spectra of lipid extracts from fish viscera could be assigned to distinct PC species, demonstrating the high selectivity of the parent scan. The assignment of PC molecules was confirmed by ESI-MS/MS fragmentation, when a protonated phosphocholine fragment was generated at m/z 184, because it is characteristic for the phosphocholine headgroup. In addition, PC molecular species could be distinguished from sphingomyelin (SM) molecular species by using the nitrogen rule, because single-charged PC has an even-numbered molecular weight, whereas

single-charged SM has an odd-numbered molecular weight. Therefore, the characteristic fragment ion of phosphocholine, m/z 184, was selected for detection of PC and lysophosphatidylcholine (LPC) in the positive-ion mode. On the other hand, fragmentation of PE and lysophosphatidylethanolamine (LPE) in the positive-ion mode exclusively yielded one peak, a $[M + H - 141]^+$ ion from the neutral loss of the phosphoethanolamine headgroup, thus chose NLS of m/z 141 in the positive-ion mode for PE and LPE detection. Similar scans can be performed to detect PI (PIS of m/z 241 in the negative-ion mode) and PS (NLS of m/z 87 in the negative-ion mode) molecules. The added ISs PC 14:0/14:0 (m/z 678.8), PE 15:0/15:0 (m/z 664.9), PI 16:0/16:0 (m/z 809.7), and PS 14:0/14:0 (m/z 678.7) are used to quantify PC, PE, PI, and PS, respectively. Using this method, at least 81 molecular species of phospholipids were identified, including 34 species of PC, 24 species of PE, 12 species of PS, and 11 species of PI. Each ample was analyzed in triplicate, and the abundance shown was the percentage of the total phospholipid classes detected, resulting in reproducible data with small standard deviations. Figure 1 shows the PIS and NLS mass spectrum of the crude lipid extract from fish viscera of *L. japonicas*.

PIS and NLS scans for the m/z of the characteristic fragments of the headgroup detect lipid species of the same class and allow calculation of the total number of carbon atoms and the total number of double bonds in fatty acid moieties. However, such scans do not reveal the aliphatic substituent. Previously, Hsu and Turk[33] reported that low-energy CID of major glycerophospholipids in the negative-ion mode might produce acyl anions of their fatty acid constituents. Ekroos et al.[34] reasoned that multiple precursor ion scanning (MPIS) experiments could detect lipids by monitoring the acyl anions of all major fatty acids, which may be present in a particular sample and thus would complement monitoring of characteristic fragments of lipid headgroups. In the current study, MPIS

Table 3. Molecular Species and Abundance (%) of Phospholipids from Fish Viscera of *L. japonicas*, *C. idellus*, and *C. auratus*[a]

PL[b]	m/z[c]	sn-1/sn-2[d]	*L. japonicas*		*C. idellus*		*C. auratus*	
			abundance	SD	abundance	SD	abundance	SD
PC 32:2	730.8	14:0/18:2	0.28	0.05	0.28	0.02	0.34	0.08
PC 32:1	732.8	16:0/16:1	1.68	0.20	1.73	0.35	1.19	0.26
PC 32:0	734.8	16:0/16:0	0.18	0.01	0.47	0.03	0.21	0.09
PC 34:4	754.8	14:0/20:4	0.36	0.01	0.13	0.02	0.3	0.03
PC 34:3	756.8	14:0/20:3	0.31	0.01	0.56	0.07	1.00	0.30
PC 34:2	758.8	16:0/18:2 and 16:1/18:1	0.90	0.05	4.10	0.18	3.60	0.36
PC 34:1	760.8	16:0/18:1	3.31	0.31	3.12	0.38	9.04	1.64
PC 36:6	778.8	14:0/22:6	3.21	0.35	0.30	0.03	0.62	0.09
PC 36:5	780.8	16:0/20:5	7.41	1.02	1.58	0.59	1.72	0.12
PC 36:4	782.8	16:0/20:4	1.10	0.02	9.36	1.64	3.60	0.19
PC 36:3	784.8	18:1/18:2 and 16:0/20:3	0.31	0.02	2.40	0.56	1.97	0.44
PC 36:2	786.8	18:0/18:2 and 18:1/18:1	0.67	0.08	1.29	0.23	2.31	0.36
PC 36:1	788.8	18:0/18:1	0.99	0.10	0.28	0.02	1.05	0.04
aPC 38:7 and pPC 38:6	790.8	a16:1/22:6[e] and p16:0/22:6[f]	0.70	0.10	0.12	0.01	0.10	0.02
PC 38:7	804.9	16:1/22:6	4.79	0.40	0.91	0.64	1.63	0.21
PC 38:6	806.9	16:0/22:6	23.52	2.49	13.18	4.16	9.75	2.01
PC 38:5	808.9	16:0/22:5 or/and 18:0/20:5	7.01	1.12	6.51	2.70	3.77	0.26
PC 38:4	810.9	18:0/20:4	0.63	0.04	1.62	0.18	2.26	0.33
PC 38:3	812.9	18:0/20:3	nd		0.76	0.05	1.49	0.10
PC 38:2	814.6	18:0/20:2	0.96	0.11	0.06	0.01	0.32	0.03
aPC 40:7 and pPC 40:6	818.9	a18:1/22:6 and p18:0/22:6	1.51	0.03	0.72	0.07	0.56	0.03
PC 40:8	830.9	18:2/22:6	0.39	0.03	0.56	0.05	0.68	0.09
PC 40:7	832.9	18:1/22:6	6.11	0.38	2.90	0.27	3.16	0.38
PC 40:6	834.9	18:0/22:6	7.16	0.32	2.04	0.26	2.50	0.21
PC 40:0	846.9	uc	0.34	0.04	0.07	0.02	0.06	0.01
PC 42:11	852.9	20:5/22:6	0.44	0.04	0.05	0.02	0.15	0.03
PC 42:10	854.9	20:4/22:6	0.25	0.02	0.41	0.08	0.30	0.02
PC 42:8	858.9	20:3/22:6	0.25	0.03	0.35	0.08	0.20	0.03
PC 42:7	860.9	20:1/22:6	1.12	0.15	0.30	0.05	0.35	0.02
PC 42:6	862.9	20:0/22:6	0.42	0.05	0.13	0.01	0.19	0.01
PC 44:12	878.9	22:6/22:6	0.50	0.07	0.10	0.02	nd	
PC 44:4	894.9	uc	0.37	0.02	nd		nd	
PC 44:1	900.9	uc	0.44	0.05	nd		0.09	0.02
PE 36:5	738.8	16:0/20:5	5.43	0.53	1.59	0.20	0.75	0.08
PE 36:4	740.7	16:0/20:4	0.84	0.05	14.41	2.88	2.85	0.23
PE 36:3	742.7	16:0/20:3 and 18:1/18:2	0.12	0.02	0.88	0.13	0.58	0.03
PE 38:7	762.8	16:1/22:6	1.70	0.21	0.55	0.11	0.63	0.05
PE 38:6	764.8	16:0/22:6 and 18:1/20:5	17.16	4.96	8.71	1.03	5.95	0.71
PE 38:5	766.8	16:0/22:5 and 18:0/20:5	10.88	1.73	15.03	2.65	9.03	1.23
PE 38:4	768.8	18:0/20:4	1.48	0.15	8.00	1.36	9.89	1.11
PE 38:3	770.8	18:0/20:3	0.13	0.02	0.52	0.04	0.47	0.02
PE 38:1	774.8	18:0/20:1	0.15	0.01	0.1	0.02	nd	
aPE 40:1 and pPE 40:6	776.8	a18:1/22:6 and p18:0/22:6	1.50	0.34	0.14	0.02	0.13	0.02
PE 40:9 and pPE 40:1	786.8	18:3/22:6 and p18:0/22:1	1.96	0.12	2.61	0.31	0.26	0.02
PE 40:8	788.8	uc	0.78	0.16	0.87	0.18	1.42	0.20
PE 40:7	790.8	18:1/22:6	14.58	0.23	4.41	0.94	8.06	1.60
PE 40:6	792.8	18:0/22:6 and 18:1/22:5	14.46	0.16	7.65	1.12	6.45	1.00
PE 40:5	794.8	18:0/22:5	0.98	0.02	4.65	0.53	3.71	0.44
PE 40:4	796.8	18:0/22:4	0.14	0.00	0.54	0.04	0.82	0.05
PE 40:0	804.8	p20:0/22:6	0.69	0.10	nd		0.14	0.01
PE 42:10	812.8	20:4/22:6	1.14	0.18	0.54	0.04	0.45	0.03
PE 42:9	814.8	20:3/22:6	1.95	0.21	0.79	0.11	0.89	0.07
PE 42:8	816.8	20:3/22:6	0.37	0.03	0.93	0.05	0.60	0.03
PE 42:7	818.9	20:1/22:6	3.45	0.40	1.79	0.27	2.35	0.34
PE 42:6	820.9	20:0/22:6	0.38	0.03	0.91	0.03	0.76	0.05
PE 44:12	836.8	22:6/22:6	0.45	0.02	nd		0.16	0.01
PE 44:11	838.8	22:5/22:6	0.22	0.01	0.23	0.01	nd	
PI 30:1	779.8	14:0/16:1	0.98	0.04	nd		nd	
PI 34:5	827.8	16:0/18:1	1.72	0.20	nd		nd	

Table 3. continued

PL[b]	m/z[c]	sn-1/sn-2[d]	L. japonicas		C. idellus		C. auratus	
			abundance	SD	abundance	SD	abundance	SD
PI 36:5	855.8	16:0/20:5	6.50	1.21	nd		nd	
PI 36:4	857.8	16:0/20:4	10.03	0.64	8.45	1.17	4.16	0.18
PI 38:6	881.8	16:0/22:6	11.72	1.45	1.91	0.14	nd	
PI 38:5	883.8	18:0/20:5	40.07	2.61	18.87	2.12	21.28	2.89
PI 38:4	885.8	18:0/20:4	22.54	3.59	56.34	3.49	34.5	4.41
PI 38:3	887.8	18:0/20:3	nd		2.48	0.24	nd	
PI 40:7	907.8	18:1/22:6	1.69	0.29	2.73	1.40	nd	
PI 40:6	909.8	18:0/22:6	2.93	0.38	2.49	0.20	nd	
PI 40:5	911.8	18:0/22:5	nd		2.24	0.36	nd	
PS 36:4	782.8	16:0/20:4	nd		4.81	0.51	nd	
PS 38:6	806.8	16:0/22:6	15.48	2.98	10.05	1.32	6.06	1.21
PS 38:5	808.6	18:0/20:5	4.77	0.68	17.00	3.49	4.07	0.76
PS 38:4	810.7	18:0/20:4	nd		9.49	1.08	6.69	0.95
PS 38:3	812.8	18:0/22:3	nd		1.53	0.29	nd	
PS 40:7	832.4	18:1/22:6	4.18	0.44	2.47	0.43	5.56	1.08
PS 40:6	834.7	18:0/22:6	40.28	9.12	11.74	2.50	39.44	6.97
PS 40:5	836.7	18:0/22:5	8.70	2.09	13.54	1.76	12.26	1.64
PS 40:4	838.6	18:0/22:4	nd		4.28	1.05	2.93	0.68
PS 42:7	860.8	uc	3.64	0.59	1.33	0.16	nd	
PS 42:6	862.7	20:0/22:6	5.33	0.75	1.89	0.37	2.55	0.25
PS 42:5	864.7	20:0/22:5	nd		0.75	0.11	1.05	0.08

[a]The concentration data of phospholipids are shown as means and SD ($n = 6$). uc, uncertain; nd, not detected. [b]The phospholipid molecular species were shown by the format of X Y:Z, where X represents the phospholipid classes, Y represents the number of total carbon atoms of the fatty acid chain, and Z represents the number of total double bonds of the fatty acid chain. [c]The m/z of PC and PE were positive, whereas those of PI and PS were negative, because they were detected in different polarity modes. [d]Fatty acid chain constituents (sn-1/sn-2) were confirmed by referring to other results already published (for PC and PE) or MPIS (for PS and PI). [e]In the notation a16:1/22:6, a means alkyl linkage (1-O-alkyl or plasmanyl). [f]In the notation p16:0/22:6, p means plasmalogen (1-O-alk-1′-enyl or plasmenyl) linkage.[26].
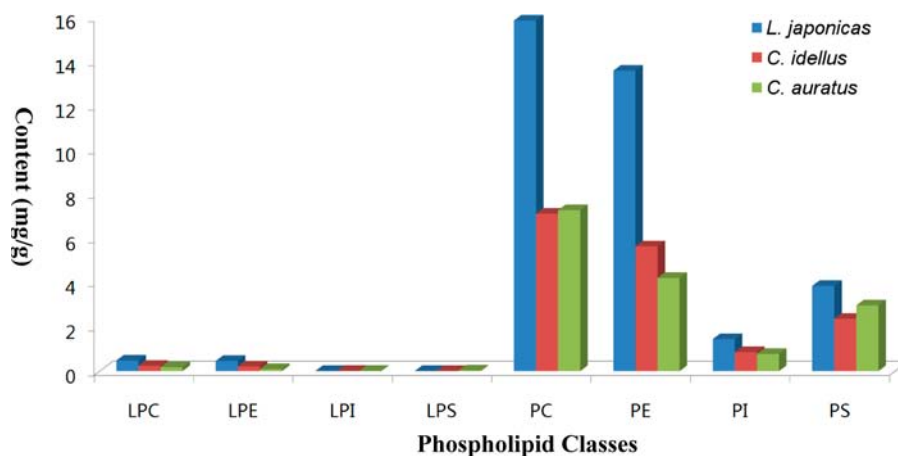

Figure 2. Total contents of each phospholipid class of viscera from three economically important fish species: *L. japonicas*, *C. idellus*, and *C. auratus*.

analysis was performed in the negative-ion mode by acquiring precursor ion spectra for the headgroup fragment ion of PI and PS, and acyl anions corresponding to FA 22:6 (PIS m/z 327.2), FA 22:5 (PIS m/z 329.2), FA 22:4 (PIS m/z 331.2), FA 20:5 (PIS m/z 301.2), FA 20:4 (PIS m/z 303.2), FA 18:2 (PIS m/z 279.2), FA 18:1 (PIS m/z 281.3), FA 18:0 (PIS m/z 283.3), and so on. The detailed identification process was according to some references elsewhere.[35] For example (Figure S-2 of the Supporting Information), m/z 806.8 was detected in NLS 87, PIS 255, and PIS 327 in the negative-ion mode simultaneously. This indicated that the molecular species of m/z 806.8 was PS 38:6 and the fatty acid chain compositions were 16:0/22:6. This led to the identification of this PS species as PS 16:0/22:6. The same was found for the other species of PS and PI. With

this method, the fatty acid chain compositions (sn-1/sn-2) of more than 80 and 60% of PS and PI molecular species, respectively, were identified. The results indicate that the molecular species of phospholipids in each fish species of this study contain great amounts of long-chain polyunsaturated fatty acid chains, defined as arachidonic acid moiety (20:4), eicosapentaenoic acid moiety (20:5), and docosahexaenoic acid moiety (22:6), which are proved to be essential to human health and development.[36] The phospholipid species shared much similarity with other aquatic products such as shark liver and Pacific oyster.[28,37]

The quantitative results showed satisfactory precision for the major species, as shown by the the standard deviation given in Tables 1−3, whereas the lower precision for the minor species
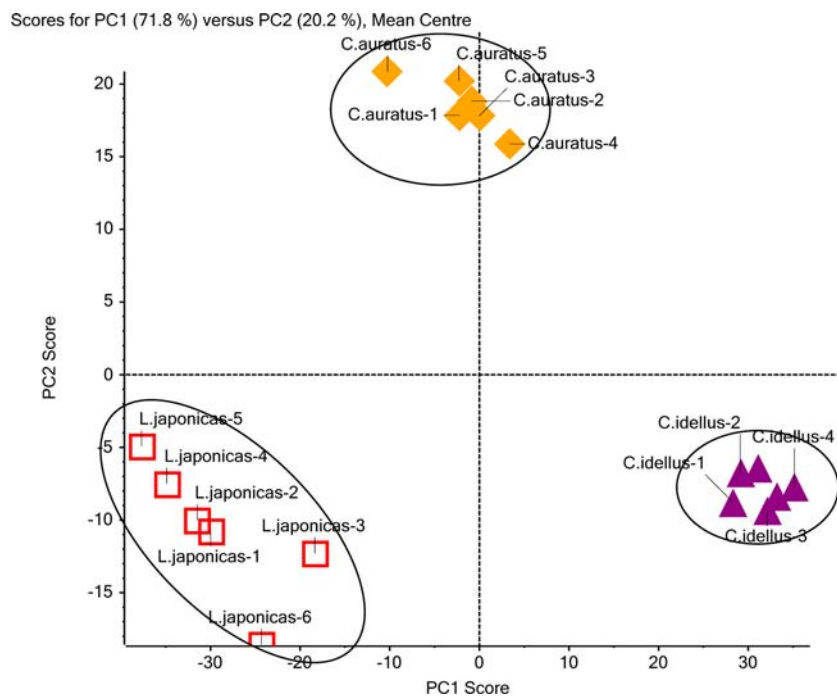
Figure 3. Principal component analysis of the identified phospholipid species of viscera from three economically important fish species: *L. japonicas*, *C. idellus*, and *C. auratus*. Eighteen fish viscera samples (6 for each fish species) were tested and can be clearly distinguished by the separated clusters.
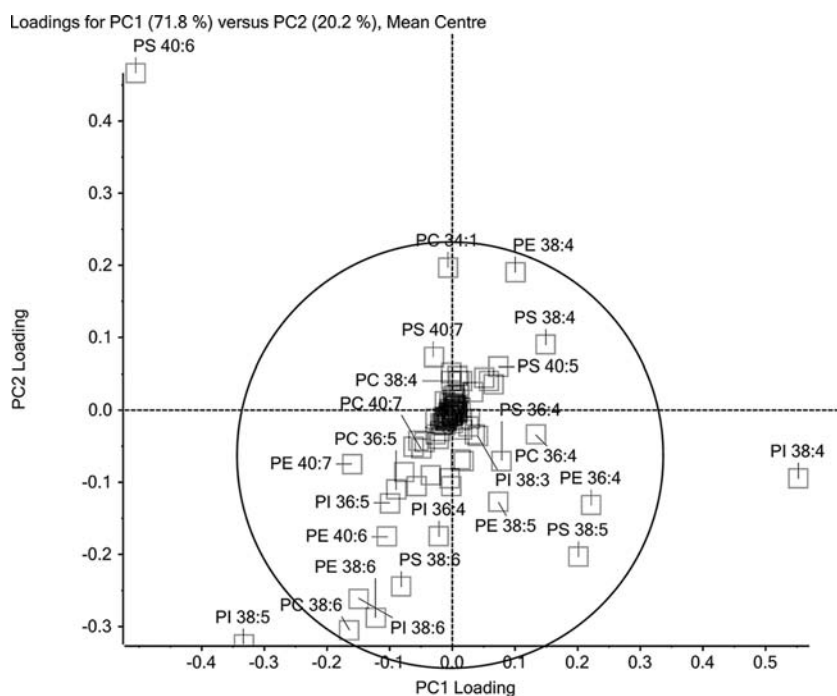

Figure 4. Loading plot on the first two principal components for the identified phospholipid species of viscera from three economically important fish species: *L. japonicas*, *C. idellus*, and *C. auratus*.

may be attributed to the concentration close to the detection limit. The contents of individual phospholipid classes are illustrated in Figure 2 and listed in Table 1. Comparisons were made of the profiles of LPC, LPE, LPI, LPS, PC, PE, PI, and PS among the viscera of the three cultured fish species. For *L. japonicas*, PC were detected as the principal phospholipid class in fish viscera with a total content 15.87 mg/g, followed by PE (13.60 mg/g) and PS (3.86 mg/g), whereas the content of PI (1.45 mg/g) was the lowest. The original amounts of individual

lysophospholipids were approximately 0.48 mg/g of LPC and 0.46 mg/g of LPE, whereas no LPI and LPS was detected in *L. japonicas* viscera sample, as shown in Table 2. The total amount of lysophospholipids (0.94 mg/g) was obviously lower than that of phospholipids (35.78 mg/g). This may be attributed to several factors, for instance, the inherent enzymatic effect in fish viscera cell, well conducted sample preparation procedure under soft conditions inhibiting phospholipids hydrolysis, or the polarity of the lipid extraction solvent. In fact, the

lysophospholipids have stronger polarity than phospholipids, which makes the methanol/aqueous methanol solvent systems more efficient for extraction of polar lipids from fish viscera sample than chloroform-containing solvent systems.[38] However, in this study, a methanol/aqueous solvent system was not tested because our main focus was on phospholipids. Fish viscera samples from *C. idellus* and *C. auratus* were also tested, and they have comparatively similar total tested phospholipids, which were 16.47 and 15.68 mg/g, respectively, but lower than that in *L. japonicas* viscera sample. Regardless of the fish species, PC is the dominant phospholipid class, with contents 7.13 and 4.29 mg/g, followed by PE at 5.65 and 3.22 mg/g for *C. idellus* and *C. auratus*, respectively. PC is the most abundant glycerophospholipid in fish viscera cell and can spontaneously organize into bilayers[39] thanks to its overall cylindrical molecular shape, which makes it very important in protecting cells. PE is synthesized by the addition of cytidine diphosphate-ethanolamine to diglyceride, releasing cytidine monophosphate. *S*-Adenosylmethionine can subsequently methylate the amine of PE to yield PC. PS was comparatively lower, with absolute contents 2.37 and 7.08 mg/g for *C. idellus* and *C. auratus*, respectively. PI is a negatively charged phospholipid and a minor component in the cytosolic side of eukaryotic cell membranes. Its total contents were even lower in *C. idellus* and *C. auratus*, about 0.86 and 0.78 mg/g, respectively.

Statistical Analysis. Despite the similar quantity of total phospholipid contents in all tested fish viscera samples, differences were observed in the proportions of lipid species of each lipid class, as shown in Tables 2 and 3. To evaluate the phospholipid species difference between the viscera samples of the tested three fish species, *L. japonicas*, *C. idellus*, and *C. auratus*, PCA was applied to normalize the relative amounts of 81 identified phospholipid species and 18 fish viscera sample (6 for each fish species) in a reduced-dimension plot. PCA is a powerful technique used for reducing the number of dimensions present in the whole data matrix with only a minimum loss of original information. It explains the variance of a large set of intercorrelated variables (phospholipid species) and transforms them into a smaller set of uncorrelated variables, namely, principal components (PC) (from PC1 to PC4 as listed in Table S-1 of the Supporting Information). The first two PCs were extracted, explaining 92.0 cumulative percent (cum %) value of the total variance of the data set, whereas PC1 explains 71.8 cum % of the variance in the initial data set and PC2 explains 20.2 cum %. In Figure 3, the first principal component (PC1) is plotted against the second (PC2), and the distribution of the samples implies that the three fish species can be differentiated well from each other from this PC1−PC2 scatter point plot.

Furthermore, the coefficient that defines the weight of the original variable in the PC was investigated to understand which phospholipid species are responsible for the difference of fish viscera samples. The study of loadings for the variables in the first two principal components is shown in Figure 4. The phospholipids that explain maximum variance in the data had higher loading values, whereas others that do not play an important role provided loading values near 0. This shows that PS 18:0/22:6, PI 18:0/20:5, and PI 18:0/20:4 were the dominant features in the first principal component with loading values of −0.51, −0.33, and 0.55, respectively. This result shows that the PI 18:0/20:5 ratio can be an indicator to distinguish the viscera sample of *L. japonicas* from those of *C. idellus* and *C. auratus*, because the relative abundance of PI 18:0/20:5 is

obviously higher than those of the latter. In the same way, PI 18:0/20:4 can act as a sign to differentiate *C. idellus* from the other two. The loading value of PS 18:0/22:6 was 0.46, which was the main contributor on PC2. This indicated that differentiation of *C. idellus* from *C. auratus* and *L. japonicas* could be well performed due to the comparatively lower abandance of PS 18:0/22:6. In conclusion, the tested viscera of three economically important fish species were shown to be rich in phospholipids with total contents of the four detected phospholipid classes ranging from 1.52 to 3.29 mg/g in the three tested fish species. An interesting discovery was that some specific phospholipids, such as PI 18:0/20:5, PI 18:0/20:4, and PS 18:0/22:6, could be used as potential makers for fish species differentiation.

In summary, in contrast to existing protocols, this assay offers an efficient strategy for the study of fish viscera phospholipid molecular species, which are usually discarded by the fishery industry, using shotgun lipidomics by direct infusion mass spectrometry. This strategy can provide information regarding not only the fatty acid chain compositions but also their positions (sn-1/sn-2) in the major molecular species of individual phospholipids classes and showed to be advanced regarding analysis time (<5 min/sample), stability of the measurement, simple sample preparation, and detailed information obtained for different phospholipid species. By using this protocol, the tested viscera from three economically important fish species were shown to be rich in phospholipids, which can be utilized by industry instead of being discarded as waste. The precision of this methodology and its high-throughput capability make it suitable for routine application. We anticipate that the method will be particularly valuable in the analysis of fish samples for quality control, making good use of wasted materials, and fish species differentiation.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Table of loadings variables and figures of lipid extraction efficiency nad representative multidimensional MS analyses of phospholipids. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

MPIS, multiple-precursor ion scanning; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PCA, principal component analysis; MDMS, multidimensional mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; PIS, precursor ion scan; NLS, neutral loss scan; QqQLIT, triple-quadrupole/linear ion trap.

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