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Characterization of Phospholipid Molecular Species in the Edible Parts of Bony Fish and Shellfish

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ABSTRACT: The phospholipid molecular species of freshwater (pangasius, Nile perch, trout), marine fish fillets (horse mackerel, European hake, common sole, European anchovy, European pilchard, Atlantic mackerel) and the edible muscle foot of bivalves (clam, mussel, oyster) commonly available in the Italian market during spring and summer were characterized by means of normal-phase high performance liquid chromatography coupled online with positive electrospray ionization ion-trap tandem mass spectrometry. From principal component analysis (PCA), it was observed that the total fatty acid profile was not suitable to differentiate among the shellfish genera. The fatty acid molecular combinations of phosphatidylcholine, the main phospholipid class, as well as phosphatidylinositol and phosphatidylethanolamine allowed for the differentiation of shellfish from the bony fishes. Phosphatidylserine and phosphatidylethanolamine plasmalogen profile allowed for the discrimination of each bony fish or shellfish genus since PS and pPE classes included a large number of fatty acid combinations that were specific for a fish genus or group.

KEYWORDS: *phospholipid molecular species, high performance liquid chromatography, marine and freshwater fish, shellfish, ω3 fatty acids*

■ INTRODUCTION

The positive effects of the consumption of fishery products on certain chronic diseases[1] are well-known since several decades and are due to the abundant concentration of long chain (LC) ω3 PUFAs, such as eicosapentaenoic acid (C20:5ω3, EPA), docosapentaenoic acid (C22:5ω3, DPA), and docosahexaenoic acid (C22:6ω3, DHA). The inability of some subjects (e.g., elderly people) to efficiently desaturate and chain elongate linolenic acid (C18:3ω3, ln) to EPA, DPA, and DHA makes the ω3 LC-PUFAs dietarily important.[2]

Polar lipids of fish include phospholipids (PLs), which are richer in ω3-PUFAs than neutral lipids (NL)[3,4] due to their functional role. The lipid composition of fish depends on several factors, such as the species, the environmental conditions of growth, the feeding composition, and the freshness of the product.[5] Consequently, the characterization of the phospholipid classes is a powerful tool in order to evaluate the nutritional properties of the fish products and of the nutraceutical or functional foods containing fish ingredients[6] or liposomes.[7] Moreover, the increased commercial use of phospholipids as ingredients for functional food, baby food, and pet food has led to the development of specific analytical methods in order to separate and identify phospholipids.[8−10] PLs extracted from fish products can play a pivotal role as an innovative food ingredient.[11,12] Recently, a lipid extract rich of ω3-containing PLs from krill (*Euphausia superba*) has been authorized by the European Commission as a novel food/food ingredient.[13]

The PL amount and the percentage of each PL class in fish have been already reported by several authors.[14,15] Instead, the characterization of the molecular species of each PL class is a still unexplored subject for many fish species.

In the present work, the molecular species of phosphatidylethanolamine (PE), plasmalogen of phosphatidylethanolamine (pPE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC) were characterized in some freshwater, marine fish and shellfish commonly found in the Italian retails' market during spring and summer. The total lipid extract was directly analyzed by using high performance liquid chromatography coupled online with electrospray ionization ion-trap tandem mass spectrometry (HPLC-ESI-MS/MS) without a preseparation step. The fragmentation of phospholipids produced the ion resulting from the loss of the polar headgroup and the ion corresponding to one of the fatty acid moieties. The phospholipid composition of different fish species and the presence of chemical markers for a peculiar species of fish was evaluated with the approach of the multivariate statistical analysis (PCA).

■ MATERIALS AND METHODS

Materials. Chloroform and methanol were HPLC grade from Lab-Scan (Dublin, Irland); ammonia solution (30%) of analysis grade was from Carlo Erba (Milano, Italy). All other chemicals, with noted exceptions, were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). PL standards (purity greater than 99%) included phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC) and were purchased from Sigma.

Samples. Three farmed freshwater fish species (Nile perch, *Lates niloticus*, n; trout, *Salmo trutta*, t; pangasius, *Pangasius hypophthalmus*, g), six fish species from the Adriatic sea (horse mackerel, *Trachurus trachurus*, m; European hake, *Merluccius merluccius*, h; common sole, *Solea vulgaris*, s; European anchovy, *Engralius encrasicolus*, a; European

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pilchard, *Sardina pilchardus*, p; Atlantic mackerel, *Scomber scombrus*, k), and three types of marine molluscs (clam, *Venus gallina*, c; mussel, *Mytilus galloprovincialis*, u; oyster, *Ostrea edulis*, y) were selected and purchased in local fish markets in Ancona (Italy).

The sampling was conducted in three different months: May, June, and July 2007. For each month, three individuals of g, n, t, m, h, s, and k were sampled together with ten individuals of p and a. The edible part of the fishes was isolated (except g, which was already purchased as fish fillet). The marine molluscs were also sampled in May, June, and July 2007; an amount of 100 g of edible part of each species was isolated each of the three months.

Lipid Extraction. The edible parts of the fishes and molluscs belonging to the same species and purchased each month were combined. An aliquot (30 g) of the edible part of each fish (fillet) and shellfish (muscle) was homogenized in chloroform−methanol (1:2, v/v) and used for lipid extraction according to Bligh and Dyer.[16]

Total Fatty Acid Analysis. Fatty acids methyl esters (FAMEs) were obtained from total lipids according to Suter at al.[17] The analysis was performed by means of gas chromatography using a CP-9002 apparatus (Chrompack, NL), equipped with a flame ionization detector (FID) and a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm i.d., film thickness 0.2 μm, Chrompack). The sample was injected on a split−splitless system. The carrier gas was helium at a flow rate of 1.6 mL min^{-1}. The temperature of the detector was 230 °C; the injector temperature was maintained at 60 °C for 6 min and then raised to 225 °C at a rate of 20 °C min^{-1}. Temperature programming started at 55 °C for 3 min, then raised to 140 °C at a rate of 4 °C min^{-1}, was held for 1 min, increased again to 225 at 2 °C min^{-1}, and was held at 225 °C for 30 min. Peaks were identified by comparison with known standard mixtures by Sigma (PUFA No. 1, Marine Source, Supelco 37, C4−C24 Unsaturates and C4−C24 Saturates FAMEs).

HPLC-MS/MS Conditions. The total lipid fraction was dissolved in chloroform−methanol (2:1, v/v), in order to obtain a 2−3 mg/mL solution, which was analyzed by means of HPLC. The HPLC-ESI-MS/MS method has been already reported in a previous work.[18] The HPLC system consisted of a pump module (Jasco PU-980) and a ternary gradient module (Jasco LG-980-02, Tokyo, Japan). The column was a Polaris Si-A 3 μ 150 mm × 4.6 mm (Varian, Middelburg, NL) protected with a Silica precolumn (4 mm × 3.0 mm i.d.) from Phenomenex (Torrance, USA). A gradient of solvent A [$CHCl_3$/MeOH/NH_4OH (30%) 70:25:1, v/v] and solvent B [$CHCl_3$/MeOH/H_2O/NH_4OH (30%) 60:40:5.5:0.5, v/v] was used. The gradient started at 100% of A, decreased to 0% A (100% B) in 10 min, then was held for 15 min; and then reached back 100% A in 5 min. The flow rate was 1.0 mL/min and the injection loop was 5 μL. The HPLC system was coupled to an LCQ ion-trap mass spectrometer (Finnigan, San Jose, CA, USA). The mass detector was equipped with an electrospray ionization source (ESI). The steel ionization needle was set at 5.0 kV, and the heated capillary was set to 200 °C. The sheath gas flow was approximately 90 arbitrary units. The ion source and the ion optic parameters were optimized with respect to the positive molecular related ions of the phospholipids standards. The molecular mass peaks from the HPLC effluent were detected using positive ion full-scan ESI-MS analysis. Mass resolution was 0.1 Da. Tandem mass (MS^2 or MS/MS) experiments were carried out with a relative collision energy of 45%. The integration was performed with the Interactive Chemical Information System (ICIS) peak detection algorithm software provided by Finnigan after correction for the contribution from the ^{13}C isotope effect.

Statistical Analysis. The statistical analysis was performed by using a multivariate statistical approach (Principal Component Analysis, PCA) through The Unscrambler software (CAMO, Corvallis, OR, USA). This has enabled to get an overall overview of the differences in the fatty acid composition among the different fish groups (marine, freshwater fish and shellfish) and fish genus. Successively, the data were statistically analyzed by using ANOVA carried out with GraphPad InStat ver.3.0 system (GraphPad Software, San Diego, CA, USA) in order to point out significant differences among the samples. Since the homogeneity of variance was confirmed, the Tukey−Kramer's test was used for comparison of the means among different fish species. Significance was accepted at a probability of 0.05 ($P < 0.05$), according to the MSD (minimum significant differences) test.

RESULTS AND DISCUSSION

Fatty Acid Profile. The fat content and the total fatty acid composition of each fish and mollusc species are reported in Table 1. The mean value and standard deviation (SD, $n = 3$) are given for each component.

Figure 1 shows the biplot reporting the loadings of the fatty acids and the scores of the samples collected in three different months (5 = May, 6 = June, 7 = July). The first principal components (PC1 and PC2) accounted for 76% (60 and 16%, respectively) of the total variance of the model.

The distribution of the fish samples in the biplot showed that the fatty acids pattern is strictly related to the environment (marine or freshwater). The fatty acids profile of freshwater fish showed high variability and was different from the fatty acid composition of sea fish and shellfish; the samples of freshwater fish were more spread in the plot than sea fishes and shellfishes. Pangasius (g5, g6, and g7) was located in the bottom/left quadrant away from the axes origin; however, Nile perch (n5, n6, and n7) was located in the same quadrant as g but near the origin. The trout (t5, t6, and t7) samples were positioned in the top/left quadrant, while European hake (h5, h6, and h7), horse mackerel (m5, m6, and m7), European anchovy (a5, a6, and a7), and Atlantic mackerel (k5, k6, and k7) were placed in the top/right quadrant. European pilchard (p5, p6, and p7) and common sole (s5, s6, and s7) were the marine fish with a fatty acid pattern more similar to that of n and molluscs and were clustered in an intermediate area of the biplot. All the mollusc samples (c, u, and y) were located in bottom/right quadrant.

To the authors' best knowledge, the fish species combination of the present experimental plan has never been analyzed so far and obviously influenced the overall PCA results.

The distribution of the variables (Figure 1) showed that EPA (e), DHA (d), oleic (o), and linoleic (l) acid explained most of the variance of the model.

The fatty acid profile of pangasius was markedly different from all the other fishes because it had the highest content of oleic acid and the lowest content of EPA and DHA (Table 1). The trout resulted richer in linoleic acid (l) than all the other fishes and poor of DHA. The samples of n and g showed a relative high content of saturated fatty acids (palmitic and stearic acids).

The sole (s) was characterized by a lower amount of DHA with respect to all the other sea fish and was richer of EPA, as well as the molluscs.

The results obtained by PCA analysis were confirmed with ANOVA (Table 1). Pangasius resulted significantly richer in MUFA, particularly in oleic acid (35.3 ± 1.6%), and poorer in DHA (2.0 ± 0.4%) than all the other samples.

The samples of n, p, and s displayed a content of DHA equal to palmitic acid, whereas the other sea fish species and molluscs showed that the DHA was the preponderant fatty acid. In addition, sea fish species, except s and p, resulted in being significantly richer in DHA with respect to all freshwater and molluscs species. Conversely, the EPA level in most of the molluscs resulted higher (significantly higher in oysters and mussels) than the bony fish species.

European anchovy presented the amount of ω3 PUFA (51.2 ± 2.2%) significantly higher than all the other species, except h and k.

Separation of the Phospholipid Classes. The total lipid fraction was injected in HPLC-MS/MS without prior cleanup in order to reduce the analysis time and the solvent amount and

Table 1. Lipid Content (on Fresh Weight) and Fatty Acid Composition (Weight % of Total Fatty Acids) of Fish Fillet and Shellfish Edible Parts[a]

	sea fish						freshwater fish			shellfish		
	European hake (h)	horse mackerel (m)	common sole (s)	European anchovy (a)	European pilchard (p)	Atlantic mackerel (k)	Nile perch (n)	pangasius (g)	trout (t)	clam (c)	mussel (u)	oyster (y)
lipid content (%)	1.0 ± 0.3	0.8 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	4.1 ± 1.2	1.0 ± 0.2	0.6 ± 0.3	1.0 ± 0.3	4.4 ± 1.0	1.1 ± 0.5	1.3 ± 0.7	2.2 ± 0.8
fatty acid (%)												
14:0	2.1 ± 0.4	2.3 ± 0.6	2.9 ± 0.8	3.2 ± 0.3	5.2 ± 0.7	1.7 ± 0.6	1.6 ± 0.4	3.3 ± 0.4	1.5 ± 0.1	1.9 ± 0.1	3.6 ± 0.8	2.8 ± 0.1
15:0	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.3	0.8 ± 0.0	0.9 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
16:0	18.3 ± 0.3	19.2 ± 0.5	15.9 ± 0.9	21.6 ± 0.6	22.2 ± 0.6	19.4 ± 0.2	22.2 ± 0.7	29.4 ± 0.3	13.7 ± 1.0	16.3 ± 0.5	20.5 ± 2.3	18.0 ± 2.4
17:0	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	1.9 ± 0.3	1.2 ± 0.1	1.3 ± 0.1
17:0-*iso*	1.1 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	tr	tr	0.2 ± 0.0	1.0 ± 0.1	tr	tr
18:0	6.2 ± 1.0	9.1 ± 0.4	5.0 ± 0.3	5.1 ± 0.1	4.9 ± 0.3	7.9 ± 0.4	9.6 ± 0.3	9.7 ± 0.0	4.1 ± 0.3	5.7 ± 0.3	4.2 ± 0.3	5.5 ± 0.0
20:0	0.5 ± 0.0	0.6 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.8 ± 0.1	0.5 ± 0.2	0.2 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	tr	tr
total SFA	28.5 ± 0.8d,e,f,g	32.7 ± 1.2b,c,d	25.9 ± 1.1g	32.1 ± 0.6b,c,d,e	35.0 ± 2.0b,c	31.0 ± 1.0c,d,e	35.2 ± 1.1b	43.0 ± 0.8a	20.7 ± 2.2h	26.1 ± 0.5f,g	30.3 ± 2.1d,e,f	28.2 ± 2.7e,f,g,
16:Δ9	2.9 ± 0.4	2.4 ± 0.4	9.0 ± 0.3	2.2 ± 0.1	4.2 ± 0.4	2.0 ± 0.3	5.1 ± 1.0	1.1 ± 0.2	2.3 ± 0.3	3.1 ± 0.2	6.4 ± 0.3	1.9 ± 0.2
16:1-*iso*	0.5 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	0.6 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.2 ± 0.1	1.1 ± 0.1	tr	0.3 ± 0.1
18:1Δ9-*trans*	0.1 ± 0.0	tr	0.5 ± 0.4	tr	0.1 ± 0.1	tr	tr	tr	tr	0.5 ± 0.1	0.5 ± 0.0	0.6 ± 0.1
18:1Δ9-*cis*	8.8 ± 1.3c,d	8.8 ± 1.9c,d	10.6 ± 1.6c	4.5 ± 0.9d,e,f	8.0 ± 1.3c,d,e	6.5 ± 1.5c,d,e,f	9.9 ± 0.6c	35.3 ± 1.6a	16.9 ± 2.9b	3.9 ± 1.3e,f	3.0 ± 0.5f	3.9 ± 1.5e,f
18:1Δ11	3.0 ± 0.1	2.7 ± 0.3	2.9 ± 0.1	2.6 ± 0.0	2.6 ± 0.3	3.6 ± 0.3	4.1 ± 0.5	1.4 ± 0.1	2.4 ± 0.3	2.5 ± 0.8	2.4 ± 0.1	3.2 ± 1.0
20:1Δ11	0.7 ± 0.1	1.1 ± 0.2	2.6 ± 1.5	0.7 ± 0.1	1.1 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	1.4 ± 0.1	2.6 ± 0.2	0.5 ± 0.0
20:1-*iso*	1.2 ± 0.3	0.8 ± 0.2	tr	tr	1.9 ± 0.3	0.7 ± 0.1	1.4 ± 0.4	0.5 ± 0.0	2.8 ± 0.6	3.9 ± 0.6	3.3 ± 0.6	5.2 ± 0.5
total MUFA	17.2 ± 1.4c,d	16.1 ± 2.7c,d,e	26.3 ± 3.5b	10.2 ± 1.0e	18.4 ± 2.6c,d	13.8 ± 1.0d,e	21.4 ± 0.6b,c	40.1 ± 1.7a	19.9 ± 1.5b,c	16.4 ± 3.4c,d,e	18.1 ± 0.4c,d	15.6 ± 1.2c,d,e
18:2Δ9,12 [ω6]	1.3 ± 0.2	1.6 ± 0.4	0.9 ± 0.1	1.4 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	1.7 ± 0.2	8.2 ± 1.0	27.4 ± 1.0	1.3 ± 0.7	2.4 ± 0.1	2.0 ± 0.2
18:3Δ6,9,12 [ω6]	tr	tr	tr	tr	tr	tr	0.2 ± 0.1	0.2 ± 0.2	0.3 ± 0.1	tr	tr	tr
18:3Δ9,12,15 [ω3]	0.2 ± 0.1	0.4 ± 0.1	2.8 ± 0.2	0.7 ± 0.1	tr	tr	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	4.1 ± 1.0	2.7 ± 0.4	2.1 ± 0.6
18:4Δ6,9,12,15 [ω3]	0.7 ± 0.3	0.7 ± 0.1	0.3 ± 0.2	0.8 ± 0.2	2.3 ± 0.3	0.6 ± 0.2	0.2 ± 0.1	Tr	0.5 ± 0.2	0.9 ± 0.3	2.7 ± 0.4	2.9 ± 0.1
20:2Δ11,14 [ω6]	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.5 ± 0.2	1.3 ± 0.2	1.0 ± 0.1	0.6 ± 0.1	
20:4Δ5,8,11,14 [ω6]	2.4 ± 0.3	2.1 ± 0.3	2.6 ± 0.7	1.5 ± 0.2	1.1 ± 0.2	2.7 ± 0.4	4.9 ± 0.8	2.2 ± 0.5	0.7 ± 0.2	3.7 ± 0.9	2.3 ± 0.5	2.9 ± 0.6
20:5Δ5,8,11,14,17 [ω3], EPA	8.7 ± 0.2c,d	6.2 ± 0.4b,c	11.3 ± 3.1d,e	7.5 ± 0.3b,c,d	9.7 ± 0.8c,d	7.8 ± 0.2b,c,d	3.8 ± 1.3a,b	0.5 ± 0.1a	4.0 ± 2.6a,b	8.8 ± 0.6c,d	13.6 ± 0.9e,f	16.4 ± 1.9f
22:2	tr	0.6 ± 0.1	tr	tr	0.8 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	tr	0.6 ± 0.2	5.1 ± 0.8	2.6 ± 0.5	4.4 ± 1.5
22:4Δ7,10,13,16 [ω6]	0.4 ± 0.1	0.5 ± 0.2	1.1 ± 0.2	tr	tr	0.2 ± 0.1	1.0 ± 0.3	0.3 ± 0.1	0.1 ± 0.0	1.3 ± 0.3	0.3 ± 0.0	tr
22:5Δ4,7,10,13,16 [ω6]	1.2 ± 0.1	1.7 ± 0.2	0.7 ± 0.6	1.2 ± 0.1	0.7 ± 0.1	1.5 ± 0.1	3.0 ± 0.2	1.1 ± 0.2	tr	2.4 ± 0.7	1.0 ± 0.3	1.1 ± 0.3
22:5Δ7,10,13,16,19 [ω3] DPA	1.5 ± 0.2	2.6 ± 0.4	7.9 ± 0.5	0.9 ± 0.1	1.3 ± 0.0	1.9 ± 0.1	5.3 ± 0.8	0.5 ± 0.1	1.7 ± 0.9	3.2 ± 0.4	1.4 ± 0.4	1.2 ± 0.1
22:6Δ4,7,10,13,16,19 [ω3]DHA	35.2 ± 0.4a	33.3 ± 2.8a,b	16.2 ± 2.7c,e	41.4 ± 1.9a	25.8 ± 4.0b,f	36.5 ± 1.0a	22.1 ± 1.0e,f	2.0 ± 0.4d	17.0 ± 2.3e,f	23.6 ± 0.8f	23.0 ± 1.0f,c	22.0 ± 1.2f,c
total PUFA	52.0 ± 0.9a,b	50.1 ± 3.7a,b	44.3 ± 1.6b,c,d	55.7 ± 2.1a	43.7 ± 4.4b,c,d	53.3 ± 1.3a	42.6 ± 1.8d	15.4 ± 2.7e	53.8 ± 2.7a	55.3 ± 3.7a	51.6 ± 2.1a,b	55.0 ± 3.8a
other peaks	1.3 ± 0.4	2.9 ± 1.0	4.4 ± 2.7	1.1 ± 0.4	1.7 ± 0.1	1.2 ± 1.0	0.8 ± 0.4	1.5 ± 0.5	0.9 ± 0.4	1.3 ± 0.7	0.1 ± 0.0	1.2 ± 0.7
ω6 PUFA	5.8 ± 0.1	5.4 ± 0.2	5.7 ± 2.0	4.4 ± 0.1	3.9 ± 0.4	6.2 ± 0.6	10.8 ± 1.6	12.3 ± 2.2	29.5 ± 1.5	9.6 ± 1.3	6.6 ± 0.6	6.1 ± 1.7
ω3 PUFA	46.2 ± 0.8a,b	43.1 ± 3.5b,c	38.5 ± 1.5c	51.2 ± 2.2a	39.0 ± 4.2c	46.8 ± 0.8a,b	31.5 ± 0.7d	3.0 ± 0.5e	23.7 ± 3.9f	40.5 ± 1.4b,c	42.4 ± 2.2b,c	44.5 ± 1.7b,c
ω3/ω6	8.0	8.0	6.7	11.6	10.0	7.5	2.9	0.2	0.8	4.2	6.4	7.3

[a]SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Results represents means ± standard deviation (n = 3); different letters (a,b,c,d,e,f) denote significant differences ($P < 0.05$), according to the MSD (minimum significant differences) with respect to the other species; tr, lower than 0.1%. Cm:n Δx; m = number of carbon atoms, n = number of double bonds, x = position of double bonds.

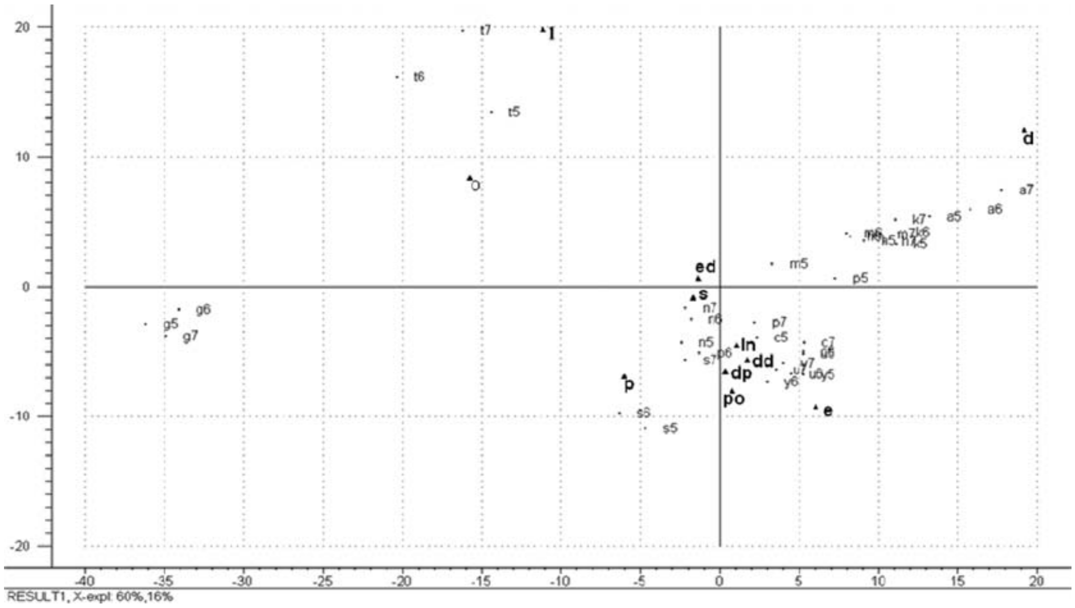

Figure 1. Principal component analysis (PCA) of the total fatty acid composition of fish fillet and shellfish edible parts. Each fish sample is marked with a dot (·) and abbreviated as follows: g5-g7, pangasius; n5-n7, Nile perch; t5-t7, trout; c5-c7, clam; u5-u7, mussel; y5-y7, oyster; k5-k7, Atlantic mackerel; s5-s7, common sole; a5-a7, European anchovy; h5-h7, European hake; p5-p7, European pilchard; m5-m7, horse mackerel. Each variable (fatty acid) is marked with a triangle (▲) and abbreviated as follows: p, C16:0; po, C16:1Δ9; s, C18:0; o, C18:1Δ9; l, C18:2ω6; ln, C18:3ω3; em, C20:1; ed, C20:2Δ11,14; a, C20:4ω6; dd, C22:2; e, C20:5ω3; dp, C22:5ω3; d, C22:6ω3.

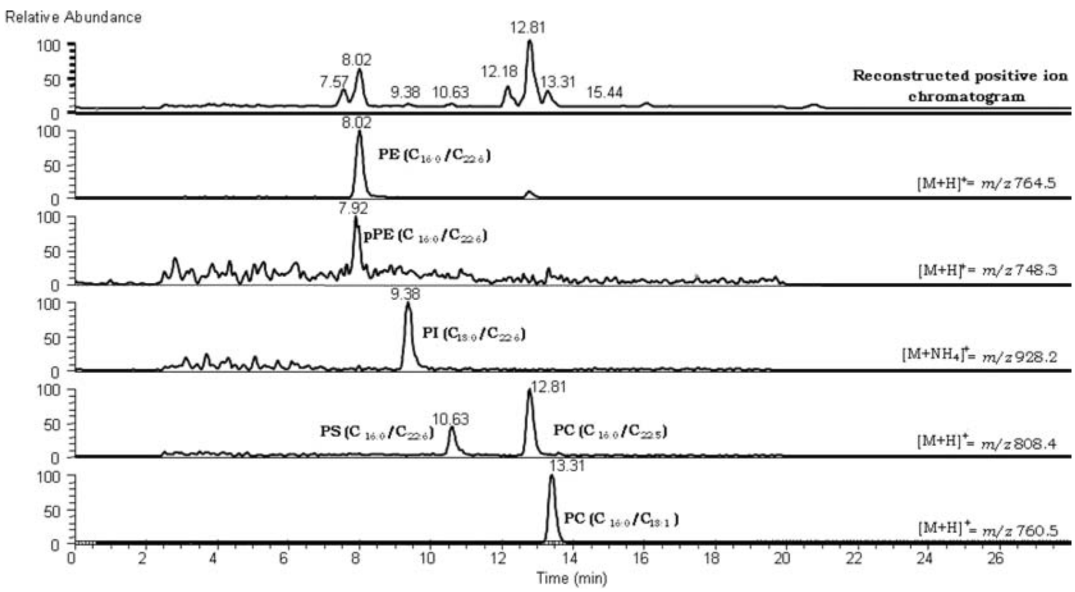

Figure 2. Positive HPLC-ESI-MS trace of PL from European anchovy with the MS operating in scanning mode.

to keep the manipulations to a minimum. The HPLC flow was directed to the waste during the first 4 min in order to prevent plugging of the heated capillary since the triacylglycerol fraction is eluted in the first 3 min from the HPLC column. Then, a switching valve automatically shifted the flow into the injection port of the mass detector. The separation of the six main phospholipid classes from fish and molluscs is reported in Figure 2. The pseudomolecular mass peaks from the different phospholipid classes were detected using positive ion full-scan ESI-MS analysis. All classes were detected as $[M + H]^+$ except for phosphatidylinositol that was detected as an ammonium adduct $[M + NH_4]^+$. The fragmentation of the PL classes has already been discussed in previous studies by different groups.[18−20] The retention time for the different classes increased in the following order: pPE < PE < PI < PS < PC. All classes eluted within 14 min.

Characterization of the Phospholipid Molecular Species. The identification of the PL molecular species was confirmed by using standard PL solutions and comparing the mass spectra with those reported in the literature.[21]

The relative abundance of individual molecular species within a phospholipid class was calculated from the single ion current responses since the peak intensity with ESI-MS among PL species of the same class is considered similar.[22,23] The molecular composition of the glycerophospholipid classes is discussed below.

Table 2. Phosphatidylethanolamine Plasmalogen (pPe) and Phosphatidylethanolamine (PE) Molecular Species Composition of Fish Fillet and Shellfish Edible Parts[a]

		sea fish						freshwater fish			mollusc		
ion (*m*/*z*)	1-alkenyl group/ fatty acid	h	m	s	a	p	k	n	t	g	c	u	y
pPE([M + H]$^+$)													
702.4	p16:0/18:1	9.7 ± 1.8a	9.1 ± 2.2a,b	4.8 ± 0.0c,d,e	6.3 ± 0.6b,c	8.9 ± 1.3a,b	6.7 ± 0.8a,b,c	4.7 ± 0.5c,d,e	6.5 ± 0.9a,b,c	5.8 ± 0.7b,c,d	6.3 ± 1.3b,c	2.8 ± 0.2d,e	1.6 ± 0.9e
704.4	p16:0/18:0 = p18:0/16:0	7.9 ± 1.4a	3.3 ± 0.1b,c	2.3 ± 0.5b,c,d,e	2.5 ± 0.6b,c,d,e	n.d.	3.8 ± 0.5b	2.2 ± 0.b,c	3.2 ± 0.3b,c,d	1.7 ± 0.3d,e	3.0 ± 0.5b,c,d	1.5 ± 0.5e	0.8 ± 0.2e
724.5	p16:0/20:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.4 ± 0.5a	4.3 ± 0.6a	11.3 ± 0.7b	n.d.	n.d.	n.d.
726.6	p16:0/20:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.3 ± 0.2a	3.2 ± 0.1b	4.1 ± 1.1b	n.d.	n.d.	n.d.
728.6	p18:0/18:2 = p18:1/18:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.2 ± 0.3a,b	n.d.	2.2 ± 0.4a,b	1.8 ± 0.4b	2.9 ± 0.2a	1.5 ± 0.5b
730.5	p18:0/18:1	8.9 ± 0.9a,b	9.5 ± 1.6a,b	4.1 ± 1.1d,e	7.2 ± 0.7b,c,d	11.1 ± 2.4a	6.6 ± 1.6c,d	7.9 ± 1.8b,c	3.6 ± 0.4d,e	2.7 ± 0.8e	5.2 ± 1.0c,d,e	4.4 ± 1.0c,d,e	2.2 ± 0.2e
732.3	p18:0/18:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.1 ± 0.3a,b	n.d.	n.d.	2.4 ± 0.4b,c	4.3 ± 1.2a	1.3 ± 0.3c
748.3	p16:0/22:6	23.3 ± 1.9b	22.0 ± 0.2b,c	15.8 ± 2.7d	18.5 ± 0.5c,d	16.8 ± 1.2d	20.7 ± 1.0b,c	10.4 ± 0.9e	46.2 ± 0.9a	24.4 ± 1.3b	n.d.	n.d.	n.d.
750.5	p18:0/20:5 = p16:0/22:5	8.3 ± 0.3c,d	7.0 ± 1.5d,e	8.5 ± 1.0c,d	10.0 ± 1.7c,d	8.4 ± 1.8c,d	9.7 ± 0.7c,d	6.4 ± 0.4d,e	16.6 ± 1.4b	18.3 ± 0.5a,b	4.2 ± 0.7e	11.8 ± 1.5c	10.8 ± 2.1c
752.4	p18:0/20:4	5.5 ± 0.6b,c	3.9 ± 1.1c,d	3.3 ± 1.5c,d	4.0 ± 0.5c,d	5.3 ± 0.9b,c	5.1 ± 0.7c	7.7 ± 0.8a,b	n.d.	8.0 ± 0.2a	1.8 ± 0.4d	4.7 ± 1.0c	5.1 ± 0.3c
754.5	p18:1/20:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.1 ± 0.4a	1.2 ± 0.3b	1.5 ± 0.3a,b	1.2 ± 0.2b
756.5	p18:0/20:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.2 ± 0.5a	17.3 ± 0.9b	2.3 ± 0.5c
758.5	p18:0/20:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.5 ± 0.5b	13.2 ± 1.3a	7.4 ± 0.8b
774.4	p18:1/22:6	11.8 ± 1.2b,c	22.6 ± 1.1a	13.3 ± 1.6b	9.7 ± 1.3c	10.3 ± 1.1b,c	22.3 ± 2.1a	4.3 ± 0.6d,e	10.9 ± 0.2b,c	5.3 ± 1.0d	3.1 ± 0.8d,e	2.0 ± 0.5e	2.2 ± 0.4d,e
776.2	p18:0/22:6 = p18:1/22:5	12.0 ± 1.1c,d	15.4 ± 1.0c	15.2 ± 1.9c	16.8 ± 2.9c	23.2 ± 2.1b	15.9 ± 2.0c	30.5 ± 1.4a	5.5 ± 0.3e	7.5 ± 0.4d,e	16.3 ± 1.0c	8.3 ± 1.0d,e	28.8 ± 2.8a
778.2	p18:0/22:5 = p18:1/22:4	12.5 ± 1.8a,b	7.0 ± 0.7c,d	8.1 ± 0.8b,c,d	25.1 ± 3.0e	16.0 ± 2.3a	9.2 ± 1.6b,c	10.1 ± 0.6b,c	n.d.	4.0 ± 0.5d	12.2 ± 1.5a,b	6.3 ± 1.0	10.1 ± 1.4b,e
780.3	p18:0/22:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.5 ± 0.2b	n.d.	1.7 ± 0.2c	4.6 ± 0.5a	2.6 ± 0.5b,c	2.1 ± 0.6c
782.2	p20:1/20:2 = p18:1/22:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5 ± 0.2b	3.7 ± 0.4a	2.1 ± 0.7b
784.4	p18:0/22:2 = p20:1/20:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13.2 ± 1.5a	7.7 ± 0.4b	11.4 ± 2.5a,b
786.5	p20:1/20:0 = p18:0/22:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.4 ± 0.3a	1.4 ± 0.3b	1.6 ± 0.4a,b
802.3	p20:1/22:6	n.d.	n.d.	18.6 ± 1.4a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.0 ± 0.7b	1.6 ± 0.4c	5.1 ± 0.9b
804.3	p20:1/22:5	n.d.	n.d.	6.0 ± 1.2a	n.d.	n.d.	n.d.	2.2 ± 0.3b,c	n.d.	0.8 ± 0.1c	3.8 ± 0.6b	1.8 ± 0.3c	2.4 ± 0.4b,c
PE ([M + H]$^+$)	fatty acids												
716.3	16:0/18:2	1.3 ± 0.3c	3.5 ± 0.8c	2.3 ± 0.1c	1.0 ± 0.3c	1.5 ± 0.6c	3.1 ± 0.1c	8.0 ± 1.0b	1.9 ± 0.2c	3.5 ± 0.4c	25.7 ± 0.8a	8.6 ± 1.9b	7.6 ± 1.5b
718.4	16:0/18:1	1.5 ± 0.3d,e	1.7 ± 0.2d,e	3.8 ± 0.5c,d	0.8 ± 0.1e	1.3 ± 0.6d,e	1.3 ± 0.3d,e	4.8 ± 0.5c	1.3 ± 0.2d,e	8.5 ± 0.2b	8.8 ± 1.7b	14.5 ± 2.3a	2.2 ± 0.4c,d,e
738.4	16:0/20:5	3.8 ± 0.7c	1.8 ± 0.4c	3.6 ± 0.3c	2.1 ± 0.7c	2.8 ± 0.2c	3.9 ± 1.0c	3.0 ± 1.1c	3.3 ± 0.3c	n.d.	3.3 ± 0.8c	8.9 ± 1.6b	17.3 ± 0.8a
740.5	18:2/18:2 = 16:0/20:4	2.6 ± 0.1c	2.3 ± 0.8c	2.3 ± 0.3c	0.8 ± 0.2d	1.5 ± 0.6c,d	2.8 ± 0.2b,c	6.7 ± 0.3a	2.1 ± 0.6c,d	5.6 ± 0.3a	2.4 ± 0.5c	4.0 ± 0.6b	6.6 ± 0.6a
742.6	18:0/18:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.7 ± 0.3d	1.3 ± 0.3d	5.8 ± 0.8b,c	4.1 ± 0.4c	13.2 ± 1.6a	6.9 ± 0.8b
744.6	18:1/18:1 = 18:0/18:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.6 ± 0.4a	1.6 ± 0.3a	8.7 ± 0.7b	10.5 ± 0.8c	19.4 ± 0.5d	7.0 ± 0.6e
746.5	18:0/18:1	1.2 ± 0.1c,d,e	1.6 ± 0.7b,c,d	2.5 ± 0.5b,c,d	n.d.	n.d.	1.1 ± 0.4d,e	1.7 ± 0.3a,b,c,	n.d.	11.3 ± 1.2f	3.4 ± 0.3a,b	8.2 ± 0.9g	2.7 ± 0.4a,b,c,d
764.3	16:0/22:6 = 18:1/20:5	17.3 ± 1.2d,e	10.3 ± 0.8f,g	13.9 ± 1.7e,f	38.5 ± 3.7a	29.9 ± 3.1b	14.0 ± 1.5e,f	7.7 ± 0.8f,g	22.4 ± 1.1c,d	5.5 ± 0.2g	11.7 ± 0.5e,f,g	8.6 ± 0.7f,g	12.2 ± 1.1e,f

Table 2. continued

		sea fish						freshwater fish			mollusc		
ion (m/z)	1-alkenyl group/ fatty acid	h	m	s	a	p	k	n	t	g	c	u	y
766.3	16:0/22:5 = 18:0/20:5	5.9 ± 0.6b,c,d	4.8 ± 0.4c,d	7.7 ± 0.6b	6.5 ± 1.0b,c,d	6.6 ± 1.4b,c,d	6.2 ± 0.4b,c,d	6.7 ± 0.7b,c,d	4.7 ± 0.1d	7.4 ± 0.5b,c	6.0 ± 0.3b,c,d	7.7 ± 1.0b	10.0 ± 1.0a
768.5	18:0/20:4 = 18:3/20:2	2.3 ± 0.2d,e	2.8 ± 0.4d	3.3 ± 0.4d	0.6 ± 0.4e	0.8 ± 0.2e	2.9 ± 0.2d	9.6 ± 1.6b	n.d.	12.1 ± 0.6a	2.3 ± 0.6d,e	n.d.	5.4 ± 0.6c
770.4	18:0/20:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.0 ± 0.8a	n.d.	12.2 ± 0.6b	n.d.	n.d.	n.d.
786.5	18:3/22:6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.5 ± 0.4	n.d.	n.d.	n.d.	n.d.
788.5	18:2/22:6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10.4 ± 2.3	n.d.	n.d.	n.d.	n.d.
790.4	18:1/22:6	10.1 ± 1.3a	4.1 ± 0.9c,d,e	7.3 ± 0.6b	2.0 ± 0.2e	2.2 ± 0.3e	4.5 ± 0.7c,d	5.0 ± 1.3c,d	8.7 ± 0.4a,b	n.d.	3.6 ± 0.3d,e	n.d.	6.4 ± 1.0b,c
792.2	18:0/22:6 = 18:1/22:5	11.6 ± 0.4a,b	24.6 ± 1.8f	12.6 ± 1.2a,b	13.5 ± 1.3a,e	10.5 ± 0.9a,b,c	20.4 ± 2.2d	12.6 ± 0.8a,b	9.7 ± 0.1b,c	10.8 ± 1.5a,b	9.6 ± 1.2b,c	n.d.	7.1 ± 1.1c
794.2	18:0/22:5 = 20:0/20:5	2.4 ± 0.8e,f	5.2 ± 0.4c	5.3 ± 0.3c	2.2 ± 0.2e,f	1.9 ± 0.5f	3.9 ± 0.1d	7.3 ± 0.3b	n.d.	9.0 ± 0.4a	3.4 ± 0.4d,e	n.d.	n.d.
796.2	18:0/22:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.3 ± 0.3
810.4	20:5/22:6	4.7 ± 0.7a,b,c	3.0 ± 0.4c,d	2.7 ± 0.3c,d	3.0 ± 0.8c,d	3.9 ± 0.2b,c,d	2.6 ± 0.2c,d	1.8 ± 0.3d	5.6 ± 1.8a,b	n.d.	4.8 ± 1.3a,b,c	6.8 ± 0.2a	2.7 ± 0.5c,d
812.3	20:4/22:6	3.1 ± 0.1a,b	1.9 ± 0.4b,c	1.9 ± 0.3b,c	1.2 ± 0.4c	1.1 ± 0.4c	2.7 ± 0.5a,b,c	4.0 ± 1.0a	3.0 ± 0.9a,b	n.d.	n.d.	n.d.	n.d.
814.3	20:4/22:5	1.7 ± 0.5a,b	2.9 ± 0.8a	2.3 ± 0.3a,b	1.4 ± 0.6b	1.1 ± 0.3b	3.1 ± 0.1a	2.5 ± 0.5a,b	2.4 ± 0.6a,b	n.d.	n.d.	n.d.	n.d.
818.3	20:1/22:6	2.0 ± 0.4b	1.6 ± 0.5b,c	6.7 ± 0.7a	n.d.	0.5 ± 0.0c	0.9 ± 0.1b,c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
836.4	22:6/22:6	21.9 ± 1.8a,b	20.4 ± 3.8a,b	9.2 ± 1.4d,e	21.2 ± 2.2a,b	26.5 ± 4.9a	18.8 ± 1.2b,c	7.8 ± 0.8e	11.8 ± 0.7c,d	n.d.	n.d.	n.d.	n.d.
838.4	22:5/22:6	5.0 ± 0.4b	5.7 ± 1.8a	8.7 ± 1.1a	4.7 ± 0.8b	5.9 ± 1.3a	6.1 ± 0.3a	n.d.	5.5 ± 1.3b	n.d.	n.d.	n.d.	n.d.
840.3	22:4/22:6	0.9 ± 0.3c,d	1.3 ± 0.4c,d	3.4 ± 0.8a	n.d.	0.6 ± 0.3d	1.5 ± 0.0b,c,d	2.4 ± 0.2a,b	1.9 ± 0.3b,c	n.d.	n.d.	n.d.	n.d.

[a]Values are given as the average internal percentage ± standard deviation (n = 3); n.d. = not detected; values in the same row with different letters are significantly different ($P < 0.05$).

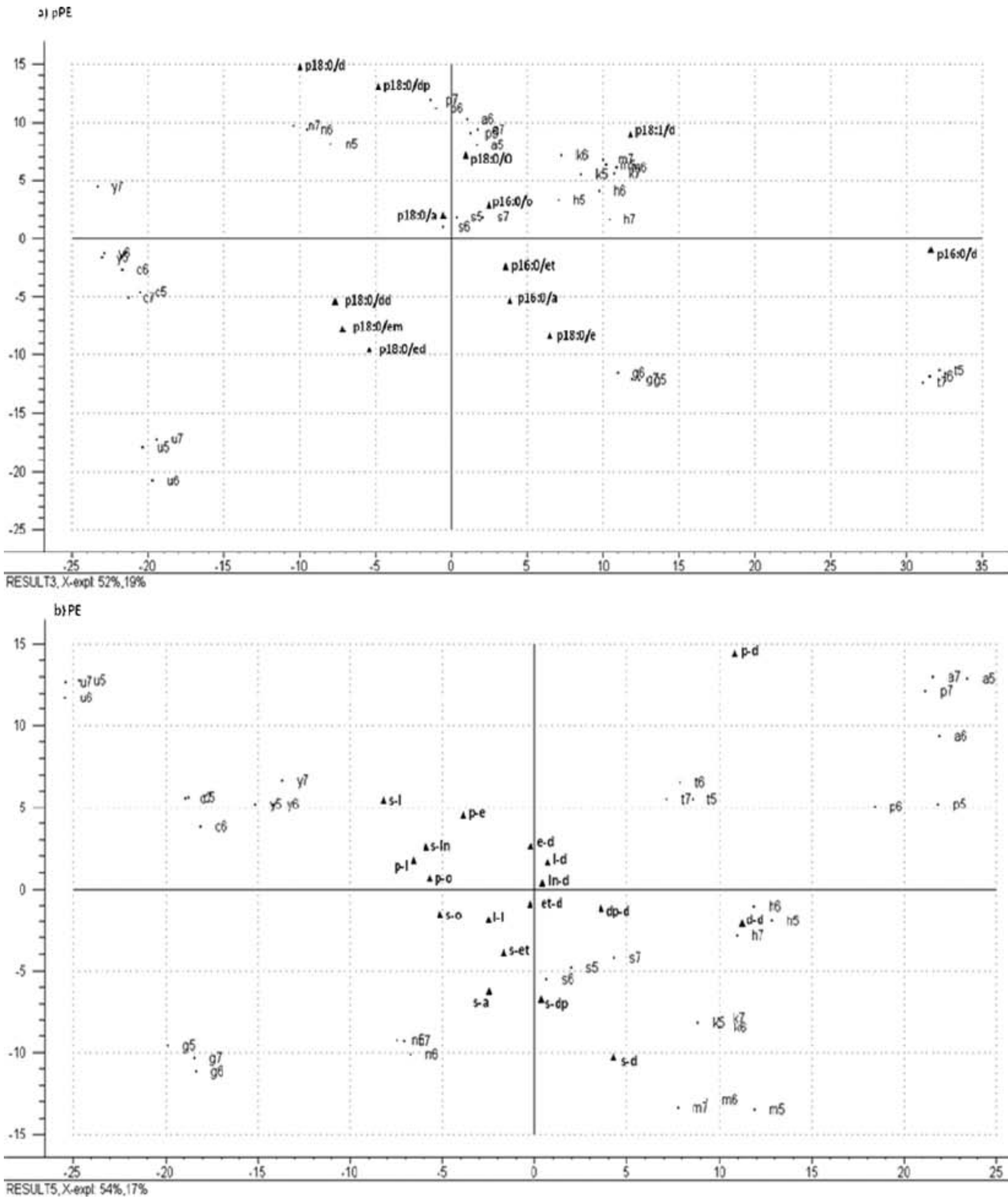

Figure 3. PCA biplot of the pPE (a) and PE (b) molecular species of the fish fillet and shellfish edible parts. Abbreviations of the fish samples are the same as those in Figure 1; abbreviations of the fatty acids (same as those in Figure 1) of the PL molecular species are reported in small characters separated by a dash (-).

Plasmalogen of Phosphatidylethanolamine. The preponderant pPE molecular species (Table 2) were different among the various bony fishes and shellfish. Shellfish did not contain pPE (p16:0/C22:6), which was preponderant in h, t, and g. The main species in shellfish was p18:0/C22:6 in c and y, whereas it was p18:0/C20:2 in u; the latest was curiously absent in all marine or freshwater fishes. Freshwater fishes were the only species containing p16:0/C20:4 and p16:0/C20:3. The most abundant molecular species in European anchovy contained octadecanoic alcohol and DPA acid. The preponderant species in s contained a monounsaturated alcohol (p20:1/C22:6). PCA showed (Figure 3a) a clear clustering of the fishes and molluscs according to the genus, unlike other phospholipid classes. Marine fishes were practically all clustered in the first quadrant due to the high fraction of p18:1/d. Among freshwater fishes, perch was localized in an opposite direction with respect to pangasius and trout due to the high presence of p18:0/d. Among molluscs, the mussel samples were localized far from the other two species due to the high percentage of pPE containing 18:0 and an unsaturated C20 (mono or diunsaturated) fatty acid.

Phosphatidylethanolamine. The molecular species composition of PE (Table 2) was a good marker of the fish and mollusc genus, as it can be observed from the biplot of PCA (Figure 3b); the preponderant molecular species depended on the fish genus. Sea fishes are clustered at positive values of PC1 due to the high content of PE molecular species containing DHA. The a and p samples were significantly richer of C16:0/C22:6 than all

Table 3. PI, PS, and PC Molecular Species Composition of Total Lipid in Fish and Shellfish[a]

		sea fish						freshwater fish			molluscs		
ion (m/z)	fatty acid	h	m	s	a	p	k	n	t	g	c	u	y
PI($[M + NH_4]^+$)													
880.2	18:0/18:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.0 ± 0.6b	16.5 ± 1.0a	n.d.	n.d.	n.d.
900.2	16:0/22:6	9.8 ± 2.2b	9.5 ± 0.9b,c	5.1 ± 0.9c	25.4 ± 1.1a	12.4 ± 3.2b	8.5 ± 0.7b,c	8.2 ± 0.8b,c	9.6 ± 1.1b,c	n.d.	n.d.	n.d.	n.d.
902.2	16:0/22:5 = 18:0/20:5	12.8 ± 1.1b,c	8.8 ± 3.1c,d	14.8 ± 2.8b	6.3 ± 0.7d	11.0 ± 0.6b,c,d	10.4 ± 0.7b,c,d	9.6 ± 0.4c,d	13.5 ± 0.9b,c	n.d.	14.9 ± 2.7b	13.8 ± 2.1b,c	20.5 ± 2.1a
904.2	18:0/20:4 = 16:0/22:4	13.2 ± 1.4e,f	11.2 ± 3.6e,f	20.1 ± 0.9d	4.0 ± 0.7g	9.8 ± 1.2f	12.7 ± 1.7e,f	38.2 ± 1.6b	21.2 ± 0.6d	50.4 ± 1.5a	26.6 ± 1.3c	15.2 ± 1.1e	15.0 ± 0.1e
906.2	18:0/20:3	2.7 ± 0.5c	4.5 ± 0.4b,c	4.1 ± 0.2b,c	3.5 ± 0.4c	2.2 ± 0.5c	2.0 ± 0.3c	6.4 ± 0.3b	n.d.	13.8 ± 2.1a	n.d.	n.d.	n.d.
926.2	18:1/22:6	13.6 ± 1.8a	10.4 ± 0.5a,b	10.1 ± 1.3a,b	5.5 ± 0.7c	4.5 ± 2.1c	7.4 ± 1.2b,c	9.5 ± 1.0b	n.d.	n.d.	5.6 ± 1.1c	n.d.	4.8 ± 0.5c
928.2	18:0/22:6 = 20:1/20:5	39.5 ± 0.4b	47.5 ± 0.9a	34.7 ± 1.6c	48.3 ± 1.2a	52.4 ± 2.1a	49.2 ± 1.4a	20.6 ± 1.2e	39.3 ± 1.1b	11.9 ± 1.1f	22.8 ± 1.9e	24.5 ± 2.5d,e	28.9 ± 1.8d
930.2	18:0/22:5 = 20:1/20:4	8.3 ± 0.7c,d	8.1 ± 0.4c,d	11.1 ± 0.8c	7.1 ± 0.7d	7.7 ± 0.9c,d	9.8 ± 1.8c,d	7.5 ± 0.4c,d	9.4 ± 0.4c,d	7.4 ± 0.7c,d	30.1 ± 0.5b	46.6 ± 3.3a	30.8 ± 1.1b
PS($[M + H]^+$)													
762.5	16:0/18:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.2 ± 0.5a	6.2 ± 0.5b	3.6 ± 0.3c	n.d.	n.d.	n.d.
788.5	18:0/18:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.1 ± 0.7b	1.5 ± 0.2c	7.2 ± 0.1a	n.d.	n.d.	n.d.
790.3	18:0/18:1	11.1 ± 0.3b,c,	9.5 ± 1.2c,d	10.9 ± 0.6b,c,	5.2 ± 0.9f	6.4 ± 1.7e,f	7.6 ± 0.7d,e,f	13.6 ± 0.8b	10.1 ± 0.7c,d	18.5 ± 1.1a	n.d.	n.d.	n.d.
808.4	16:0/22:6	7.4 ± 0.1a	10.0 ± 1.9c,d	2.7 ± 0.4e	37.3 ± 2.2a	30.9 ± 4.6b	15.2 ± 0.8c	n.d.	25.8 ± 2.2b	n.d.	13.8 ± 0.2c	15.4 ± 1.4c	12.0 ± 2.9c,d
810.4	18:0/20:5 = 18:1/20:4	5.2 ± 0.4d	7.0 ± 0.2d	8.5 ± 0.6c,d	8.9 ± 1.1c,d	12.4 ± 2.2c	8.9 ± 0.8c,d	n.d.	n.d.	n.d.	7.0 ± 0.2b	20.4 ± 1.4d	25.3 ± 3.3a
812.5	18:0/20:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.8 ± 0.5b	4.1 ± 0.7b	4.9 ± 0.9b	n.d.	n.d.	11.4 ± 1.3a
814.5	18:0/20:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.4 ± 0.4b	n.d.	11.7 ± 1.1a	n.d.	n.d.	n.d.
816.5	18:1/20:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.7 ± 1.3	7.9 ± 2.7
818.5	18:0/20:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	15.1 ± 0.9	n.d.	n.d.
836.3	18:0/22:6 = 18:1/22:5	49.6 ± 0.5a	49.4 ± 1.9a	39.6 ± 1.6c	26.2 ± 2.8e	32.0 ± 3.3d	44.9 ± 0.7a,b	31.0 ± 1.4d,e	41.2 ± 0.7b,c	17.2 ± 1.2f	30.3 ± 0.6a,b	46.2 ± 2.9d,e	16.0 ± 1.2f
838.2	18:0/22:5 = 18:1/22:4	10.9 ± 0.6c,d,e	14.6 ± 3.0b,c	28.2 ± 1.8a	7.0 ± 0.7e	8.5 ± 1.8d,e	12.1 ± 1.2c,d,e	27.1 ± 1.7a	9.2 ± 0.9d,e	24.2 ± 1.3a	13.2 ± 1.2b	17.9 ± 2.0b,c,d	9.8 ± 2.9c,d,e
840.4	18:0/22:4	n.d.	n.d.	10.0 ± 0.6a,c	n.d.	n.d.	n.d.	7.9 ± 1.6c	1.8 ± 0.4b	12.7 ± 1.3a	n.d.	n.d.	n.d.
844.5	18:2/22:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.9 ± 0.8b	n.d.	17.8 ± 1.4a
882.4	22:6/22:5	4.8 ± 0.3ab	5.7 ± 3.0ab	n.d.	3.7 ± 0.5b	n.d.	8.1 ± 0.7a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
880.4	22:6/22:6	11.1 ± 0.3a	3.9 ± 0.7b	n.d.	11.7 ± 1.2a	9.8 ± 1.9a	3.2 ± 0.3b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PC($[M + H]^+$)													
732.5	16:0/16:1 = 14:0/18:1	4.9 ± 1.3b,c	2.5 ± 0.4c,d	8.0 ± 1.1b	3.3 ± 1.3c,d	2.9 ± 1.4c,d	2.6 ± 0.4c,d	3.2 ± 0.7b,c	1.2 ± 0.5d	4.1 ± 0.7c,d	5.5 ± 1.3b,c	15.8 ± 2.3a	5.2 ± 1.3b,c
734.5	16:0/16:0	2.2 ± 0.4c,d	1.4 ± 0.3d	2.1 ± 0.4c,d	1.3 ± 0.4d	1.3 ± 0.6d	1.8 ± 0.4c,d	5.4 ± 0.8b	0.7 ± 0.3d	4.8 ± 1.0b,c	2.8 ± 0.4b,c,d	9.9 ± 2.8a	4.8 ± 1.0b,c
758.5	16:0/18:2	3.3 ± 0.5c,d	2.4 ± 0.3d	3.5 ± 0.3c,d	2.1 ± 0.2d	3.6 ± 0.4c,d	2.5 ± 0.3d	2.4 ± 0.3d	6.4 ± 0.5b	10.0 ± 1.0a	3.8 ± 1.1c,d	6.6 ± 0.7b	4.5 ± 1.0c
760.5	16:0/18:1	18.0 ± 0.8b	12.6 ± 0.7c	24.1 ± 0.9a	12.1 ± 1.7c	11.8 ± 0.8c	11.8 ± 0.9c	20.4 ± 2.2a,b	7.8 ± 1.1e,f	34.0 ± 2.5g	10.4 ± 1.0c,d	5.6 ± 0.6f	7.6 ± 0.6d,e
780.5	16:0/20:5	9.3 ± 0.6b,c	9.1 ± 1.3c	11.2 ± 0.6a,b,c	11.0 ± 1.5a,b,c	13.0 ± 1.6a,b	11.9 ± 2.0a,b,c	4.4 ± 0.9d	9.4 ± 0.2c	2.6 ± 0.6d	11.4 ± 0.5a,b,c	14.5 ± 2.5a	22.1 ± 0.6e
782.5	16:0/20:4	4.0 ± 0.6d	5.2 ± 0.8c,d	5.4 ± 1.6c,d	3.2 ± 1.3d	3.8 ± 1.2d	6.2 ± 0.9b,c,d	8.6 ± 1.8a,b	4.2 ± 0.5d	9.0 ± 0.7a,b	7.3 ± 0.8a,b,c	5.9 ± 0.6b,c,d	9.1 ± 0.7a
788.5	18:0/18:1	1.5 ± 0.4d,e	1.4 ± 0.1d,e	1.5 ± 0.2d,e	0.8 ± 0.2e	0.7 ± 0.0e	1.0 ± 0.0d,e	3.6 ± 0.3b	0.6 ± 0.1e	7.4 ± 1.0a	3.2 ± 0.6b,c	2.2 ± 0.1c,d	1.5 ± 0.1d,e
790.5	18:0/18:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.9 ± 1.4a	0.4 ± 0.2c	3.9 ± 0.8b	n.d.	n.d.	n.d.
806.4	16:0/22:6	21.0 ± 2.0d,e	26.9 ± 4.0b,c,d	12.9 ± 1.1e,f	32.0 ± 4.7a,b	24.2 ± 3.4b,c,d	22.7 ± 3.1b,c,d	12.9 ± 0.3e,f	38.3 ± 6.9a	5.9 ± 1.0f	31.4 ± 1.9a,b,c	23.4 ± 1.0b,c,d	22.1 ± 2.6c,d,e
808.4	16:0/22:5	5.2 ± 0.8d,e	7.3 ± 0.2b,c,d	8.6 ± 0.8a,b	5.8 ± 0.7c,d,e	4.4 ± 0.9e	5.6 ± 0.2c,d,e	7.3 ± 0.1b,c,d	6.7 ± 0.8b,c,d	7.7 ± 0.7b,c,d	10.9 ± 0.7a	6.6 ± 1.7b,c,d,e	8.0 ± 0.7b,c

Table 3. continued

ion (m/z)	fatty acid	sea fish						freshwater fish			molluscs		
		h	m	s	a	p	k	n	t	g	c	u	y
818.6	18:0/20:0	2.2 ± 0.1a,b	1.5 ± 0.4b,c,d	1.8 ± 0.1a,b,c	1.6 ± 0.3a,b,c,d	0.9 ± 0.2c,d	2.6 ± 0.3a	0.6 ± 0.1d	1.1 ± 0.3c,d	1.0 ± 0.2c,d	1.2 ± 0.1b,c,d	1.7 ± 1.0a,b,c,d	1.5 ± 0.4b,c,d
830.4	18:2/22:6	1.0 ± 0.2c	1.0 ± 0.4c	1.2 ± 0.3c	1.1 ± 0.5c	0.8 ± 0.3c	2.1 ± 0.4c	3.9 ± 1.5b	6.6 ± 0.7a	1.2 ± 0.2c	0.6 ± 0.1c	0.5 ± 0.1c	0.9 ± 0.1c
832.4	18:1/22:6	5.9 ± 0.5a	4.2 ± 0.4a,b,c,d	3.7 ± 0.2b,c,d	1.7 ± 0.1e	2.5 ± 0.3d,e	4.5 ± 0.8a,b,c	3.1 ± 0.7b,c,d,e	4.8 ± 1a,b	3.5 ± 0.5b,c,d,e	3.8 ± 1.0b,c,d	2.8 ± 0.8c,d,e	4.4 ± 0.3a,b,c
834.4	18:0/22:6	3.1 ± 0.4c,d,e	4.3 ± 0.6a,b,c	3.3 ± 0.1b,c,d	1.4 ± 0.1f	2.3 ± 0.5d,e,f	3.1 ± 0.4c,d,e	3.4 ± 0.4b,c,d	2.0 ± 0.6e,f	3.1 ± 0.3c,d,e	4.1 ± 0.5a,b,c	4.4 ± 0.8a,b	4.9 ± 0.3a
852.4	20:5/22:6	4.6 ± 1.0b	4.3 ± 0.8b	2.8 ± 0.4b,c,d	5.1 ± 1.0b	8.5 ± 1.1a	4.4 ± 0.7b	1.8 ± 0.5c,d	4.1 ± 1.8b,c	n.d.	0.8 ± 0.3d	n.d.	1.2 ± 0.2d
854.4	20:5/22:5	2.7 ± 0.9a,b	2.3 ± 0.5a,b	3.1 ± 0.5a	1.9 ± 0.6a,b,c	3.0 ± 0.1b,c	3.4 ± 0.6a	2.6 ± 0.2a,b	2.5 ± 1.5a,b	n.d.	1.1 ± 0.2b,c	n.d.	0.5 ± 0.1c
878.5	22:6/22:6	8.1 ± 1.4b,c	10.2 ± 0.5a,b	2.9 ± 0.3d,e	12.8 ± 2.2a	13.0 ± 3.3a	10.7 ± 0.3a,b	4.0 ± 0.6d,e	6.0 ± 0.7c,d	1.0 ± 0.7e	1.1 ± 0.6e	0.2 ± 0.1e	1.1 ± 0.2e
880.5	22:6/22:5	2.9 ± 0.3a	3.5 ± 0.3a	3.7 ± 0.9a	2.8 ± 0.7a,b	3.3 ± 0.7a	3.2 ± 0.1a	3.4 ± 0.1a	1.6 ± 0.6b,c	0.9 ± 0.2c,d	0.5 ± 0.1c,d	0.2 ± 0.0d	0.6 ± 0.4c,d

[a]Values are given as the average internal percentages ± standard deviation (n = 3). n.d. = not detected; values in the same row with different letters are significantly different ($P < 0.05$).

other species. On the contrary, molluscs were clustered at the top/left of the score plot because of the low content of ω-3 LC-PUFA (the only ω-3 LC-PUFA was EPA).

Freshwater fishes were splitted into opposite quadrants. The trout had a composition close to a and p, whereas g and n were characterized by C18:0/C20:4, C18:0/C18:1, C18:0/C20:3, and C16:0/C20:4 (or C18:2/C18:2).

The PE species containing DHA/DHA was only present in marine fishes, in n and t, and was the preponderant species in h. PE with C16:0/C18:2 was significantly higher in c than all the other fish and mollusc samples. Some of the PE molecular species were typical of a particular genus, such as C18:0/C22:4 for y and C18:3/C22:6 and C18:2/C22:6 for t, respectively.

Phosphatidylinositol. The fish samples could be classified through the PI molecular species (Table 3) into separated groups according to the fish class with the exception of t, which was similar to the marine fishes (Figure 4a). The main molecular species of marine fishes and t contained DHA. The main molecular species of molluscs contained DPA and stearic acid, which was significantly higher than all the other fishes. Freshwater fish, such as g and n contained stearic combined with arachidonic acid, as the predominant molecular species. In conclusion, the preponderant PI species always contained stearic acid combined with different LC-PUFAs according to the fish classes. In molluscs and in g, C16:0/C22:6 was absent, whereas it reached 25.4% in a.

Phosphatidylserine (PS). The profile of the molecular species of PS (Table 3) were homogeneous within a fish genus; however, the clustering of the samples (Figure 4b) did not follow a predictable distribution. Freshwater fish clustered both in the top/right (t), in the bottom/left quadrant (g), or along the negative values of the PC1 axis (n). Also marine fish genera were spread in three different quadrants. The molluscs were distributed in the two quadrants with positive values of PC1. The main molecular species was C18:0/C22:6 in all fishes except a, g, and y, where the main species were C16:0/C22:6, C18:0/C22:5, and C18:0/C20:5, respectively. Only freshwater fishes contained C16:0/C18:1 and C18:0/C18:2. The molecular species C18:0/C22:4 was only present in s and freshwater fishes. The molecular species containing C20:1 were only present in molluscs. Mussel and oysters were the only genera containing C18:1/C20:1, whereas oysters and clams were the only genera containing C18:2/C22:2. Moreover, only clams contained C18:0/C20:1. Pangasius and Nile perch were the only genera lacking of C16:0/C22:6.

Phosphatidylcholine (PC). The PC molecular species composition of fish and shellfish samples was reported in Table 3. The main PC molecular species contained palmitic and docosahexaenoic acid (PC 16:0/22:6) in all fish and shellfish samples except in sole, Nile perch and pangasius where the preponderant PC molecular species resulted the PC (C16:0/C18:1). The molecular species containing exclusively stearic acid (C18:0/C18:0) was detected only in freshwater fish.

The PCA biplot obtained using the molecular species profile of PC (Table 3) is reported in Figure 4c. PC1 and PC2 accounted for 77% of the total variance of the model; the majority of the variance was explained by PC1 (58%).

The PC molecular species composition of molluscs (particularly oysters) was markedly different from that of all the fish species since it resulted being richer of PC 16:0/20:5. The fish species can be clustered in two groups: the first group was formed by t and all marine fish species except common sole (s). These fish species

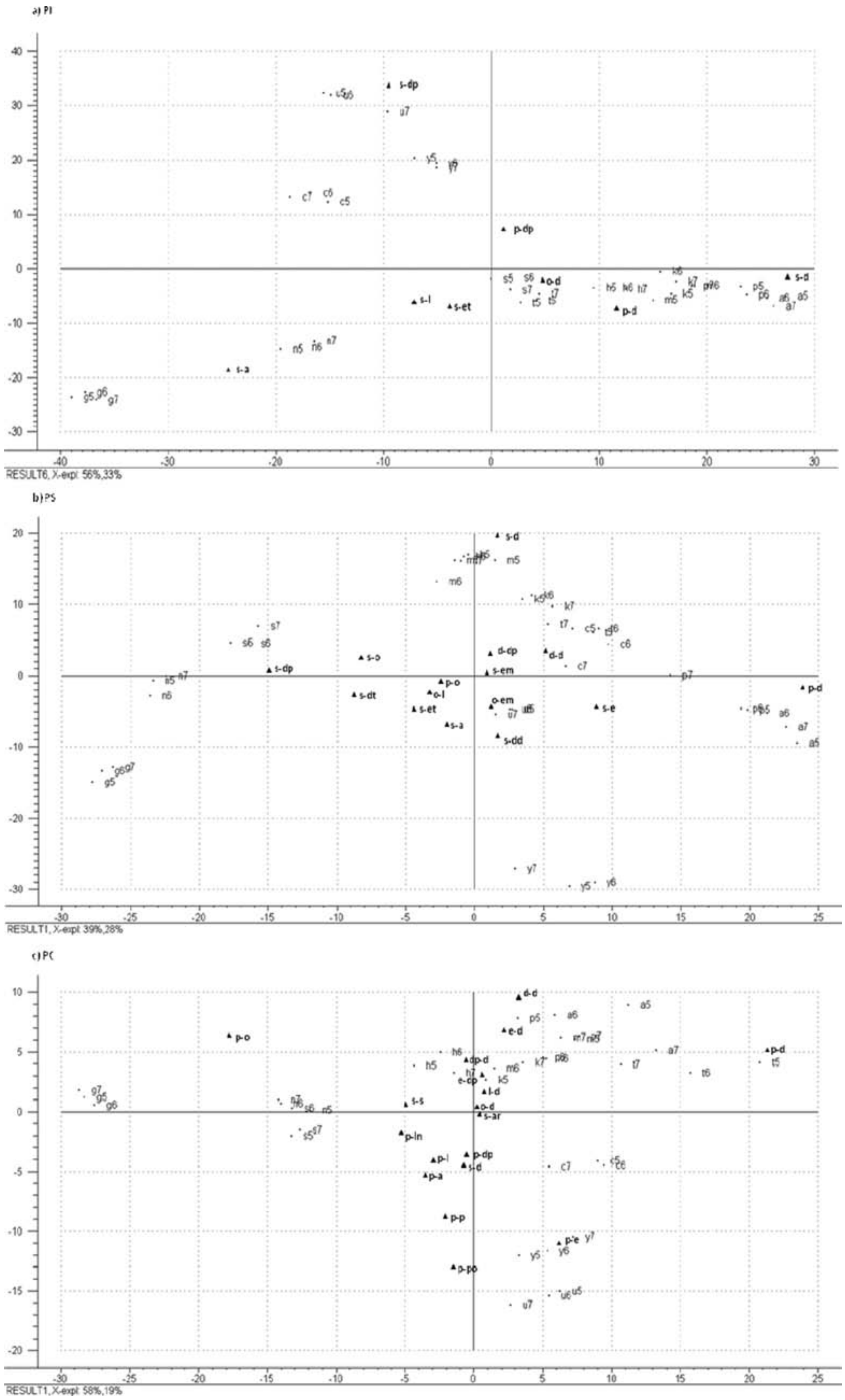

Figure 4. PCA biplot of PI (a), PS (b), and PC (c) molecular species of the fish fillet and shellfish edible parts. Abbreviations of the fish samples are the same as those in Figure 1; abbreviations of the fatty acids (same as those in Figure 1) of the PL molecular species are reported in small characters separated by a dash (-).

were rich of 16:0/22:6 and 22:6/22:6. The second group was formed by the common sole and the remaining freshwater fish (g and n), which were rich in 16:0/18:1. The ANOVA confirmed these results (Table 3). The PC 16:0/18:1 amount in g (34.0 ± 2.5% of total PC molecular species) was significantly higher than all the other samples, including common sole and Nile perch. The opposite trend was shown by PC 16:0/22:6 content; it resulted in being significantly low in g and high in t, a, and c.

Moreover, marine fish species (except common sole) showed the highest significant amount of PC 22:6/22:6 with respect to all freshwater fish and molluscs. The exception was detected in common sole where PC22:6/22:6 (2.9 ± 0.3% of PC molecular species) resulted in being weakly lower than n and t (4.0 ± 0.6% and 6.0 ± 0.7%, respectively) and weakly higher than g and molluscs.

Molluscs were characterized by a high content of PC 16:0/20:5, which resulted in being significantly greater in the oyster (22.1 ± 0.6%) than all the other samples, including clam and mussel.

In addition, the phosphatidylcoline composition of the mussel was characterized by a significantly high content of PC 16:0/16:1 with respect to all the other species of fish and molluscs. Curiously, PC 20:5/22:6 and PC 20:5/22:5 were absent both in g and u.

General Remarks. In summary, a HPLC-ESI-MS/MS method previously developed for the characterization of pork meat and egg phospholipids[18,24] was successfully applied to the qualitative and quantitative analysis of the phospholipid molecular species of marine and freshwater fish and shellfish collected in different periods without prior separation of the polar lipid fraction.

Freshwater fishes showed the highest heterogeneity of the fatty acid combinations in the PL; moreover, the composition of the trout was more similar to marine fish. This can be due to the different geographical origin of the freshwater fish samples since phospholipids are considered the lipid fraction less affected by the seasonal and dietary variability[25] with respect to triacylglycerols. While pangasius was of Asian origin and Nile perch was from Africa, the trout came from Italian fish farms.

Common sole was distanced from marine fishes probably due to the different living conditions; while sole is a benthic fish, all the other bony fishes swim in the water column above the bottom.

Regarding the identification of chemical markers for peculiar species of fish it must be reported that the phospholipid classes could be subdivided in two categories. For PC, the main phospholipid class, the fatty acid combination was the tool for differentiating shellfish from the bony fishes. The same conclusion can be drawn for PI and PE. Conversely, PS and pPE allowed for the discrimination of the several bony fish or shellfish genus because these PL classes include a large number of fatty acid combinations, which were specific for a fish genus or a group of genera, as previously discussed. Freshwater fishes were the only species containing the pPE combinations p16:0/C20:4 and p16:0/C20:3.

From the comparison of the different PCA models, it can be concluded that the differentiation among the shellfish genera is achievable only through the profile of pPE and PS and not by using the total fatty acid profile. However, the PC molecular profile was partially similar to that of total fatty acids, presumably because PC is the main PL in animal fat.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ANOVA, analysis of variance; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; HPLC-ESI-MS/MS, electrospray ionization ion-trap tandem mass spectrometry; LC-PUFA, long chain polyunsaturated fatty acids; NL, neutral lipids; PC, phosphatidylcholine; PCA, Principal Component Analysis; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; pPE, plasmalogen of phosphatidylethanolamine; PS, phosphatidylserine; SD, standard deviation

REFERENCES

(1) Ruxton, C. H. S.; Reed, S. C.; Simpson, M. J. A.; Millington, K. J.; Ruxton, C. The health benefits of ω-3 polyunsaturated fatty acids: a review of the evidence. Commentary. *J. Hum. Nutr. Diet.* **2007**, *20*, 275−287.

(2) Véricel, E.; Calzada, C.; Chapuy, P.; Lagarde, M. The influence of low intake of ω-3 fatty acids on platelets in elderly people. *Atherosclerosis* **1999**, *147*, 187−192.

(3) Pacetti, D.; Hulan, H. W.; Schreiner, M.; Boselli, E.; Frega, N. G. Positional analysis of egg triacylglycerols and phospholipids from hens fed diets enriched with refined seal blubber oil. *J. Sci. Food Agric.* **2005**, *85*, 1703−1714.

(4) Lei, L.; Li, J.; Li, G.; Hu, J.; Tang, L.; Liu, R.; Fan, Y.; Deng, Z. Stereospecific analysis of triacylglycerol and phospholipid fractions of five wild freshwater fish from Poyang Lake. *J. Agric. Food Chem.* **2012**, *60*, 1857−1864.

(5) Luzia, A. L.; Sampaio, G. R.; Castellucci, C. M. N.; Torres, E. A. The influence of season on the lipid profiles of five commercially important species of Brazilian fish. *Food Chem.* **2003**, *83*, 93−97.

(6) Cohn, J. S.; Wat, E.; Kamili, A.; Tandy, S. Dietary phospholipids, hepatic lipid metabolism and cardiovascular disease. *Curr. Opin. Lipidol.* **2008**, *19*, 257−262.

(7) Henna, L. F. S.; Nielsen, N. S.; Timm-Heinrich, M.; Jacobsen, C. Oxidative stability of marine phospholipids in the liposomal form and their applications. *Lipids* **2010**, *46*, 3−23.

(8) Le Grandois, J.; Marchioni, E.; Zhao, M.; Giuffrida, F.; Ennahar, S.; Bindler, F. Investigation of natural phosphatidylcholine sources: separation and identification by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS^2) of molecular species. *J. Agric. Food Chem.* **2009**, *57*, 6014−6020.

(9) Standal, I. B.; Axelson, D. E.; Aursand, M. ^{13}C NMR as a tool for authentication of different gadoid fish species with emphasis on phospholipid profiles. *Food Chem.* **2010**, *121*, 608−615.

(10) Winther, B.; Hoem, N.; Berge, K.; Reubsaet, L. Elucidation of phosphatidylcholine composition in Krill oil extracted from *Euphausia superba*. *Lipids* **2010**, *46*, 25−36.

(11) Dumay, J.; Allery, M.; Donnay-Moreno, C.; Barnathan, G.; Jaouen, P.; Carbonneau, M. E.; Bergé, J. P. Optimization of hydrolysis of sardine (*Sardina pilchardus*) heads with Protamex: enhancement of lipid and phospholipid extraction. *J. Sci. Food Agric.* **2009**, *89*, 1599−1606.

(12) Commission Decision 2000/195/EC authorising phospholipides (85% and 100%) to be placed on the market in the Community, Official Journal L 061, 08/03/2000, p 12.

(13) Commission Decision 2009/752/EC authorising the placing on the market of a lipid extract from Antarctic Krill (*Euphausia superba*) as a novel food ingredient under Regulation (EC) No 258/97 of the

European Parliament and of the Council, OJ L268 of 13 October 2009, p 33.
(14) Kinsella, J. E.; Shimp, J. L.; Mai, J. The proximate and lipid composition of several species of freshwater fishes. *N. Y. Food Life Sci. Bull.* **1978**, *69*, 1−19.
(15) Weihrauch, J. L.; Son, Y. S. The phospholipid content of foods. *J. Am. Oil Chem. Soc.* **1983**, *60*, 1971−1978.
(16) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* **1959**, *37*, 911−917.
(17) Suter, B.; Grob, K.; Pacciarelli, B. Determination of fat content and fatty acid composition through 1min transesterification in the food sample; principles. *Z. Lebensm. Unters. Forsch. A* **1997**, *204*, 252−258.
(18) Boselli, E.; Pacetti, D.; Curzi, F.; Frega, N. G. Determination of phospholipid molecular species in pork meat by high performance liquid chromatography-tandem mass spectrometry and evaporative light scattering detection. *Meat Sci.* **2008**, *78*, 305−313.
(19) Zemski Berry, K. A.; Murphy, R. C. Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1499−1508.
(20) Pacetti, D.; Boselli, E.; Lucci, P.; Frega, N. G. Simultaneous analysis of glycolipids and phospholipids molecular species in avocado (*Persea americana* Mill.). *J. Chromatogr., A* **2007**, *1150*, 241−251.
(21) Pacetti, D.; Boselli, E.; Frega, N. G. Characterization of phospholipid molecular species by means of HPLC-Tandem Mass Spectrometry. In *Tandem Mass Spectrometry*; Prasain, J., Ed.; Intech: Rijeka, Croatia, 2012; pp 655−690.
(22) Han, X.; Gross, R. W. Electrospray ionization mass spectroscopic analysis of human erythrocyte plasma membrane phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91* (22), 10635−10639.
(23) Han, X.; Gubitosi-Klug, R. A.; Collins, B. J.; Gross, R. W. Alterations in individual molecular species of human platelet phospholipids during thrombin stimulation: electrospray ionization mass spectrometry-facilitated identification of the boundary conditions for the magnitude and selectivity of thrombin-induced platelet phospholipid hydrolysis. *Biochemistry* **1996**, *35* (18), 5822−5832.
(24) Pacetti, D.; Boselli, E.; Hulan, H. W.; Frega, N. G. High performance liquid chromatography-tandem mass spectrometry of phospholipid molecular species in eggs from hens fed diets enriched in seal blubber oil. *J. Chromatogr., A* **2005**, *1097*, 66−73.
(25) Joensen, H.; Steingrund, P.; Fjallstein, I.; Grahl-Nielsen, O. Discrimination between two reared stocks of cod (*Gadus morhua*) from the Faroe Islands by chemometry of the fatty acid composition in the heart tissue. *Mar. Biol.* **2000**, *136*, 573−580.