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Effect of soybean oil and fish oil on individual molecular species of Atlantic salmon head kidney phospholipids determined by normal-phase liquid chromatography coupled to negative ion electrospray tandem mass spectrometry

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Abstract

The effect of soybean oil (SO) and fish oil (FO) on the relative molecular species distribution of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) in Atlantic salmon head kidney was studied using normal-phase liquid chromatography coupled with negative ion electrospray tandem mass spectrometry. The conformation of identity of the phospholipid species was based on retention time, the mass of the $[M-H]^-$ ($[M-15]^-$ for PC) molecular ions and the carboxylate anion fragments in the product ion spectrum. The intensity ratio of *sn*-1/*sn*-2 fragment ions increased with increasing number of double bonds in the *sn*-2 acyl chain but was not affected by increasing number of double bonds in the *sn*-1 acyl chain of the species examined. The relative distribution of the molecular species was determined by multiple reaction monitoring of the carboxylate anion fragment from the *sn*-1 position. A total of 68 different phospholipid species were determined in the head kidney and the largest amount was found in PE (22 species). Depending on the diet, the main species identified in the different phospholipid classes were; PC 16:0/18:1, PE 16:0/22:6, PI 18:0/20:4 and PS 16:0/22:6. The SO diet significantly increased the 18:2, 20:3 and most 20:4 containing species and significantly reduced the 14:0 and most 20:5 and 22:6 fatty acid containing species. The increase of the 20:4 and the decrease of the 20:5 and 22:6 containing species were dependent on the fatty acid combination of the species

Keywords: Molecular species; Phospholipids

1. Introduction

Glycerophospholipids are the major constituents in membranes. Phospholipids consist of several distinct molecular classes characterised by the alcohol group attached to the phosphate moiety of the molecule. Numerous molecular species are found in a single phospholipid class characterised by the combination of fatty acyl residues attached to the *sn*-1 and *sn*-2 positions of the molecule and in the chemical linkage

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of the fatty acid chain to the *sn*-1 position of the glycerol backbone. Acyl, ether, and vinyl-ether bonds are common.

With the recent coupling of HPLC to mass spectrometry (MS), new detection opportunities have been opened for the structural analysis of phospholipid species [1]. Several ionisation techniques have been used for this purpose such as particle beam [2,3], thermospray [4–7], plasmaspray [8] and modern electrospray (ES) [9–12]. ES is a soft atmospheric ionisation technique that can accommodate flow-rates up to 1000 μl/min and is increasingly becoming the most popular ionisation technique for LC–MS. Using triple–quadrupole ES-MS instruments, the lipids have also been introduced into the MS without a previous chromatographic separation, i.e. loop injection [13–18]. The disadvantage of loop injection into MS is, of course, the possibility of mass overlap of the different species. Triple–quadrupole ES-MS instruments (ES-MS–MS) facilitate the structural determination of the individual species in one single analysis. The molecular ion of interest is selected by MS1 and fragmented by collision induced dissociation (CID). The resulting product ion spectrum in MS2 show fragments which can identify the phospholipid species.

Plant oils represent interesting candidates for replacing fish oil in fish feed. Fish oils typically contain significant levels of highly unsaturated long chain fatty acids (C_{20}–C_{22}) of the n-3 family. In contrast, plant oils typically have 18-carbon atoms, and fatty acids belonging to the n-6 family are more common than n-3 fatty acids. Both n-3 and n-6 fatty acids are considered essential to fish, but the requirement for n-3 fatty acids is much higher than for n-6 fatty acids in salmonids [19,20]. The fatty acid composition of fish tissue strongly reflects the dietary lipids ingested [21–24] and may influence some physiological parameters. Thompson et al. reported that replacing fish oil with sunflower oil in diets to Atlantic salmon might result in less resistance to infection [25].

We have recently developed a normal-phase HPLC method coupled with negative ion ES-MS, which separates the phospholipids into their respective classes [26]. Using the normal-phase HPLC method coupled with negative ion ES-MS–MS, we have also studied the mechanism involved in the collision induced dissociation of carboxylate anions from glycerophospholipids [27]. In the present study the normal-phase HPLC method coupled with negative ion ES-MS–MS was used to determine individual molecular species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) from Atlantic salmon head kidney. The effect of feeding salmon 100% capelin oil (FO), 100% soybean oil (SO) or a 50:50 mixture of the two oils (50SO–50FO) on the relative distribution of the molecular species in the different phospholipid classes was also studied. Since the macrophages (of head kidney) are important cells in Atlantic salmons immune response, one of the aims of this study was to describe how soybean oil diets influence the phospholipid composition of macrophages.

2. Experimental

2.1. Materials

Chloroform, formic acid (98–100%) and ammonia (25%) were pro-analysis grade, methanol was LiChrosolv grade all from Merck (Darmstadt, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-(phospho-L-serine) (D-PPS) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) were from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Diheptadecanoyl-*sn*-glycero-3-phosphocholine (DHPC) and soybean L-α-phosphatidylinositol (PI) were from Sigma (St. Louis, MO, USA). Metacain 222 (MS 222) was from Norsk Medisinaldepôt (Oslo, Norway). 2, 6-Di (*tert.*-butyl)-*p*-cresol (BHT) was from Sigma.

2.2. Animals and diets

Triplicate groups of 40 Atlantic salmon (*Salmo salar L.*) were fed three experimental diets for 3 months. The fish were kept in nine cylindro-conical tanks (0.75 m diameter) and supplied with seawater at a temperature of 12°C. The experimental diets provided by Nutreco ARC (4 mm extruded feed), were fishmeal based and differed only in the type of oil, 100% capelin oil (FO), 100% soybean oil (SO) or a 50:50 mixture of the two oils (50SO–50FO). The diets contained 30% fat and the dietary fatty

acid compositions was measured by extracting total lipids from homogenised feed, using the method described by Folch et al. [28]. The chloroform–methanol phases were dried under nitrogen, dissolved in hexane and the total lipid fractions were *trans*-methylated with 2,2-dimethoxypropane, methanolic–HCl and benzene according to Mason and Waller [29]. The methyl esters were separated on a non-polar fused capillary column by gas chromatography and quantitated according to Røsjø et al. [24]. The dietary fatty acid compositions are shown in Table 1. The fish grew from an average initial mass of 100 g to a final average mass of 320 g.

Table 1
Fatty acid composition of the diets (% of total fatty acid content)

	FO	50SO–50FO	SO
$C_{14:0}$	5.7	3.1	0.9
$C_{16:0}$	14.0	13.2	12.7
$C_{18:0}$	1.6	2.7	3.7
Saturated not listed	0.4	0.7	0.9
$C_{16:1}$ n-7	7.5	4.2	1.3
$C_{18:1}$ n-7	2.8	2.2	1.6
$C_{18:1}$ n-9	10.3	15.4	20.3
$C_{20:1}$ (sum isomers)	12.8	6.8	1.1
$C_{22:1}$ (sum isomers)	15.9	8.4	1.0
$C_{24:1}$ n-9	0.7	0.5	0.3
Monoenes not listed	0.5	0.3	0.0
$C_{18:3}$ n-3	0.8	3.1	5.3
$C_{18:4}$ n-3	3.2	1.8	0.5
$C_{20:4}$ n-3	0.4	0.2	NQ[a]
$C_{20:5}$ n-3 (EPA)	9.0	5.5	2.1
$C_{22:5}$ n-3	0.6	0.4	0.2
$C_{22:6}$ n-3 (DHA)	7.0	4.9	2.8
n-3 not listed	0.3	0.2	0.0
$C_{16:2}$ n-6	0.5	0.3	NQ[a]
C_{18}:2 n-6	4.4	25.3	45.4
$C_{20:4}$ n-6	0.2	0.1	NQ[a]
n-6 not listed	0.2	0.0	0.0
Others	1.4	0.6	0.2
Sum saturated	21.6	19.6	18.2
Sum monoenes	50.5	37.9	25.4
Sum n-6	5.3	25.7	45.4
Sum n-3	21.3	16.2	10.9
n-6/n-3 ratio	0.2	1.6	4.2

[a] NQ: not quantified.

2.3. Preparation of head kidney samples

The fish were anaesthetized in MS 222 and killed by a blow to the head. The abdomen was cut open and the head kidney was frozen by pouring liquid nitrogen on the kidney. The head kidney was then taken out, put on dry ice and stored in a freezer (−70°C) prior to extraction. The phospholipids were extracted by the method of Folch et al. [28]. The solutions used for lipid extractions contained BHT (50 mg/ml). Prior to analysis the extracted samples were redissolved in 0.3 ml of chloroform–methanol–water (65:25:4, v/v/v) and stored in a freezer (−20°C).

2.4. HPLC system

The chromatographic system consisted of a Waters 2690 mobile phase pump (Waters, Milford, MA, USA) equipped with an autosampler. The column was a LiChroCART, LiChrospher 100 Diol, 250×2 mm (5 μm) with a LiChrospher 100 Diol, 2×2 mm (5 μm), precolumn from Merck. The lipids were separated similar to previously reported methods [26,27]. Briefly, the mobile phase consisted of chloroform (mobile phase A) and methanol with 0.2% (v/v) formic acid titrated to pH 5.3 with ammonia (mobile phase B). To obtain class separation of the lipids, a gradient run was started at 95% mobile phase A, decreasing to 64% A in 7.75 min. and further decreasing to 40% A in 2.25 min, and then back to 95% A in 2.5 min (gradient 1). Some molecular species of PC showed peak splitting using gradient 1, this was improved by a slight adjustment of the gradient; the gradient run was started at 95% mobile phase A isocratic for 5 min, decreasing to 75% A in 3.3 min and further decreasing to 40% A in 2 min, and then back to 95% A in 2.5 min (gradient 2). Total run time for each sample was set to 20.5 min. The flow-rate was 0.3 ml/min, and the analyses were performed with the column kept at ambient temperature. The samples were kept at room temperature, and 2.5 or 10 μl was injected for each analysis.

2.5. Tandem mass spectrometry

The HPLC system described above was coupled on-line to a Quattro LC–MS–MS triple–quadrupole mass spectrometer (Micromass, Altrincham, UK)

equipped with a pneumatically assisted electrospray ionisation source. Data acquisition and processing were performed using a MASSLYNX NT 3.1 data system. The HPLC effluent entered the MS through an electrospray capillary set at 4.0 kV at a source block temperature of 80°C and a desolvation gas temperature of 150°C. Nitrogen was used both as drying gas and nebulizing gas at flow-rates of ~1000 and ~80 l/h, respectively. The ion-source parameters were optimised with respect to the negative molecular ions of the phospholipids. For ionisation the cone voltage was set to 50 V for all the phospholipids except for PC, which yielded a better response with a cone voltage of 80 V, resulting in the loss of a methyl ($-CH_3$) group from the choline moiety. The mass spectra, between 600 and 1000, were obtained at a scan speed of 200 m/z units/s, with a mass resolution corresponding to 1 u at half peak height. Argon was used as collision gas for collision induced dissociation (CID) at a pressure of $1.0 \cdot 10^{-3}$ mbar. The collision energy was set to 40 eV. The product ion spectra, between m/z 200 and 350 (400), were obtained at a scan speed of 200 m/z units/s, with a mass resolution corresponding to 1 u at half peak height. For determination of the carboxylate anion peak areas, the instrument was operated in the multiple reaction monitoring (MRM) mode where MS1 was set to the mass of the deprotonated phospholipid $[M-H]^-$ (except for PC were MS1 was set to the mass of the $[M-15]^-$ -ion) and MS2 was set to transmit the masses of the carboxylate anions.

When applicable, the peak areas of the carboxylate anions were corrected for the isotopic contribution of the ^{13}C and ^{18}O isotopes. In addition, some molecular species of PC and PE were found to contain fatty acid combinations with the same fatty acid in the *sn*-1 and *sn*-2 position. In these cases, the carboxylate anion peak area was corrected for 0.46 and 0.27 for PC and PE species, respectively. This correction was based on the estimated ratio of *sn*-1 to *sn*-2 determined from 16:0/16:1 and 18:0/18:1 species of PC and PE, respectively. After adjusting the carboxylate anion peak areas where necessary, the relative quantity of the molecular species in each phospholipid class and diet group was estimated.

The MS–MS instrument was calibrated with sodium iodide (NaI) prior to analysis.

2.6. Data handling

Microsoft EXCEL 2000 was used for estimation of mean and standard deviations (SD).

The differences in phospholipid species composition between dietary groups (Tables 2–5) were analysed for statistical significance using the 95% Duncan interval method, UNISTAT v. 4.53.

3. Results

3.1. Separation of the phospholipid classes

Fig. 1 A shows the chromatographic separation of the phospholipid classes studied in this work. The different phospholipid classes were well separated except for PI and PS. The m/z values for the $[M-H]^-$ molecular ions of PI and PS are, however, not identical and easily distinguished. Some molecular species of PC showed peak splitting (molecular species containing long chain polyunsaturated fatty acids) in the original chromatographic method (gradient 1 in Experimental). This was more or less eliminated by slightly changing the gradient of the chromatographic method (gradient 2 in Experimental) and reducing the injection volume from 10 to 2.5 μl. In addition, preliminary results show that the chromatography of the PC species is improved by dissolving the samples in a less polar solvent, e.g. chloroform [Å. Larsen, personal communication].

3.2. Identification of the phospholipid species from the different phospholipid classes

Extracted salmon head kidney was analysed by negative-ion LC–ES-MS–MS set in the scanning mode (Fig. 1B). The reconstructed negative ion chromatogram (RIC) in Fig. 1B shows that there are several extractable compounds with similar retention time to PC and PE (from 7 to 11 min in Fig. 1B). The MS software, however, offers the possibility of making chromatograms of individual molecular ions that are found in a spectrum. Thus from the analysis in Fig. 1B, RICs of representative $[M-H]^-$ ions of the different phospholipid classes are shown in Fig. 1C, demonstrating the separation of endogenous phospholipids and the specifity of the MS detector.

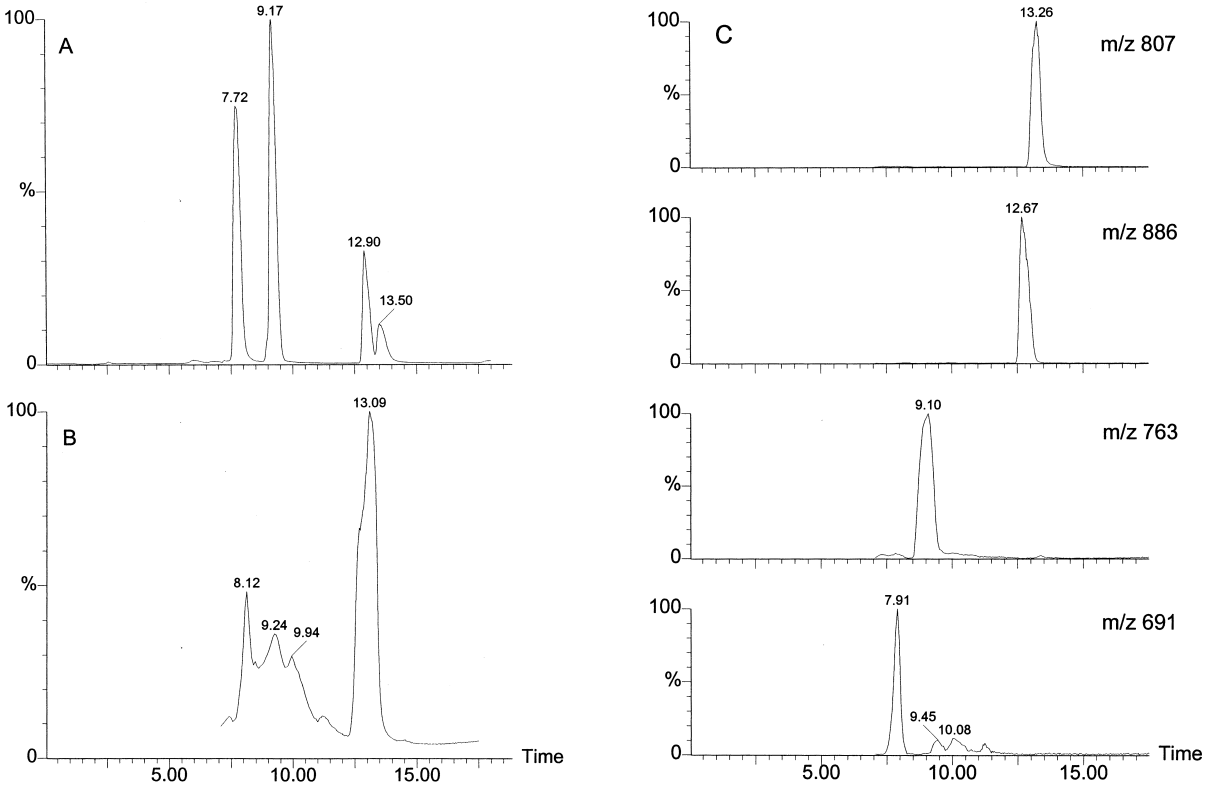

Fig. 1. Reconstructed negative ion chromatograms after LC–ES-MS–MS analysis of; (A) phospholipid mixture with the MS1 set to single ion monitoring of the molecular ions of DHPC (7.7 min), DSPE (9.2 min), PI (12.9 min) and DPPS (13.5 min) (≈5 μg/ml of each); (B) extracted Atlantic salmon head kidney with MS1 operated in the scanning mode; (C) same as in (B) showing representative $[M-H]^-$ ions of the different phospholipid classes at m/z 807 (16:0/22:6 PS, 13.3 min), m/z 886 (18:0/20:4 PI and 18:1/20:3 PI, 12.7 min), m/z 763 (16:0/22:6 PE and 18:1/20:5 PE, 9.1 min) and m/z 691 (16:0/14:0 PC, 7.9 min). For compound abbreviations, see Section 2.1. The mobile phase is described in Section 2.4 (gradient 1). For other conditions see the Experimental section.

The RIC of the $[M-H]^-$ ions at m/z 691 in Fig. 1C shows, in addition to the PC peak at 7.9 min, peaks at 9.5 min and 10.1 min. No attempts at identifying these peaks have been made. The mass spectrum under the peaks corresponding to the retention time of synthetic PC, PE, PI and PS, showed $[M-H]^-$ ($[M-15]^-$ for PC) ions that corresponded to molecular species of the respective phospholipid classes (Fig. 2). The mass spectrum of PC showed in addition ions with an m/z increase of 60 compared to the $[M-15]^-$ ions, e.g ions at m/z 750.7, 776.7 and 804.8 (Fig. 2). These ions were probably an adduct formation of formic acid with intact PC species, i.e. $[M+45-H]^-$. Product ion spectrum of the $[M+45-H]^-$ PC ions were shown to contain the same carboxylate anion fragments as the corresponding $[M-15]^-$ PC ions (not shown). Since there are several isobaric species in each phospholipid class, the identity conformation of the species were acquired in an MS–MS experiment where the molecular ions were selected by MS1 and fragmented by CID. As previously described [26,27], the product ion spectrum from MS2 showed the carboxylate anion fragments that were used for species conformation. This is illustrated in Fig. 3 where the product ion spectrum of the major molecular ion for each phospholipid class is presented as an example. In Fig. 3A the molecular ion at m/z 806.6 show carboxylate anion fragments at m/z 255.2 (16:0) and 327.4 (22:6). The retention time of the RIC peak in Fig. 3A corresponded to synthetic PS (not shown), confirming the identity of this species to 16:0/22:6

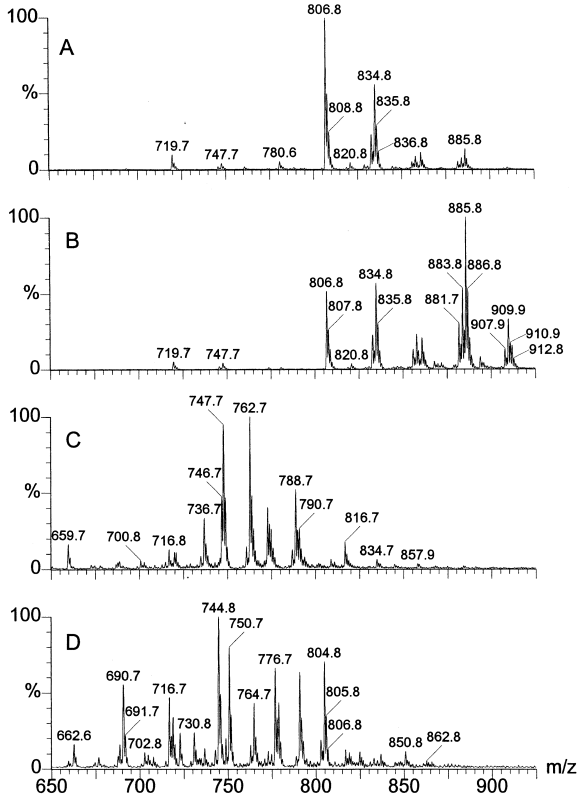

Fig. 2. Negative ion LC–ES-MS–MS mass spectra obtained from the reconstructed negative ion chromatogram in Fig. 1B. The molecular ions of the following Atlantic salmon head kidney phospholipid classes are shown; (A) PS; (B) PI; (C) PE; (D) PC. The mass spectra were acquired under peaks with retention times corresponding to the respective synthetic phospholipid classes. Conditions are described in the Experimental section.

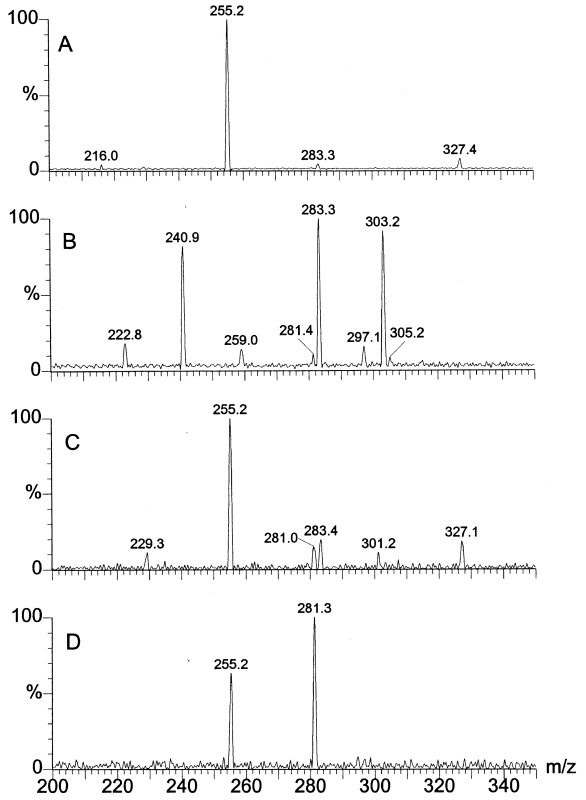

Fig. 3. Negative ion LC–ES-MS–MS product ion spectra of the major molecular ions from each of the four Atlantic salmon head kidney phospholipid classes studied. The product ion spectra are from the molecular ions at; (A) *m*/*z* 806.6 (PS); (B) *m*/*z* 885.7 (PI); (C) *m*/*z* 762.6 (PE); (D) *m*/*z* 744.7 (PC). The different molecular ions were selected by MS1 and fragmented by CID and the resulting product ions were detected by MS2. The product ion spectra were acquired under peaks with retention times corresponding to the respective synthetic phospholipid classes. Conditions are described in the Experimental section.

PS. Similarly, the product ion spectra of the molecular ions at *m*/*z* 885.7 (Fig. 3B), *m*/*z* 762.6 (Fig. 3C) and *m*/*z* 744.7 (Fig. 3D) show carboxylate anion fragments that, together with the retention time of the respective RIC peaks (not shown), confirmed the identity of the following species; 18:1/20:3 PI and 18:0/20:4 PI (based on fragments at *m*/*z* 281.4/305.2 and *m*/*z* 283.3/303.2 in Fig. 3B), 16:0/22:6 PE and 18:1/20:5 PE (based on fragments at *m*/*z* 255.2/327.1 and *m*/*z* 281.0/301.2 in Fig. 3C), and 16:0/18:1 PC (based on fragments at *m*/*z* 255.2/281.3 in Fig. 3D). The additional fragments found in Fig. 3B (*m*/*z* 222.8, *m*/*z* 240.9, *m*/*z* 259.0 and *m*/*z* 297.1) are characteristic for the product ion spectra of PI molecular ions [10]. In both Fig. 3A and C the carboxylate anion fragment at *m*/*z* 283.3 was found. We have no explanation for the presence of this fragment, since from the retention time of the RIC peak and the mass of the respective molecular ions, this indicates 18:0/20:6 PS and PE species. As far as we know this is impossible.

As shown in Fig. 3 and as previously reported [14–16,27,30], the abundance of the carboxylate anion peaks from the *sn*-1 and *sn*-2 position may differ in the product ion spectrum of a phospholipid molecular ion. Due to this fact and the low abundance of several of the phospholipid molecular ions

(see Fig. 2), some product ion spectra showed only one carboxylate anion fragment (not shown). In these cases the identity of the molecular species was confirmed on the basis of a single carboxylate anion fragment. This is a valid assumption since the retention time, molecular mass and one carboxylate anion fragment supports the conformation of identity. The retention time repeatability of the different molecular species was ≈0.2% relative standard deviation. Furthermore, to verify the identity of each molecular species, the carboxylate anion at the *sn*-2 position was determined by MRM (not shown). This was possible since MRM analysis is more sensitive than product ion spectrum analysis. Tables 2–5 show the salmon head kidney phospholipid species identified in the four phospholipid classes.

3.3. Assignment of the fatty acids to the sn-1 and sn-2 positions

Under the present conditions, for PC and PE species, the intensity of the *sn*-1 carboxylate anion peak was less than that of the *sn*-2 when the carbon chain length of both fatty acids were below 20. The *sn*-1 to *sn*-2 abundance ratios of e.g. 16:0/16:1 PC and 18:0/18:1 PE species from salmon head kidney, were determined to be 0.85 (0.01) and 0.37 (0.02) (mean (SD), $n=3$), respectively. This is of course based on the assumption that 16:1 and 18:1 is in the *sn*-2 position. The intensity ratio of 18:0/18:1 PE estimated in this study was similar to the intensity ratio of the same species in human blood measured in a previous study [27]. When the carbon chain length of the *sn*-2 fatty acid was≥20 the intensity ratio of *sn*-1 to *sn*-2 was above 1 for PC and PE species (not shown for PC; see Table 6 for some PE data). For PC and PE species, therefore, the carboxylate anion with the less intense peak was assigned to the *sn*-1 position when the carbon chain length of both fatty acids were <20. Otherwise, when the carbon chain length of one (or both) of the fatty acids was ≥20, the carboxylate anion with the less intense peak was assigned to the *sn*-2 position.

The assignment of the fatty acids to the *sn*-1 and *sn*-2 positions for PI was more straightforward. All the species identified contained fatty acids with carbon chain length of 16 or 18 (saturated or with one double bond) combined with carbon chain lengths of 20 or 22 (varying number of double bonds) (see Table 4). The *sn*-1 to *sn*-2 abundance ratio of the 16:0/20:4 PI species was approximately 1 (see Fig. 3B). This is similar to what has previously been reported [27]. With increasing number of double bonds this ratio increased as for the other phospholipid classes (not shown for PI, see Table 6 for PS and PE data). For PI the fatty acids with carbon chain lengths of ≥20 were, therefore, always assigned to the *sn*-2 position.

In contrast to the other phospholipid classes and as previously reported [27,30], the *sn*-1 to *sn*-2 abundance ratios of the PS species were always above 1 (see Table 6 for some results on the abundance ratios of PS). The carboxylate anion with the less intense peak in the product ion spectrum of the PS species was, therefore, always assigned to the *sn*-2 position.

3.4. Variations of the sn-1 to sn-2 abundance ratio

Previous observations have shown that the degree of dissociation of the *sn*-2 fatty acid relative to the *sn*-1 fatty acid was dependent on the *sn*-2 acyl substituent in the phospholipids [15,27,30]. Some of the PS and PE species identified in the salmon head kidney were used to systematically study whether the variation of the product ion peak area ratio of the *sn*-1 to *sn*-2 carboxylate anion was more affected by chain length than the number of double bonds in the acyl substituents. When the carbon chain length of the *sn*-1 and *sn*-2 fatty acids remained the same, e.g. 16:0 or 18:0 in *sn*-1 and 20 or 22 with varying number of double bonds in *sn*-2, the *sn*-1 to *sn*-2 abundance ratio increased for both PS and PE species with an increase in the number of double bonds in the *sn*-2 fatty acid (Table 6). This was not evident in the opposite situation i.e. when comparing the *sn*-1 to *sn*-2 abundance ratios of 18:0/22:6, 18:1/22:6 and 18:2/22:6 PS and PE species, surprisingly little difference was found between them (Table 6). Since the abundance ratio of the 16:0/20:5 species was larger than the abundance ratio of the 16:0/22:5 species, no evidence was found for an increase in the abundance ratio with an increase of the *sn*-2 chain length (Table 6). However, when comparing the *sn*-1 to *sn*-2 abundance ratios of 16:0/20:5 with 18:0/20:5 and 16:0/22:6 with 18:0/22:6, the abundance ratios were higher in the species

with 16:0 in the *sn*-1 position (Table 6). Indicating that the carbon chain length of the fatty acid in the *sn*-1 position to some extent affects the degree of dissociation of this fatty acid relative to the *sn*-2 fatty acid.

3.5. Relative molecular species distribution of the different phospholipid classes

The relative distribution of the phospholipid species identified in the phospholipid classes was determined by MRM of the *sn*-1 carboxylate anion peak area as described in the Experimental section. Tables 2–5 show that there was a large difference in the phospholipid molecular species distribution between the different phospholipid classes. The major molecular species in PC was the 16:0/18:1 species (Table 2). In addition, PC also had, depending on the diet, a large amount of 16:0/18:2, 16:0/16:0 and 16:0/16:1 species. PC was the only phospholipid class that was found to contain species with the 14:0 fatty acid (14:0/14:0, 14:0/16:1, 16:0/14:0, and 14:0/22:6). PC also contained some species with the 20:5 and 22:6 fatty acids. The main 20:5 and 22:6 containing species were 16:0/20:5 and 16:0/22:6, respectively (Table 2).

Table 3 shows that there was more molecular species identified in the PE class compared to the other phospholipid classes. The major molecular species in PE was the 16:0/22:6 species. In addition, PE also contained, depending on the diet, a large amount of other 22:6 fatty acid containing species, namely 18:2/22:6, 18:1/22:6, 18:0/22:6 and 20:1/22:6. Of the 20:5 fatty acid containing species in PE, the most prominent were 16:0/20:5, 18:2/20:5, 18:1/20:5 and 18:0/20:5 (Table 3). Similar to PC, PE also contained a large amount of 16:0/18:1 and 16:0/18:2.

Table 4 shows that PI was the only phospholipid class containing major amounts of the 20:4 fatty acid species, namely 18:0/20:4, which was the main phospholipid species in PI. PI also contained some 18:1/20:4 and 16:0/20:4. The 20:5 and 22:6 fatty acid containing species in PI were found as 16:0/20:5, 18:1/20:5 and 18:0/20:5, and 18:0/22:6, 16:0/22:6 and 18:1/22:6, respectively. As opposed to the other phospholipid classes, no 18:2 fatty acid containing species was found in PI (Table 4).

Table 5 shows that, similar to PE, the most prominent molecular species in PS was found to be 16:0/22:6. In addition, there was a great deal of the 18:0/22:6 species. The 22:6 fatty acid species dominated in PS and like PE, the 20:1/22:6 species was also found in PS. In addition to the 22:6 containing

Table 2
Molecular species of PC from extracted salmon head kidney after 3 months on the experimental diets

Molecular species	m/z[d] $[M-15]^-$	50SO–50FO Mean (SD)	SO Mean (SD)	FO Mean (SD)
14:0/14:0	663	3.4[b] (0.9)	0.85[c] (0.3)	9.6[a] (0.7)
14:0/16:1	689	1.9[b] (0.2)	0.65[c] (0.1)	4.3[a] (0.4)
16:0/14:0	691	13.0[b] (1.8)	8.2[c] (2.3)	19.6[a] (0.2)
16:0/16:1	717	7.7[b] (0.3)	5.4[c] (0.1)	9.8[a] (0.5)
14:0/18:1	717	4.0[b] (0.2)	2.0[c] (0.2)	6.0[a] (0.2)
16:0/16:0	719	12.1[b] (0.4)	16.4[a] (1.6)	8.8[c] (0.5)
16:0/18:2	743	10.8[b] (0.7)	19.1[a] (0.8)	2.1[c] (0.1)
16:0/18:1	745	28.0[a] (1.4)	30.8[a] (3.4)	21.5[b] (0.6)
16:0/18:0	747	0.73[b] (0.08)	1.1[a] (0.1)	0.43[c] (0.06)
14:0/22:6	763	1.6[b] (0.1)	0.61[c] (0.08)	2.6[a] (0.1)
16:0/20:5	765	4.7[b] (0.03)	3.2[c] (0.1)	5.7[a] (0.8)
18:1/18:2	769	1.4[b] (0.1)	2.3[a] (0.1)	0.28[c] (0.01)
16:0/22:6	791	9.5[a] (2.1)	8.5[a] (1.5)	7.7[a] (0.1)
18:1/20:5	791	1.4[a,b] (0.2)	1.1[b] (0.3)	1.6[a] (0.2)

[a,b,c] Extracted head kidney from three salmons in each feeding group was analysed. The values are the mean of the relative amount (in %) and the standard deviation is given in parentheses (SD). Values marked with different superscript letters (a, b and c) are significantly different, based on 95% confidence interval. See Section 2.6 for the statistical method used.

[d] The mass of all ions was rounded to nearest integer.

Table 3
Molecular species of PE from extracted salmon head kidney after 3 months on the experimental diets

Molecular species	$[M-H]^-$ m/z[d]	50SO–50FO Mean (SD)	SO Mean (SD)	FO Mean (SD)
16:0/18:2	715	3.9[b] (0.7)	6.9[a] (0.5)	1.0[c] (0.1)
16:0/18:1	717	5.9[a] (1.1)	5.9[a] (0.6)	6.7[a] (0.3)
16:0/20:5	737	8.5[b] (0.7)	5.5[c] (1.0)	13.9[a] (0.6)
16:0/20:4	739	1.2[b] (0.1)	2.8[a] (0.6)	1.6[a] (0.1)
18:2/18:2	739	1.0[b] (0.1)	2.8[a] (0.2)	0.07[c] (0.02)
18:1/18:2	741	3.2[b] (0.6)	4.6[a] (0.6)	1.1[c] (0.1)
18:1/18:1	743	0.78[b] (0.2)	0.59[b] (0.13)	1.1[a] (0.2)
18:0/18:2	743	0.70[b] (0.03)	1.5[a] (0.1)	0.15[c] (0.02)
18:0/18:1	745	0.47[a] (0.13)	0.46[b] (0.01)	0.41[c] (0.07)
18:0/18:0	747	0.59[a] (0.04)	0.45[b] (0.06)	0.69[a] (0.06)
18:2/20:5	761	4.1[a] (0.1)	4.2[a] (0.2)	1.5[b] (0.1)
16:0/22:6	763	26.6[b] (0.7)	22.4[c] (0.7)	29.7[a] (1.3)
18:1/20:5	763	4.3[b] (0.3)	2.6[c] (0.2)	7.3[a] (0.2)
16:0/22:5	765	1.4[b] (0.1)	1.1[c] (0.1)	1.9[a] (0.1)
18:0/20:5	765	2.9[a] (0.03)	2.4[c] (0.1)	2.7[b] (0.05)
18:1/20:3	767	0.52[b] (0.07)	1.3[a] (0.1)	0.20[c] (0.05)
18:1/20:2	769	0.23[b] (0.05)	0.37[a] (0.02)	ND
18:2/22:6	787	11.6[b] (1.2)	15.5[a] (1.8)	3.5[c] (0.1)
18:1/22:6	789	11.8[b] (0.5)	9.0[c] (0.2)	15.7[a] (0.8)
18:0/22:6	791	7.4[a] (0.8)	8.5[a] (0.8)	5.5[b] (0.4)
18:0/22:5	793	0.26[a] (0.06)	0.34[a] (0.06)	0.27[a] (0.04)
20:1/22:6	817	2.6[b] (0.8)	0.77[c] (0.20)	4.9[a] (0.9)

[a,b,c] Extracted head kidney from three salmons in each feeding group was analysed. The values are the mean of the relative amount (in %) and the standard deviation is given in parentheses (SD). Values marked with different superscript letters (a, b and c) are significantly different, based on 95% confidence interval. See Section 2.6 for the statistical method used.

[d] The mass of all ions was rounded to nearest integer.

Table 4
Molecular species of PI from extracted salmon head kidney after 3 months on the experimental diets

Molecular species	$[M-H]^-$ m/z[d]	50SO–50FO Mean (SD)	SO Mean (SD)	FO Mean (SD)
16:0/20:5	856	3.1[b] (0.6)	1.2[c] (0.1)	5.4[a] (0.5)
16:0/20:4	858	7.1[b] (0.8)	5.9[c] (0.6)	10.0[a] (0.3)
16:0/20:3	860	2.4[a] (0.2)	1.8[b] (0.2)	1.1[c] (0.1)
16:0/22:6	882	6.7[b] (0.3)	4.9[c] (0.2)	8.6[a] (0.2)
18:1/20:5	882	2.3[b] (0.4)	0.88[c] (0.14)	3.9[a] (0.2)
18:0/20:5	884	9.4[b] (0.4)	4.4[c] (0.8)	10.6[a] (0.6)
18:1/20:4	884	5.0[b] (0.4)	5.8[b] (0.7)	7.0[a] (0.2)
18:0/20:4	886	42.3[b] (0.8)	55.3[a] (1.1)	36.0[c] (1.0)
18:1/20:3	886	2.1[a] (0.3)	2.0[a] (0.3)	0.88[b] (0.19)
18:0/20:3	888	5.5[a] (0.4)	6.5[a] (1.5)	1.6[b] (0.1)
18:1/22:6	908	3.5[b] (0.1)	2.0[c] (0.1)	4.6[a] (0.2)
18:0/22:6	910	9.7[a] (1.7)	8.6[a] (0.7)	9.2[a] (0.3)
18:0/22:5	912	0.92[a,b] (0.18)	0.68[b] (0.09)	0.99[a] (0.15)

[a,b,c] Extracted head kidney from three salmons in each feeding group was analysed. The values are the mean of the relative amount (in %) and the standard deviation is given in parentheses (SD). Values marked with different superscript letters (a, b and c) are significantly different, based on 95% confidence interval. See Section 2.6 for the statistical method used.

[d] The mass of all ions was rounded to nearest integer.

Table 5
Molecular species of PS from extracted salmon head kidney after 3 months on the experimental diets

Molecular species	m/z[d] $[M-H]^-$	50SO–50FO Mean (SD)	SO Mean (SD)	FO Mean (SD)
16:0/18:2	759	0.19[b] (0.04)	0.46[a] (0.07)	0.073[c] (0.016)
16:0/18:1	761	0.56[a] (0.18)	0.67[a] (0.18)	0.57[a] (0.07)
16:0/20:5	781	1.3[b] (0.1)	1.0[b] (0.2)	2.4[a] (0.1)
16:0/20:4	783	0.23[b] (0.01)	0.62[a] (0.14)	0.37[b] (0.03)
16:0/20:3	785	0.35[b] (0.002)	1.1[a] (0.1)	0.10[c] (0.01)
16:0/20:2	787	0.076[b] (0.004)	0.18[a] (0.06)	0.034[b] (0.011)
18:0/18:2	787	0.31[b] (0.04)	0.69[a] (0.12)	0.043[c] (0.011)
18:0/18:1	789	0.42[a] (0.24)	0.45[a] (0.12)	0.27[a] (0.08)
16:0/22:6	807	40.4[b] (1.1)	35.7[b] (2.9)	46.4[a] (3.4)
16:0/22:5	809	3.1[b] (0.2)	2.5[c] (0.1)	4.1[a] (0.1)
18:0/20:5	809	1.3[a] (0.1)	1.1[a] (0.1)	1.3[a] (0.04)
18:0/20:4	811	0.41[b] (0.09)	0.98[a] (0.21)	0.37[b] (0.02)
18:0/20:3	813	0.54[b] (0.04)	1.5[a] (0.1)	0.099[c] (0.008)
18:2/22:6	831	1.7[b] (0.2)	2.2[a] (0.2)	0.70[c] (0.03)
18:1/22:6	833	7.9[b] (0.3)	5.0[c] (0.2)	11.8[a] (0.5)
18:0/22:6	835	34.8[b] (0.6)	41.9[a] (3.3)	21.9[c] (3.0)
18:1/22:5	835	0.37[a] (0.10)	0.21[a] (0.02)	0.48[a] (0.17)
18:0/22:5	837	2.4[a] (0.1)	2.4[a] (0.4)	2.1[a] (0.05)
20:1/22:6	861	3.6[b] (0.1)	1.3[c] (0.2)	6.9[a] (0.7)

[a,b,c] Extracted head kidney from three salmons in each feeding group was analysed. The values are the mean of the relative amount (in %) and the standard deviation is given in parentheses (SD). Values marked with different superscript letters (a, b and c) are significantly different, based on 95% confidence interval. See Section 2.6 for the statistical method used.

[d] The mass of all ions was rounded to nearest integer.

species, there was some 20:5 fatty acid containing species, mainly as 16:0/20:5 and 18:0/20:5. There was also some 18:2 fatty acid containing species in PS but only as minor products, the most dominant was the 18:2/22:6 species (Table 5).

3.6. Effect of diet on the molecular species distribution

Tables 2–5 show a similar pattern of the overall effect of the diets on the relative amount of the molecular species in the different phospholipid classes, i.e. with a relative increase in one molecular species, in e.g. the SO diet group, there was a corresponding decrease in the same species in the FO diet group with the 50SO/50FO diet group in between. Therefore only the effects of the SO and FO diets on the molecular species distribution are considered.

Tables 2–5 show that the main effects of the SO diet was a significant increase in the 18:2 fatty acid containing species when compared to the FO diet group for all the phospholipid classes. In fact all the 18:2 fatty acid containing species increased significantly in the SO diet group. In addition, the SO diet also significantly increased the 18:0/20:4 species found in PI and PS (Tables 4 and 5). However, as Table 4 shows the 16:0/20:4 and 18:1/20:4 species in PI did not increase in the SO diet group, on the contrary the two species decreased when compared to the FO diet group. Furthermore, all the 20:3 containing species (found in all phospholipid classes except for PC) was significantly increased in the SO diet group compared to the FO diet group.

Most of the 20:5 and 22:6 fatty acid containing species decreased significantly in the SO diet group when compared to the FO diet group. However, as Tables 2– 5 show, this was not a general trend. The 18:2/20:5 (PE), 18:2/22:6 (PE and PS) and 18:0/22:6 (PE, PI and PS) species did not decrease in the SO diet group. On the contrary, these species were significantly increased in the SO diet group compared to the FO diet group, except for 18:0/22:6 (PI) (Table 4), where no significant difference was found between the diet groups. In addition to the decrease

of most of the 20:5 and 22:6 fatty acid containing species in the SO diet group, there was also a significant decrease of the PC 14:0 fatty acid containing species compared to the FO diet group (Table 2).

4. Discussion

We have previously reported that the ratio of the *sn*-1 to *sn*-2 carboxylate anion fragments differ depending on the phospholipid class, the intensity of the collision energy and the fatty acids attached to the *sn*-2 position [27]. Other authors have also reported similar observations [15,30]. In this study we show that with increasing number of double bonds in the *sn*-2 fatty acid, the extent of dissociation to form this carboxylate anion decreased relative to the dissociation of the *sn*-1 fatty acid (Table 6). The same was not found with an increase in the chain length of the *sn*-2 fatty acid (Table 6). An increasing number of double bonds in a fatty acid generate a change in the shape of the hydrocarbon chain, which clearly has a strong influence on the degree of dissociation of the *sn*-2 fatty acid. With an increase in the number of double bonds in the *sn*-1 fatty acid, there was no change in the dissociation of this fatty acid relative to the *sn*-2 fatty acid (Table 6). Although there was some variation of the *sn*-1 fatty acid dissociation depending on the carbon chain length of the fatty acid, it is safe to say that the dissociation of the *sn*-1 fatty acid is more consistent than that of the *sn*-2 fatty acid. To determine the relative distribution of the individual molecular species in a specific phospholipid class by negative ion LC–ES-MS–MS and multiple reaction monitoring of the carboxylate anions it is, therefore, important to follow the carboxylate anion in the *sn*-1 position.

As far as we know there is only one previous publication on the distribution of phospholipid molecular species in fish tissue determined by tandem mass spectrometry [31]. Liquid secondary ion mass spectrometry combined with tandem mass spectrometry and other analytical techniques were used to study the main phospholipid species in rainbow trout liver [31]. The main phospholipid species were reported to be; 16:0/22:6 (PC); 20:1/22:6 (PE), 18:0/22:6 (PE), 18:1/22:6 (PE), 18:2/22:6 (PE), 16:0/22:6 (PE), 16:0/22:6 (PS), 18:0/22:6 (PS) and no molecular species containing 22:6 was found in PI [31]. The molecular species distribution of the phospholipid classes in salmon head kidney determined in the present study was surprisingly similar to that found in rainbow trout liver. We found that the main molecular species in PE were 16:0/22:6,

Table 6
Product ion peak area ratio of *sn*-1 to *sn*-2 carboxylate anion from different salmon head kidney molecular species of PS and PE[a]

Molecular species of PS	Mean ratio (SD)	Molecular species of PE	Mean ratio (SD)
16:0/20:5 (n=4)	9.6 (0.6)	16:0/20:5 (n=3)	3.1 (0.2)
16:0/20:4 (n=4)	5.6 (0.8)	16:0/20:4 (n=3)	1.2 (0.1)
16:0/20:3 (n=4)	3.4 (0.1)		
16:0/20:2 (n=4)	2.6 (0.1)	16:0/22:6 (n=9)	6.7 (0.4)
		16:0/22:5 (n=9)	1.4 (0.2)
16:0/22:6 (n=9)	16.7 (0.7)		
16:0/22:5 (n=9)	5.2 (0.3)	18:2/20:5 (n=3)	2.3 (0.2)
		18:1/20:5 (n=3)	2.4 (0.2)
18:0/20:5 (n=3)	6.2 (0.6)	18:0/20:5 (n=3)	2.6 (0.4)
18:0/20:4 (n=3)	3.9 (0.7)		
18:0/20:3 (n=3)	2.8 (0.2)	18:2/22:6 (n=3)	4.9 (0.8)
		18:1/22:6 (n=3)	4.8 (0.9)
18:2/22:6 (n=3)	11.2 (1.7)	18:0/22:6 (n=9)	5.7 (0.5)
18:1/22:6 (n=3)	11.6 (1.2)		
18:0/22:6 (n=9)	11.5 (0.5)		

[a] Results are expressed as the mean ratio and the standard deviation is given in parentheses (SD). The number of independent analysis for each species is indicated in parentheses (n=).

18:2/22:6, 18:1/22:6 and 18:0/22:6, and in addition, 16:0/18:2, 16:0/18:1 and 16:0/20:5 (Table 3). The main molecular species in PS were 16:0/22:6 and 18:0/22:6 (Table 5). In contrast to rainbow trout liver, we found that the main molecular species in salmon head kidney PC was 16:0/18:1 but, in addition, we also found substantial amounts of 16:0/22:6 (Table 2). As opposed to rainbow trout liver, we also found some 22:6 containing species in salmon head kidney PI, namely 16:0/22:6, 18:1/22:6 and 18:0/22:6 (Table 4). The main species in salmon head kidney PI were, however, 20:4 containing species. This has also previously been shown in different tissues of the Atlantic salmon when the fatty acid distribution in the phospholipids was determined [32–35].

The individual molecular species distribution of the different phospholipid classes in the Atlantic salmon head kidney showed some interesting differences (Tables 2–5). PC was the only phospholipid class that was shown to incorporate 14:0 into phospholipid species. The major phospholipid species in PC was 16:0/18:1. This species was also found in PE but not in the other two classes. The 18:2 fatty acid was mainly incorporated into species found in PE and PC. Some minor species with 18:2 was also found in PS while no species with 18:2 was found in PI. The majority of the 22:6 containing species was found in PE and PS with some in PC and PI. The major phospholipid species in PE and PS was the same, namely 16:0/22:6. The 20:5 containing species was found in all phospholipid classes in relative minor amounts except for PE, where the 20:5 containing species accounted for approximately 20% of the total amount.

As Tables 2–5 show there was a significant change in the molecular species distribution in salmon head kidney depending on the diet. The SO diet contained substantial amounts of the 18:2 n-6 (LA) fatty acid (Table 1). This was also reflected in the molecular species distribution with a significant increase of all the 18:2 containing species in the SO diet groups (Tables 2, 3 and 5). The same effect of diets containing a high amount of the 18:2 n-6 fatty acid has also previously been reported in different Atlantic salmon tissues when the fatty acid distribution in the phospholipids was determined [32–35]. They also found an increase of the 20:3 n-6 fatty acid, probably due to the elongation and desaturation (Δ-6-desaturase) of the 18:2 n-6 fatty acid, and an increase of the 20:4 n-6 in PC, PE and PS. The 20:4 n-6 is a Δ-5-desaturase product of 20:3 n-6. We also found a significant increase of the 20:3 containing species in the SO diet group compared to the FO diet group. The same was found for the 20:4 containing species in PE and PS. In PI the SO diet significantly increased the 18:0/20:4 species while the 16:0/20:4 and the 18:1/20:4 species were significantly decreased compared to the FO diet. The decrease of the 18:1/20:4 species is difficult to explain since the SO diet contained more of these fatty acids compared to the FO diet. However, it has previously been shown that there was no increase of the 18:1 n-7 and 18:1 n-9 fatty acids in Atlantic salmon gill cells and leucocytes with increasing amount of these fatty acids in the diet [33].

The SO diet significantly decreased all the 20:5 containing species, except for the PE 18:2/20:5 species when compared to the FO diet, while the 22:6 containing species did not show the same general trend. This confirms previous results [32–35].

5. Conclusion

We have successfully determined 68 phospholipid molecular species from the Atlantic salmon head kidney. The effect of different diets on the relative distribution of these species show the same general trend as reported previously in other Atlantic salmon tissues. The LC–ES-MS–MS method applied makes it possible to determine the phospholipid species directly on line without a previous purification of the phospholipid classes. However, using this method it is not possible to assign the correct position of the double bond in the carbon chain of the fatty acids.

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