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Tracking Phospholipid Profiling of Muscle from *Ctennopharyngodon idellus* during Storage by Shotgun Lipidomics

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ABSTRACT: This paper aims to study phospholipid (PL) profiling of muscle from *Ctenopharyngodon idellus* during room-temperature storage for 72 h by direct-infusion electrospray ionization tandem mass spectrometry (ESI−MS/MS). Five classes of PLs, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM), were analyzed. At least 110 molecular species of PLs were identified, including 32 species of PC, 34 species of PE, 24 species of PS, 18 species of PI, and 2 species of SM. The result showed that oxidation and hydrolysis are the two main causes for the deterioration of PLs in fish muscle during storage. Most content of PL molecular species increased and then decreased gradually. However, some special PE molecular species with former low abundance, such as PE 32:1, PE 34:2, and PE 34:1, emerged during the storage in quantity. It indicated that those PE molecular species may come from the microbe bred in the muscle. This phenomenon was found and discussed for the first time. The possible relevance between the emergence of these special PE molecular species and the freshness of the fish muscle during storage will be investigated in further studies.

KEYWORDS: Phospholipid profiling, muscle, storage, *Ctenopharyngodon idellus*, shotgun lipidomics

INTRODUCTION

Fish freshness is fundamental to fish quality and closely related to the microbe flora, storage temperature, handling, and physiological conditions of the fish.[1] As the higher abundance membrane lipid in muscle, phospholipid (PL) changes during storage are one of the most important post-mortem changes for fish freshness. Oxidation and hydrolysis are the two obvious reactions in fish PLs for quality deterioration.[2] They result in a range of substances, among which some have unpleasant taste or smell. Some may also contribute to texture changes by binding covalently to fish muscle proteins. For example, hydrolysis may release the free fatty acid (FFA), which could then cause denaturation of proteins in the muscle.[3,4] The various reactions are either non-enzymatic, enzymatic catalyzed by microbial enzymes, or intracellular digestive enzymes from the fish. The relative significance of these reactions mainly depends upon fish species and storage temperature.[2]

There are numerous papers[3−11] about PL profiling of fish muscle, while most of them have been devoted to analyze the total content of PLs as well as the individual PL fractions. When the fatty acid chain compositions were involved, hydrolysis was taken for individual PL fractions, which were separated by thin-layer chromatography (TLC). The result is the total fatty acid chain compositions of individual PL fractions or PL classes and not the individual PL molecular species. Some researchers[4,5,7,9] have investigated the changes of the PL classes and their fatty acid chain compositions from fish muscle during storage at different temperatures. However, the evolution during the storage about the individual PL molecular species, especially the fatty acid chain compositions of individual PL molecular species at sn-1 and sn-2 positions of glycerol backbone, has not yet been studied.

Typically, the analytical procedure for PLs involves lipid extraction, followed by chromatography separation of lipid classes, and then detection by mass spectrometry or other detectors.[12] The traditional method is time-consuming, and its major drawback is the large amount of lipids required; moreover, chromatography methods often suffer from poor lipid recoveries and changes to the distribution of molecular species.[13] Recently, soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI)[14] techniques, coupled with tandem mass spectrometry [tandem triple quadrupole (Q-Q-Q) or quadrupole-time-of-flight (Q-TOF)] have been developed to comprehensively analyze lipid composition,[15−21] and it has enabled the development of shotgun lipidomic approaches. Shotgun lipidomic approaches provide for rapid and sensitive monitoring of the molecular compositions and abundances of individual lipid species in unfractionated lipid extracts.[22] It only needs simple sample preparation and little samples for direct-infusion analysis. After collision-induced dissociation (CID), the majority of PL classes possess some unique headgroup fragmentation that is usually diagnostic for the lipid class of interest, with two powerful tandem MS techniques [neutral loss scan (NLS) and precursor ion scan (PIS)].[23] These headgroup-specific fragmentations are exploited not for identification but for quantification by comparing the peak intensities of PLs molecularly detected to those of the selected internal standards (ISs).

The purpose of the present study was to track PL profiling of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM) from *Ctennopharyngodon idellus* muscle during

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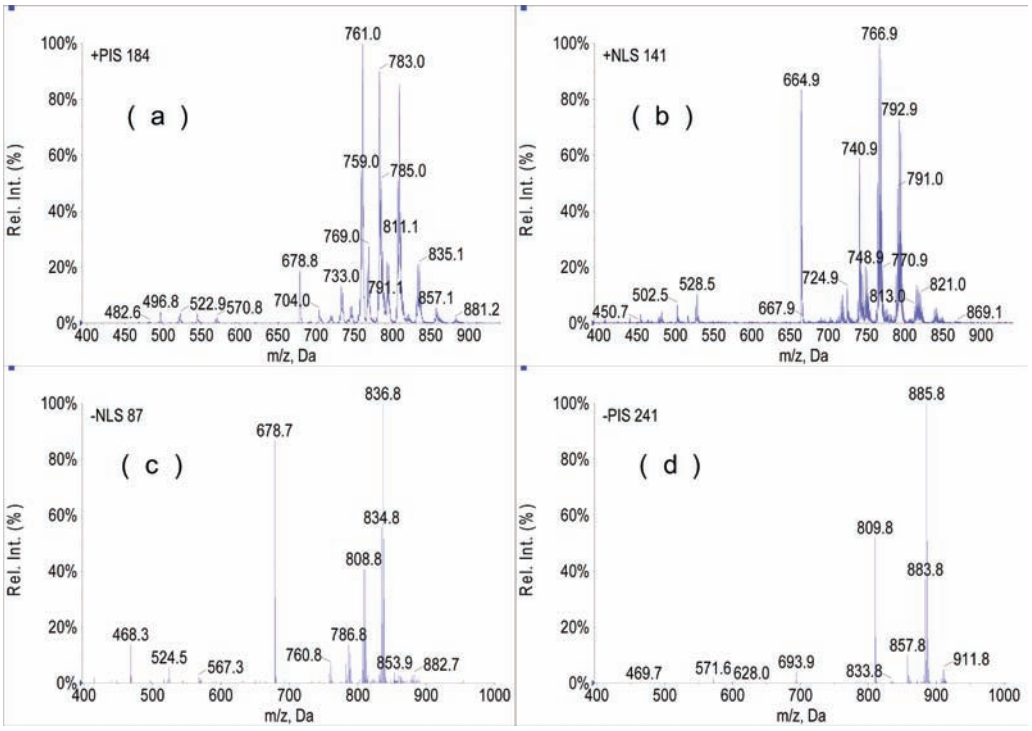

Figure 1. PIS and NLS mass spectrum of PL from *C. idellus* muscle: (a) PIS of *m/z* 184 in positive mode, identifying PC and SM species; (b) NLS of 141 Da in positive mode, identifying PE species; (c) PIS of *m/z* 241 in negative mode, identifying PI species; and (d) NLS of 87 Da in negative mode, identifying 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 (SM), PE, PI, and PS, respectively.

room-temperature storage by shotgun lipidomics. This study could be useful to further understand changes of PL profiling during storage for another material.

MATERIALS AND METHODS

Reagents and Materials. High-performance liquid chromatography (HPLC)-grade methanol (MeOH) and chloroform ($CHCl_3$) were purchased from Merck (Darmstadt, Germany). ISs, 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) and prepared in MeOH/$CHCl_3$ (1:1, v/v) to a final concentration of 1 μg/mL. Aqueous solutions were prepared using a Millipore Milli-Q system. *C. idellus* were newly caught and purchased from a local aquatic market (Jingjiang, Hangzhou, China).

Equipment. Analyses were performed on a 4000 Q-Trap triple quadrupole/linear ion trap (QqQLIT) 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 Analyst 1.5.1 software (AB Sciex, Toronto, Canada). The analysis was taken directly into the ion source by a sample pump (Harvard Pump 11 Plus Syringe Pump, Harvard Apparatus, Inc., Holliston, MA).

Lipid Extraction from Muscle. Muscle tissue was removed from carcasses and stored at room temperatures for 72 h. Samples were taken regularly and homogenized at 1500 rpm using an Ultra Turrax (T25, IKA-Werka, Germany). Lipids were extracted from muscle samples according to a modified version by the Bligh and Dyer[24] method. Briefly, about 0.1 g of muscle sample was mixed with 3 mL of $CHCl_3$/MeOH (2:1, v/v) solution in a glass tube and extracted by an ultrasonic cleaning device for 15 min. Then, 1 mL of water was added to separate the phase. After centrifugation at 3000*g* for 10 min, the lower organic phase was recovered and transferred to a new glass tube. The aqueous phase was re-extracted with 2 mL of $CHCl_3$ for another 2 times and then centrifuged as before. The organic phase was collected and evaporated under nitrogen. 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. ISs were added before sample injection.

MS/MS Parameters. The TurboIon-Spray interface was operated in both positive and negative ionization modes. Nitrogen was used as a curtain gas and nebulizer. The source parameters were the following: 5.5 and 4.5 kV capillary voltage in positive and negative modes, respectively; temperature, 425 °C; curtain gas, 10 psi; GS1, 25 psi; CAD, medium. The MS/MS experiments combining PIS and NLS were performed to monitor the different PL classes (Figure 1). 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). The scans were acquired in multiple-channel acquisition (MCA) mode, which improves the ion statistics by summing the number of scans.[25] The injected rate was set at 5 μL min^{-1}, and the MS scan range was from *m/z* 350 to 1150.

Identification and Quantitation of PLs. With the data acquired, we performed peak identification and correction for ^{13}C isotope effects using the LipidView software, version 1.1 (AB Sciex, Toronto, Canada). Only the peaks that comprise more than 0.5% intensity and a signal-to-noise ratio (SN) > 3 were identified and quantified. Assignment of fatty acid chain constituents was achieved by consulting with other results already published[16−21,26,27] (for PC and PE) or multidimensional PIS to monitor the formation of specific *m/z* product ions corresponding to deprotonated FA ions in negative-ion mode (for PS and PI). 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. PLs was quantified using normalization intensity of the corrected ions to selected ISs.

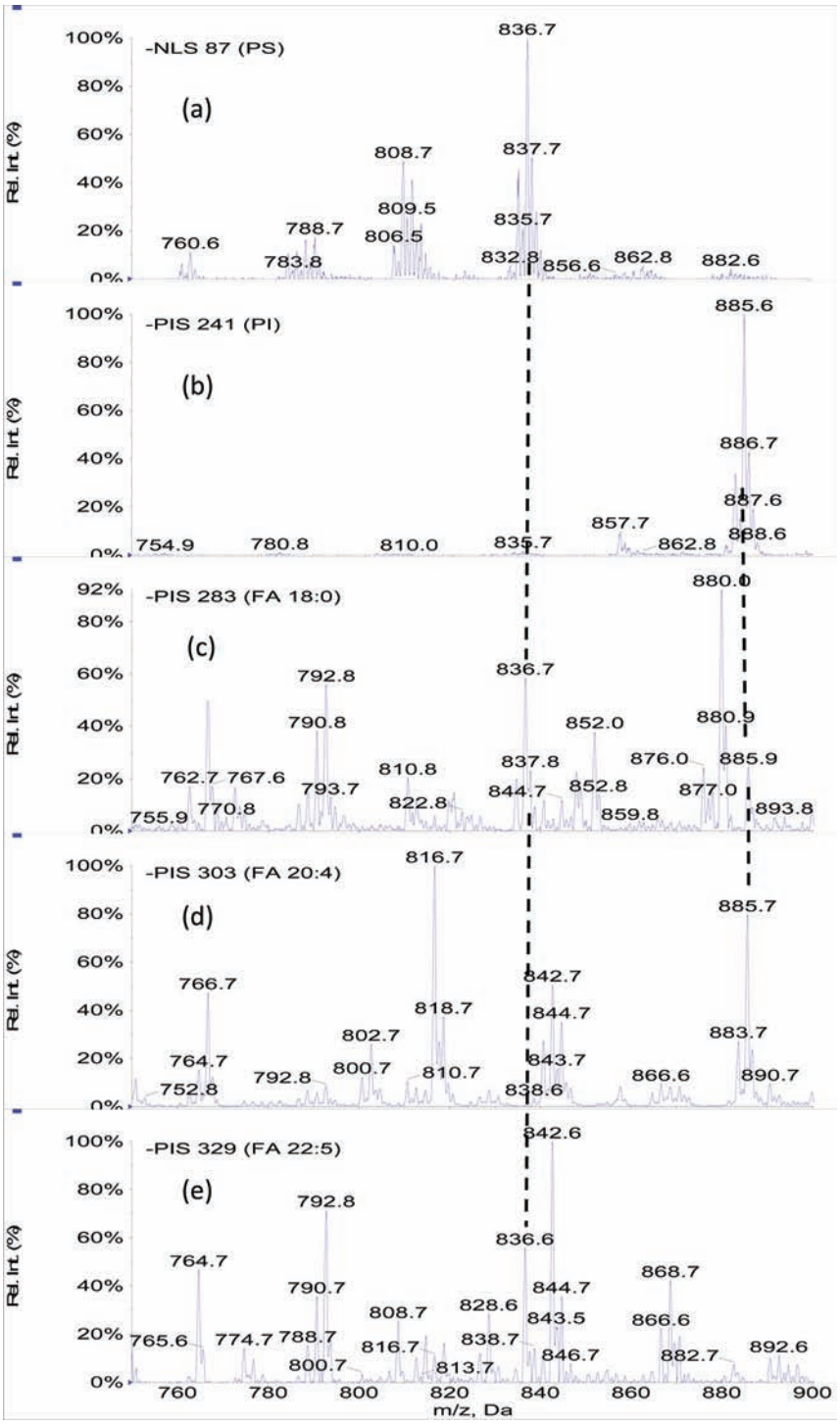

Figure 2. Multi-dimensional mass spectrometric analysis of PLs from *C. idellus* muscle: (a) NLS of 87 Da in negative mode (PS species), (b) PIS of *m/z* 241 in negative mode (PI species), (c) PIS of *m/z* 283 in negative mode (FA 18:0-containing PLs), (d) PIS of *m/z* 303 in negative mode (FA 20:4-containing PLs), and (e) PIS of *m/z* 329 in negative mode (FA 22:5-containing PLs).

RESULTS AND DISCUSSION

Identification and Quantitation of PL Molecular Species. Headgroup fragmentation scanning by PIS and NLS mass spectrum has been discussed in detail.[18,28] Each PL class shows fragmentation during CID at the phosphordiester linkage, resulting in a headgroup fragmentation.[17] For PC, scanning for precursor ions that produce the +*m/z* 184 from the lipid extracts will identify and characterize PC and SM molecules only because the headgroup fragmentation of PC is phosphocholine $[(HO)_2POOCH_2CH_2N(CH_3)_3^+$, +*m/z* 184]. Similar scans can be performed to detect PE, PI, and PS molecules. Figure 1 shows the PIS and NLS mass spectrum of the crude lipid extract from *C. idellus* muscle. The +PIS 184, +NLS 141, −PIS 241, and −NLS 87 scan modes were used to identify and characterize PC

Table 1. Molecular Species and Abundance (%) of LPLs from *C. idellus* Muscle

	LPC		LPE		LPS		LPI	
LPLs[a]	$[M + H]^+$	abundance	$[M + H]^+$	abundance	$[M - H]^-$	abundance	$[M - H]^-$	abundance
14:0	*b*	*b*	*b*	*b*	468.2	1.06 ± 0.05	*b*	*b*
16:0	496.3	1.53 ± 0.06	454.2	0.59 ± 0.05	496.3	1.29 ± 0.12	571.3	0.91 ± 0.08
18:2	520.3	0.31 ± 0.02	478.4	0.23 ± 0.02	*b*	*b*	*b*	*b*
18:1	522.3	0.51 ± 0.03	480.2	0.49 ± 0.04	522.3	1.09 ± 0.07	*b*	*b*
18:0	524.4	0.15 ± 0.01	482.2	0.56 ± 0.03	524.3	1.12 ± 0.11	599.4	0.48 ± 0.02
20:4	544.3	0.27 ± 0.02	502.3	0.64 ± 0.03	*b*	*b*	619.4	0.29 ± 0.03
20:3	546.3	0.13 ± 0.01	504.2	0.21 ± 0.02	*b*	*b*	*b*	*b*
20:1	*b*	*b*	508.4	0.14 ± 0.01	*b*	*b*	*b*	*b*
22:6	*b*	*b*	526.3	0.72 ± 0.05	568.3	1.43 ± 0.04	*b*	*b*
22:5	570.3	0.21 ± 0.01	528.3	1.17 ± 0.08	570.3	1.04 ± 0.01	*b*	*b*
22:4	*b*	*b*	530.3	0.14 ± 0.01	572.3	1.21 ± 0.09	*b*	*b*
sum of LPLs		3.11 ± 0.16		4.89 ± 0.34		8.24 ± 0.49		1.68 ± 0.13

[a] The LPL molecular species were shown by the format of *X*:*Y*, where *X* represents the number of total carbon atoms of the fatty acid chain and *Y* represents the number of total double bonds of the fatty acid chain. [b] Undetected.

(SM), PE, PI, and PS, respectively. 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 (SM), PE, PI, and PS, respectively. Using this method, at least 110 molecular species of PLs were identified, including 32 species of PC, 34 species of PE, 24 species of PS, 18 species of PI, and 2 species of SM. Samples were analyzed in triplicate, and the abundance shown was the percentage of the total PL classes detected, resulting in reproducible data with small standard deviations.

Figure 2 shows the multi-dimensional mass spectrometric analysis of PS and PI molecular species from *C. idellus* muscle. The fatty acid chain compositions were then determined by multi-dimensional PIS, which scan the deprotonated fatty acids ions in negative-ion mode. As shown, *m*/*z* 836.7 was detected in NLS 87, PIS 283, and PIS 329 in negative mode simultaneously. It indicated that the molecular species of this PS was PS 40:5 and the fatty acid chain compositions were 18:0/22:5. This led to the identification of this PS species as PS 18:0/22:5. The same was found for the *m*/*z* 885.6, and it was identified as PI 18:0/20:4. With this method, the fatty acid chain compositions (sn-1/sn-2) of PS and PI were confirmed and listed in Table 2.

Tables 1 and 2 list the molecular species and abundance of PL and lysophospholipid (LPL) classes from fresh *C. idellus* muscle. Obvious differences could be observed between the different PL classes about the major molecular species and abundance. Major molecular species of PC were PC 16:0/18:1, PC 16:0/20:4, and PC 18:0/20:5 (19.53, 15.05, and 14.38%, respectively). Major molecular species of PE were PE 18:0/20:5, PE 18:0/22:6, and PE 18:0/20:4 (18.49, 12.71, and 11.41%, respectively). Major molecular species of PS were PS 18:0/22:5, PS 16:0/20:4, and PS 18:0/20:5 (23.65, 11.53, and 10.25%, respectively). Major molecular species of PI were PI 18:0/20:4, PI 18:0/20:5, and PI 18:0/20:3 (54.43, 19.17, and 6.54%, respectively). The two molecular species of SM identified were SM d18:1/16:0 and SM d18:1/24:1 (not shown in the tables). Some LPL molecular species, such as lysophosphatidylcholine (LPC) 16:0 (1.53%), lysophosphatidylethanolamine (LPE) 22:5 (1.17%), and lysophosphatidylserine (LPS) 14:0 (1.04%) are detected in this study. The abundance of total LPLs in fresh muscle is relatively low, with LPC (3.11 ± 0.16%), LPE (4.89 ± 0.34%), LPS (8.24 ± 0.49%), and lysophosphatidylinositol (LPI, 1.68 ± 0.13%). It indicated that the main saturated fatty acid (SUFA) chains of PLs are myristic acid (FA 14:0), palmitic acid (FA 16:0), and stearic acid (FA 18:0), while the main unsaturated fatty acids are polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA, FA 22:6), eicosapentaenoic acid (EPA, FA 20:5), and arachidonic acid (ARA, FA 20:4). The fatty acid chain profiling of PL and LPL classes from *C. idellus* muscle was similar to some results already published.[5−9] Nevertheless, it is well-known[29] that the PL composition of a particular muscle sample depends upon many factors, such as animal species, feed compositions, rearing conditions, and intrinsic factors of muscle.

Changes of PC, PS, PI, and SM Profiling during Storage. Figures 3 and 4 show the content changes of PL and LPL classes from *C. idellus* muscle during the room-temperature storage. The content was quantified by normalizing the intensity of the acquired peak to the selected ISs after corrected ^{13}C isotope effects. The content of PL and LPL classes is expressed as nanograms of PL or LPL molecular species per milligram of fish muscle. It is shown that, in fresh *C. idellus* muscle, PC is the largest quantity of PLs (2172.16 ng/mg) and SM is the smallest (35.29 ng/mg), while PE (182.02 ng/mg), PI (201.03 ng/mg), and PS (227.36 ng/mg) lie in the middle. For LPLs, as discussed above, the content was very low, with the following breakdown: LPC (55.48 ng/mg), LPE (12.25 ng/mg), LPS (8.78 ng/mg), and LPI (2.03 ng/mg). This agrees well with some studies reported before.[7,11]

As shown in Figure 3, during storage at room temperature, the content changes of PLs (PE will be discussed later) from *C. idellus* muscle were similarly decreasing gradually overall. In the early storage time (t < 12 h), the content increased slightly, and in midterm storage time (12 < t < 52 h), the content decreased gradually, while PLs were almost exhausted in the late storage time (t > 52 h). LPLs have similar trends with PLs, but there is some slight differences. It can be seen from Figure 4 that LPLs gradually increased first, at the 24th hour, reached a maximum, and after that, decreased slowly. The increasing of PL content

Table 2. Molecular Species and Abundance (%) of PLs from *C. idellus* Muscle

	PC		PE		PS		PI	
sn-1/sn-2[a]	$[M + H]^+$	abundance	$[M + H]^+$	abundance	$[M - H]^-$	abundance	$[M - H]^-$	abundance
14:0/18:2	730.6	0.35 ± 0.03	*b*	*b*	*b*	*b*	*b*	*b*
16:0/16:1	732.6	2.12 ± 0.13	690.5	0.45 ± 0.02	*b*	*b*	*b*	*b*
16:0/16:0	734.6	1.32 ± 0.01	692.5	0.28 ± 0.02	*b*	*b*	*b*	*b*
14:0/20:4	754.6	0.52 ± 0.02	712.6	0.36 ± 0.02	*b*	*b*	*b*	*b*
14:0/20:3	756.6	1.28 ± 0.03	714.6	0.39 ± 0.03	*b*	*b*	*b*	*b*
16:0/18:2 and 16:1/18:1	758.7	7.82 ± 0.24	716.6	1.68 ± 0.10	758.5	1.84 ± 0.09	833.5	0.43 ± 0.02
16:0/18:1	760.7	19.53 ± 0.22	718.6	2.28 ± 0.21	760.5	1.96 ± 0.09	835.6	0.53 ± 0.04
16:0/18:0	*b*	*b*	*b*	*b*	*b*	*b*	837.6	0.38 ± 0.01
14:0/22:6	778.6	0.26 ± 0.01	*b*	*b*	*b*	*b*	*b*	*b*
16:0/20:5	780.7	2.19 ± 0.17	738.6	1.41 ± 0.06	*b*	*b*	855.5	0.32 ± 0.01
16:0/20:4	782.7	15.05 ± 0.49	740.6	9.42 ± 0.43	782.5	2.29 ± 0.02	857.6	5.14 ± 0.04
18:1/18:2 and 16:0/20:3	784.7	6.94 ± 0.26	742.6	3.12 ± 0.20	784.5	1.72 ± 0.04	859.5	1.39 ± 0.11
18:0/18:2 and 18:1/18:1	786.7	3.75 ± 0.10	744.6	2.37 ± 0.22	786.5	3.34 ± 0.21	861.5	0.93 ± 0.02
18:0/18:1	788.7	1.40 ± 0.09	746.7	0.83 ± 0.06	788.5	3.39 ± 0.03	*b*	*b*
a16:1/22:6[c] and p16:0/22:6[d]	790.7	0.65 ± 0.05	748.6	2.80 ± 0.14	790.6	1.46 ± 0.12	*b*	*b*
16:0/22:6	806.7	7.54 ± 0.03	764.6	10.88 ± 0.28	806.5	3.77 ± 0.11	881.5	1.96 ± 0.07
18:0/20:5	808.7	14.38 ± 0.44	766.6	18.49 ± 0.32	808.5	10.24 ± 0.21	883.6	19.17 ± 0.28
18:0/20:4	810.6	4.42 ± 0.08	768.7	11.41 ± 0.52	810.5	8.94 ± 0.76	885.5	54.43 ± 0.13
18:0/20:3	812.7	0.95 ± 0.04	770.7	2.20 ± 0.07	812.5	3.83 ± 0.21	887.4	6.54 ± 0.45
18:0/20:2	*b*	*b*	772.7	0.53 ± 0.05	814.6	2.54 ± 0.10	889.5	1.70 ± 0.15
18:1/20:0 and 18:0/20:1	816.7	0.22 ± 0.02	774.7	0.75 ± 0.06	*b*	*b*	*b*	*b*
a18:1/22:6 and p18:0/22:6	818.7	0.40 ± 0.01	776.7	0.79 ± 0.05	*b*	*b*	*b*	*b*
18:0/22:6	834.7	3.60 ± 0.06	792.7	12.71 ± 0.32	834.5	11.53 ± 0.68	909.5	2.32 ± 0.02
18:0/22:5	836.8	1.43 ± 0.10	794.7	8.97 ± 0.44	836.5	23.65 ± 0.16	911.7	2.50 ± 0.20
18:0/22:4	838.7	0.29 ± 0.02	796.7	0.80 ± 0.04	838.6	6.19 ± 0.52	913.4	0.57 ± 0.03
20:0/22:6	862.7	0.34 ± 0.03	820.7	1.60 ± 0.05	862.6	3.05 ± 0.27	*b*	*b*
20:0/22:5	864.7	0.12 ± 0.01	822.7	0.57 ± 0.03	864.6	2.04 ± 0.11	*b*	*b*

[a] Fatty acid chain constituents (sn-1/sn-2) were confirmed by referring to other results already published (for PC and PE) or multi-dimensional PIS (for PS and PI). [b] Undetected. [c] In the notation a16:1/22:6, a means alkyl linkage (1-*O*-alkyl or plasmanyl). [d] In the notation p16:0/22:6, p means plasmalogen (1-alk-1′-enyl or plasmenyl) linkage.[26,27]

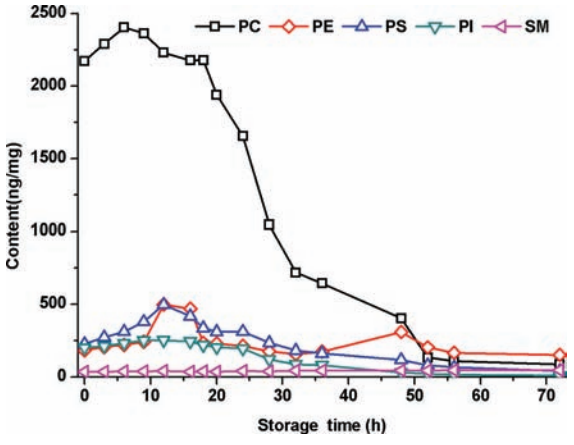

Figure 3. Content changes of PL classes (PC, PE, PS, PI, and SM) from *C. idellus* muscle during room-temperature storage.

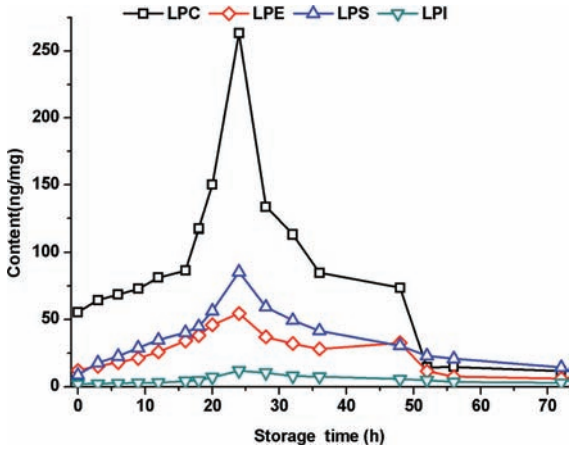

Figure 4. Content changes of LPL classes (LPC, LPE, LPS, and LPI) from *C. idellus* muscle during room-temperature storage.

during the early storage time may be due to the decomposition of the biofilm in the muscle tissue. As well-known, PL is the constitute unit of the biological membrane in cells.[10] It includes not only the cell membrane externally but also the organelles within the biofilm. During the storage, with the gradual muscle autolysis, the biofilm starts to decompose and release PLs. However, because PLs in the cell organelles are marginal, the increment is limited.

Relevant studies have shown that two distinct reactions in fish for PL deterioration are oxidation and hydrolysis, caused by a non-enzymatic effect or catalyzed by microbial and intracellular digestive enzymes from the fish themselves.[2] For one thing, the large amount of PUFAs found in PLs (Tables 1 and 2) makes them highly susceptible to oxidation by an autocatalytic mechanism. For another, phospholipase could play an important role in hydrolysis of the PLs. Phospholipases in animal cells mainly include phospholipase A1, phospholipase A2, phospholipase C, and phospholipase D. Phospholipase A1 and phospholipase A2 specifically catalyze the hydrolysis of ester bonds at sn-1 and sn-2, releasing FFA and LPLs.[31,32] During the midterm and late storage times, the reason for the rapid decline for PL content may be mainly due to the occurrence of the phospholipase-catalyzed hydrolysis and oxidation reactions. LPLs come from the hydrolysis of PLs, and their content may be affected by the deterioration of PLs. For this reason, the content of LPLs increased first and reached a maximum after 24 h, when the PLs had started to decrease rapidly (Figures 3 and 4). However, noticeably, the content of SM increased unobviously during the whole storage (CV < 10%). It suggested that SM may be relatively stable to the phospholipase effect.

Changes of PE Profiling during Storage. The changes of PE profiling were different from other PLs (PC, PS, PI, and SM) during the storage. It can be seen from Figure 5 that, before the 9th hour, the content increased slightly. After that, it started to increase suddenly and then decreased sharply during the next 9 h. After 13.5 h, the content reached a maximum, which was 2.5 times more than the original. Then, it decreased gradually. However, some low abundant PE molecular species, such as PE 32:1, PE 34:2, or PE 34:1, show a gradual upward trend in content during the storage. The increase of the PE content at the initial 13.5 h may be due to the decomposition of the biofilm and the demethylation of PC or the decarboxylation of PS,[35] but the repeated increase is not because the molecular species of the latter were totally different from the original. Many previous studies[30−33] only focused on the changes of the total content of PL fractions without describing any changes of individual PL molecular species; therefore, this phenomenon had never been discovered and discussed before.

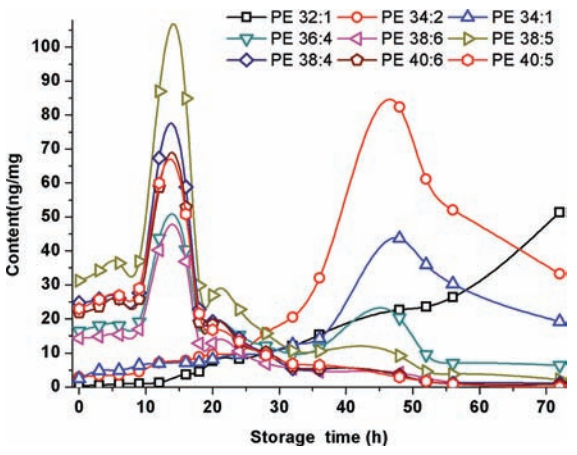

Figure 5. Content changes of major PE molecular species (content > 25 ng/mg) from *C. idellus* muscle during room-temperature storage.

The specific PE molecular species emerging during the storage were confirmed by mass spectrum (Figure 6). The molecular species of PE 32:1, PE 34:2, and PE 34:1 were confirmed simultaneously by NLS 141 in positive-ion mode (Figure 6a) and PIS 196[28] (Figure 6b). Figure 6c shows the fragmentation spectrum of m/z 714.7 (PE 34:2) in negative mode. The ion m/z 281 indicates the 18:1 fatty acid chain, and the ion m/z 253 indicates the 16:1 fatty acid chain, which fragmentated from glyceride bonds at sn-1 and sn-2 positions. It shows that the fatty acid chain of those PE molecular species (sn-1/sn-2) are 16:0/16:1 (PE 32:1), 16:1/18:1 (PE 34:2), and 16:0/18:1 (PE 34:1). As we know, the lipids of fish include up to 40% long-chain fatty acids (14−22 carbon atoms), while the majority are highly unsaturated (more than three double bonds).[2] Because the molecular species of PE have varied from the flash fish muscle, we infer they may come from the microbe breeding in the muscle. This result is in good agreement with published results by some researchers,[34,35] who found that the major PE molecular species in membranes of *Salmonella enterica*, *Bacillus thuringiensis*,[34] and *Escherichia coli*[35] were PE 16:0/16:1, PE 16:1/18:1, and PE 16:0/18:1. The possible relation between these special PE molecular species and microbe species will be investigated in further studies.

In summary, the direct infusion combined with ESI−MS/MS was proven to be an effective method for qualitative and quantitative analyses of PLs from the *C. idellus* muscle. It can provide information regarding not only the fatty acid chain

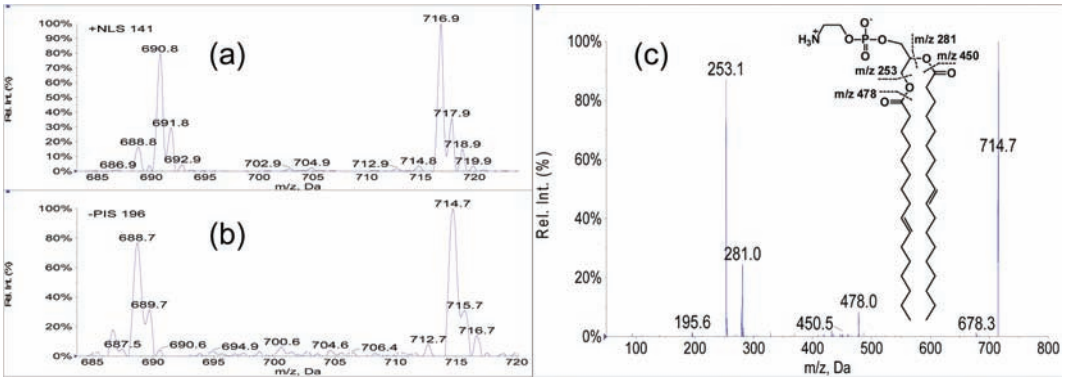

Figure 6. Mass spectrum of the emerging PE molecular species during the storage. (a) NLS of 141 Da in positive mode, with the ions m/z 716.9 and 690.8 indicating PE 34:2 and PE 32:1, respectively. (b) PIS for m/z 196[28] in negative mode, with the ions m/z 714.7 and 688.7 indicating PE 34:2 and PE 32:1, respectively. (c) Fragmentation spectrum of the ion m/z 714.7 in negative mode. The ion m/z 281 indicates the 18:1 fatty acid chain, and the ion m/z 253 indicates the 16:1 fatty acid chain, fragmentated from glyceride ester bonds in sn-1 and sn-2.

compositions but also their relative positions (sn-1/sn-2) in individual PL classes. It indicated that oxidation and hydrolysis are the two main causes for the deterioration of PLs in fish muscle during storage. Most content of PL molecular species increased and then decreased gradually. However, some special PE molecular species with former low abundance, such as PE 16:0/16:1, PE 16:1/18:1, and PE 16:0/18:1, emerged in abundance during the storage. We inferred that those PE molecular species may come from the microbe breeding in the muscle, and they could be used as an assessment of fish quality. This study is useful to further understand changes of PLs during storage at room temperature.

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ABBREVIATIONS USED

PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; LPL, lysophospholipid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; LPI, lysophosphatidylinositol; FFA, free fatty acid; PUFA, polyunsaturated fatty acid; ESI−MS/MS, electrospray ionization tandem mass spectrometry; NLS, neutral loss scan; PIS, precursor ion scan

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