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Structure and cardioprotective activities of polar lipids of olive pomace, olive pomace-enriched fish feed and olive pomace fed gilthead sea bream *(Sparus aurata)*

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Abstract

Total lipids of olive pomace (OP), olive pomace diet (OP diet), fish oil diet (FO diet) and fish fillets of farmed gilthead sea bream (fish fed with FO diet and OP diet respectively) were extracted and separated into polar (TPL) and neutral (TNL) lipids. All samples were assessed for their *in vitro* activity against washed rabbit platelets aggregation induced by Platelet Activating Factor (PAF) and they were further analyzed by electrospray-mass spectrometry. The high levels of palmitic (16:0), oleic (18:1 cis ω-9), linoleic (18:2 ω-6) and docosapentanoic acid (DPA 22:5 ω-3) contained in both OP and FO diets are reflected to the gilthead sea breams fed with the individual diet respectively, while the gilthead sea bream fed with FO diet displays a decrease in DPA. All samples contained various glycerophospholipids species. Two PE species were identified in OP, OP diet and fish fed with OP diet and not in FO diet, while that might be an indication that these substances are likely to be the key polar phospholipids that have the ability to be *in vitro* PAF inhibitors, i.e. inhibit the formation of atherosclerotic plaques in blood arteries.

KEYWORDS: Gilthead sea bream (*Sparus aurata*); olive pomace; cardioprotective properties; tandem mass spectrometry; glycerophospholipids.

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1. Introduction

The requirement within the aqua feed industry to find and implement sustainable alternatives to fish oils (FO)[1] is gradually increasing. Mostly due to the fact that the worldwide availability of fish, which is the main source of fishmeal and FO, is expected to remain stable or even to decrease in future decades. Meanwhile the consumption of fish and fish-derived products has considerably increased over recent decades partly due to their omega-3 (*ω*-3) fatty acids that confer benefits for the risk reduction of cardiovascular disease (CVD) (Hu et al., 2002; Levitan, Wolk, & Mittleman, 2009; Mozaffarian, 2008), making them very important components of adult nutrition, while there is an evidence that fish oil has the ability to decelerate the formation of plaque in the arteries (Wang et al., 2006). The use of FO in aquaculture is the key impediment on the future growth and sustainability of the industry. FO, being the most widely available dietary source of health-beneficial omega-3, long-chain polyunsaturated fatty acids (ω-3 LC-PUFA), ranges remarkably in supply and cost. The most severe product from a food security point of view, is that FO is extracted unsustainably from world oceans (Bimbo, 2015). As a result, in aquaculture today, we are facing this paradox: for the production of FO we need wild sardines whose overfishing is probably not sustainable. Current trends in aquaculture are now towards the lowering of our dependence on FO; research is now thus focusing towards

[1] **Abbreviations.** OP, olive pomace; FO, fish oil; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; TL, total lipids; TPL, total polar lipids; TNL, total neutral lipids; PL, phospholipid; TAG, triacylglycerol; DAG, diacylglycerol; FAME, fatty acid methyl ester; PAF, platelet activating factor; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentanoic acid; IC50, inhibitory concentration at fifty percent; HPLC-UV, high performance liquid chromatography-ultra violet; GC-FID, gas chromatography-flame ionization detector; GC-MS, gas chromatography-mass spectrometry; ES-MS, electrospray-mass spectrometry; CVD, cardiovascular disease.

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alternative oil-sources in global level, in order to reduce the aquaculture industry's dependence on FO and the corresponding overfishing of sardines' stocks.

The state of the world fishery resources, which provides the raw material for fish meal and fish oil production, has been a bone of contention in many quarters (De Silva, Francis, & Tacon, 2011). Recent data show that the proportion of fisheries production used for direct human consumption increased from about 71% in the 1980s to more than 86% (136 million tons) in 2012, with the remainder 14% (21.7 million tons) destined to non-human food uses (FAO (Food and Agriculture Organization), 2014) (e.g. fishmeal and fish oil). FO from small pelagic fish represents a finite fishery resource and is not expected to be able to sustain the rapid expansion of the global aquaculture industry. The overwhelming evidence that ω-3 LC-PUFA have a significant impact on many aspects of human health means that lipids/fatty acids derived from fish have an important role in consumer perception. This is the reason why managing the lipid resources that we use in fish aquaculture more sustainably is vital for the future of aquaculture production, as well as consumer perception that it is a product with health benefits.

Therefore, over the past few years there is a currently great urgency within the aqua feed industry for identifying and using alternative plant-derived oils in formulated fish feeds in order to reduce dependence upon marine FO as well as to reduce costs (Alexis, 1997; Izquierdo et al., 2005; Nasopoulou & Zabetakis, 2012; Wassef, Saleh, & El-Abd El-Hady, 2008). The most common vegetable oils used previously for fish feed production have been soybean, linseed, rapeseed, sunflower, palm oil and olive oil. Partial replacement of FO by these vegetable oils is only possible when the fatty acids present in the diets in sufficient quantities to meet the essential fatty requirements of the fish and ultimately the human.

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A promising alternative plant lipid source is olive pomace (OP), which is a natural agricultural by-product of olive oil production, that contains constituents with atheroprotective activity such as PAF (Platelet Activating Factor) inhibitors (Demopoulos, Pinckard, & Hanahan, 1979; Karantonis et al., 2008; Nasopoulou & Zabetakis, 2013) according to "The PAF implicated atherosclerosis theory"(Demopoulos, Karantonis, & Antonopoulou, 2003). Extensive research has been carried out in our laboratory on olive oil by-products and fish feeds supplemented with these products, with respect to their capacity to prevent or delay the process of atherogenesis and thus prevent the subsequent development of cardiovascular diseases (CVDs). Therefore, the presence of PAF-inhibitors or PAF-antagonists in OP highlights the importance of this resource in terms of cardio-protection. In addition, olive oil (Karantonis et al., 2006) and olive pomace polar lipids (Tsantila et al., 2007) possess *in vivo* antiatherogenic properties, while a separate *in vivo* study (Nasopoulou et al., 2010) confirmed that phospholipids from gilthead sea breams (*Sparus aurata*) can reduce the thickness of atherosclerotic lesions in hypercholesterolemic rabbits. In addition, it has been demonstrated that, the feeding of OP supplemented fish feeds to fish results in an improvement in its ability to prevent atherogenesis and therefore heart diseases (Nasopoulou, Stamatakis, Demopoulos, & Zabetakis, 2011).

Taking into consideration the benefits of fish oil replacement by alternative plant sources in fish feeds as reviewed recently by our team (Nasopoulou & Zabetakis, 2012) and the capacity of OP to prevent atherogenesis and therefore the onset of CVDs, our group has further focused to the use of OP in fish feeds. The effect of OP in fish feed (OP-diet) and in gilthead sea bream muscle (*Sparus aurata* fed with OP-diet) by HPLC-UV (Nasopoulou et al., 2013) has been studied. In the

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current paper, we have extended that work by carrying out ES-MS-MS lipidomic analysis of OP, OP-diet and gilthead sea bream fed with OP-diet with the scope to identify in fish feed and fish anti-atherogenic compounds of OP origin. Such data would suggest to upscale OP usage in the sustainable production of aquacultured gilthead sea bream (*Sparus aurata*); a fact that would lead to the improvement of the aquatic food security.

2. Materials and methods

2.1. Reagents and instrumentation

All reagents and solvents were of analytical grade and purchased by Merck (Darmstadt, Germany). Fatty acid methyl ester (FAME) standards were of gas chromatographic (GC) quality and supplied by Sigma-Aldrich (St. Louis, MO, USA), as well as bovine serum albumin (BSA) and PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine). Platelet aggregation was measured in a Chrono-Log (Havertown, PA, USA) aggregometer (model 400-VS) coupled to a Chrono-Log recorder (Havertown, PA, USA). The lipidomic analysis was conducted with an Absceix 4000 QTrap, a triple quadrupole mass spectrometer equipped with a nanoelectrospray source while the FAME products were analysed by gas chromatography-flame ionization detector (GC-FID) on an Agilent Technologies J&W with a DB-23 fused silica capillary column (60 M x 0.251 mm i.d., 0.25 μm; Agilent, Santa Clara California, USA) and by gas chromatography-mass spectrometry (GC–MS) on an Agilent Technologies (GC-6890 N, MS detector-5973) with a ZB-5 column (30 M x 0.25 mm i.d., x 0.25 μm film thickness, Phenomenex).

2.2. Fish diets analysis

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Olive pomace (OP), the experimental fish feed enriched with OP (OP diet), compounded by adding 8% OP prior to the extrusion, following the principles of fish nutrition (Gatlin, 2010) and the reference fish feed – fish oil diet (FO diet) – containing 100% fish oil (anchovy oil), were analyzed for a number of nutritional parameters. Protein digestibility determination took place according to van Leeuwen *et al*. (1991) (Leeuwen, Kleef, Kempen, Huisman, & Verstegen, 1991) and energy determination took place according to the following equation (Gatlin, 2010):

Energy (MJ/Kg) = {(CPg * 23.6KJ) + (CFg * 39.5KJ) + ([CFig.+NFEg] * 17.4KJ)}/1000, where CP: crude protein; CF: crude fat; CFi: crude fiber; NFE=1000-(CP+CF+Ash+Moist).

OP originated from a local olive oil production. Both diets were formulated at the facilities of the marine farm where the dietary experiment took place using a twin-screw extruder creating pellets, followed by the addition of oil mixture. The pellets were dried, sealed and kept in air-tight bags until use. The analysis of OP and both diets is given in Supplemental table 1.

2.3. Fish sampling

Cultured gilthead sea bream (*Sparus aurata),* raised with different diets and of initial mean body weight 350-400g were obtained from a commercial marine farm located in the suburbs of Athens. In total, two types of samples were used (gilthead sea breams fed with OP diet and FO diet). Approximately 100 fish specimens were collected per each dietary treatment and transported in ice to the laboratory. The mass of 30 fish samples was weighted and 5 of them were beheaded, chopped and filleted and only the fillets were used in the subsequent experiments.

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2.4. Isolation of lipids extracts

Total lipids (TL) of OP, OP diet, FO diet and fish fillets of aquacultured gilthead sea bream fed with OP and FO diet, were extracted according to the Bligh - Dyer method (Bligh & Dyer, 1959). One tenth of the TL was weighed and stored at -20° C while the rest of it was further separated into polar lipids (PL) and neutral lipids (NL) by counter-current distribution (Galanos & Kapoulas, 1962). The upper phase of petroleum ether contained the total neutral lipids while the lower phase of ethanol with total polar lipids were selected in a glass-stoppered flask, evaporated at 30 °C on the rotary evaporator, weighted, dissolved in chloroform/methanol (1:1), and stored at −20 °C until further analysis.

2.5. Biological assay

Purified polar lipid fractions of OP, OP diet, FO diet and fish fillets of aquacultured gilthead sea bream fed with OP and FO diet respectively, were tested for their biological activity according to the washed platelet aggregation assay (C A Demopoulos et al., 1979). PAF as well as the examined samples were dissolved in 2.5 mg of bovine serum albumin (BSA) mL^{-1} of saline. The inhibitory activity of TPL was expressed as IC_{50} value (in μg of TPL) against PAF-induced aggregation (Nasopoulou, Nomikos, Demopoulos, & Zabetakis, 2007) (10^{-8} mol/L final concentration in the cuvette).

2.6. Gas chromatographic analysis with GC-FID

Fatty acid methyl esters of OP, both diets (OP diet and FO diet) and both fish fillets of farmed gilthead sea bream (fish fed with OP diet and FO diet respectively), were prepared using a solution of 0.5 mol/L KOH in CH_3OH (KOH–CH_3OH method)

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and extracted with n-hexane. The fatty acid analysis was carried out using the internal standard method. Three injections of 1,5 µL of each solution were analyzed with a Shimadzu CLASS-VP (GC-17A) (Kyoto, Japan) gas chromatograph equipped with a split/splitless injector and flame ionization detector.

Separation of fatty acid methyl esters was achieved on an Agilent J&W DB-23 fused silica capillary column as described above. The oven temperature program was: initially 120 ºC for 5 min, raised to 180 ºC at 10 ºC / min, then to 220 ºC at 20 ºC/min and finally isothermal at 220 ºC for 30 min. The injector and detector temperatures were maintained at 220 and 225 ºC, respectively. The carrier gas was high purity helium with linear flow rate of 1 ml/min and split ratio 1:50. Fatty acid methyl esters were identified using fatty acid methyl esters standards (Sigma, St. Louis, Mont, USA) by comparison of the retention times of the relative peaks. Heptadecanoic (17:0) acid methyl ester was used as internal standard.

2.7. Lipidomic analysis

Total polar lipid extracts were dissolved in 15 µl of chloroform/methanol (1:2) mixture and 15 µl of acetonitrile/isopropanol/water (6:7:2) mixture and analysed with an Absceix 4000 QTrap, a triple quadrupole mass spectrometer equipped with a nanoelectrospray source. Samples were delivered using a Nanomate interface in direct infusion mode (~125 nl/min). The lipid extracts were analysed in both positive and negative ion modes using a capillary voltage of 1.25 kV. MS/MS scanning (daughter, precursor and neutral loss scans) were performed using nitrogen as the collision gas with collision energies between 35 and 90 V. Each spectrum encompasses at least 50 repetitive scans. Tandem mass spectra (MS/MS) were obtained with collision energies as follows: 35-45V, PC in positive ion mode, parent-ion scanning of m/z 184; 35-55 V, PI in negative ion mode, parent-ion scanning of m/z 241; 35-65 V, PE in negative

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ion mode, parent-ion scanning of m/z 196; 20-35 V, PS in negative ion mode, neutral loss scanning of m/z 87; and 40-90 V, for all glycerophospholipids (including PA and PG) detected by precursor scanning for m/z 153 in negative ion mode. MS/MS daughter ion scanning was performed with collision energies between 35-90 V. Assignment of phospholipid species is based upon a combination of survey, daughter, precursor and neutral loss scans. The identity of phospholipid species were verified using the LIPID MAPS: Nature Lipidomics Gateway (www.lipidmaps.org).

2.8. Quantification of fatty acid content with GC-MS

Full characterisation and quantification of the fatty acids was conducted by conversion to the corresponding fatty acid methyl esters (FAME) followed by GC-MS analysis. Briefly, the samples were spiked with an internal standard fatty acid 17:0 (20 µl of 1 mmol/L) and dried under nitrogen. The fatty acids from the lipids (neutral and phospholipid) were released by base hydrolysis to release fatty acids were released by base hydrolysis using 500 µl of concentrated ammonia and 50% propan-1-ol (1:1), followed by incubation for 5 h at 50 ^{o}C. After cooling the samples are evaporated to dryness with nitrogen and dried twice more from 200 µl of methanol:water (1:1) to remove all traces of ammonia. The protonated fatty acids are now extracted by partitioning between 500 µl of 20 mmol/L HCl and 500 µl of ether, the aqueous phase is re-extracted with fresh ether (500 µl) and the combined ether phases are dried under nitrogen in a glass tube.

The fatty acids are converted to methyl esters, by adding an ethereal solution of diazomethane (3 x 20 µl aliquots) to the dried residue, while on ice. After 30 min the samples were allowed to warm to RT and left to evaporate to dryness in a fume hood.

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The FAME products are dissolved in 10-20 μl dichloromethane and 1-2 μl analysed by GC-MS on a Agilent Technologies (GC-6890N, MS detector-5973) with a ZB-5 column (30M x 25mm x 25mm, Phenomenex), with a temperature program of at 70 ^{o}C for 10min followed by a gradient to 220 ^{o}C at 5 ^{o}C /min and held at 220 ^{o}C for a further 15min. Mass spectra were acquired from 50-500 amu. The identity of FAMEs was carried out by comparison of the retention time and fragmentation pattern with a Bacterial FAME standard (Supelco).

2.9. Statistical analysis

Chemical analyses were carried out in triplicates and all results were expressed as mean value ± SD. The Wilcoxon sign test was used to determine significant differences in the same group. Differences were considered significant for $p < 0.05$. The data were analyzed using a statistical software package (IBM SPSS Statistics 19.0, SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Total (TL), total polar (TPL) and total neutral (TNL) lipid content of fish feeds and fish fillets

TL, TPL, and TNL content of OP, OP diet, FO diet, and gilthead sea bream fillets fed with OP and FO diet is shown in Table 1.

Table 1

According to Table 1, OP diet exhibited significant elevated levels of TL in comparison to FO diet. This upward trend is also reflected in TPL and TNL levels of OP diet. However, both fishes (fish fed with OP and FO diet respectively) exhibited not statistically different levels of TL, TPL and TNL.

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3.2. Biological activity of TPL of fish feeds and fish fillets

An equal amount of TPL of OP, two experimental diets (OP and FO diet) and gilthead sea bream fillets (fed with OP and FO diet respectively), were examined for their ability to induce washed rabbit platelet aggregation or inhibit the PAF-induced platelet aggregation. Inhibitory activity was detected in all samples and IC_{50} value expressed in μg of TPL is shown in Table 1.

The IC_{50} value is inversely proportional to the biological activity of the sample (PAF inhibition). This implies that the lowest IC_{50} value of a sample displays better biological activity and therefore better cardioprotective effect. Regarding the inhibitory activity against the PAF-induced platelet aggregation OP diet and FO diet exhibited similar potent inhibitory activity while the IC_{50} value of gilthead sea bream TPL fed with the OP diet significantly decreased (57.7μg as opposed to 179μg) in comparison with the gilthead sea bream fed with the FO diet (Table 1). The experimental data indicate that OP reinforces the anti-PAF biological activity of gilthead sea bream, probably due to the fact that this olive oil production industry by-product contains anti-PAF lipid components (Tsantila et al., 2007). It can be suggested that an important enrichment of the biological activity of polar lipids of gilthead sea bream fed with OP as opposed to the corresponding polar lipids of gilthead sea bream fed with FO has taken place. This biological improvement of gilthead sea bream fed with OP could be attributed to components of OP that probably were transferred to the OP diet and thus to fish maintaining their strong biological activity. This result is in accordance with results from our team (Karantonis et al., 2008; Nasopoulou et al., 2014; Nasopoulou et al., 2013), which indicate that OP inhibits the potent PAF activity.

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3.3. Fatty acid profile of fish diets and fish fillets lipids

The fatty acids profile of OP, OP diet and FO diet and gilthead sea bream muscle TPL, are presented in Table 2. The abundant fatty acids in OP were palmitic (16:0), oleic (18:1) and linoleic (18:2) acid. Both diets (OP diet and FO diet) exhibited high levels of palmitic (16:0), oleic (18:1), linoleic (18:2) acids whereas in fish muscle fed with OP and FO diet the most abundant acids were 16:0, 18:1 cis and the omega-3 DHA (22:6) fatty acid, while in the OP diet showed stearic (18:0) acid has also been found at high levels.

Regarding the gilthead sea breams fed with OP and FO diet respectively, we have found high levels of palmitic (16:0), oleic (18:1), and DHA (22:6) acids, while the fish fed with FO diet exhibited also high levels of linoleic acid (18:2) acid when compared to the sea bream fed with OP diet.

Table 2

3.4. Fatty acid analysis

The fatty acid content of the lipids present in fish feeds and fish fillets have been determined by GC-MS and are presented as: OP fatty acid content and the data for two fish feeds (i.e. OP diet and FO diet) are given in Fig. 1 (Fig. 1A corresponds to the OP, Fig. 1B corresponds to the OP-diet and Fig. 1C describes the FO-diet), whereas the data for fish fillets (i.e. fish fed with FO diet and OP diet, respectively) are given in Fig. 2 (Fig. 2A corresponds to fish fed with OP diet and Fig. 2B describes the fish fed with FO-diet).

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Samples of OP and both diets (OP diet and FO diet) contain low levels of myristic (14:0), palmitelaic (16:1) and stearic (18:0) acids and high levels of palmitic (16:0), oleic (18:1) and linoleic (18:2) acids. However OP diet and FO diet also contained rather high levels of AA (20:4 ω-6) and DPA (22:5).

Fig. 1

The fatty acid contents of gilthead sea bream fillets (fish fed with FO and OP respectively), are given in Fig. 2 (Fig. 2A corresponds to the fish fed with OP-diet and Fig. 2B describes the fish fed with FO-diet). Both fish contain low levels of myristic (14:0), palmitelaic (16:1) and eicosenoic (20:1 ω-9) acids and high levels of palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids. However, gilthead sea bream fed with OP diet also contained low levels of α-linoleic (18:3 ω-3) and adrenic (22:4 ω-6) acids which were not detected in gilthead sea bream fed with FO diet, as well as higher levels of AA (20:4 ω-6) and DPA (22:5).

Fig. 2

The phospholipid composition of all samples OP, OP diet, FO diet and both gilthead sea breams (fed with OP and FO respectively) were investigated in detail using electrospray mass spectrometry. ES-MS-MS daughter ion fragmentation analysis allowed full characterization of all the major lipid species. In positive ion survey scans of OP (Fig. 3A), the lipid species were predominantly diacylglycerols and triacylglycerols. In negative ion mode, survey scans between 600-1000 m/z, of

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(Fig. 3B) showed molecular species mainly of PE, and PI while a diacylclycerol and a PG species were also identified.

Fig. 3

In positive ion survey scans of OP-diet (Fig. 4A), we have found mainly PC species and very few low abundance of triacylglycerols. In negative ion mode, survey scans between 600-1000 m/z (Fig. 4B), molecular species of PA, PE, PI and a TAG have been found while PE was the most predominant class of species.

Fig. 4

In positive ion survey scans of gilthead sea bream fed with OP diet (Fig. 5A), only PC species have been detected. In negative ion mode, survey scans between 600-1000 m/z (Fig. 5B), molecular species mainly of PE and PG have been identified while PI species have been identified as well but in lower abundance. The majority of PE species found in all samples are in good correlation with our previous work where OP was used in the fish diet of sea bass (Nasopoulou et al., 2014).

Fig. 5

In OP sample (negative ion ES-MS survey scan is shown in Fig.. 3B), two PE species with 748 m/z and 764 m/z have been identified as PE (18:0, 18:0) and PE (18:2, 20:4), respectively and these two species have been found to be also present in OP-diet (Fig. 4B) with 747 m/z and 763 m/z and in gilthead sea bream fed with OP diet as well (Fig. 5B) with 747 m/z and 777 m/z respectively. These substances have been identified at significant lower levels in FO diet and sea bream fed with FO diet (Supplemental Figs 1 and 2).

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The species identified in this work as PE (18:0, 18:0) in OP, OP-diet and fish fed with OP-diet could be derived from PE (18:1, 18:2) that has been reported with m/z 741 in olives and olive oil (Shen et al., 2013). The fragment 748 m/z is probably of olive origin since it has been identified as PG (16:0, 18:1) in olive oil (Verardo et al., 2013) and also in olives (Montealegre, Sánchez-Hernández, Crego, & Marina, 2013).

Also, in OP sample (Fig. 3B), another PE species with 794 m/z has been identified as PE (18:0, 22:4) and this compound has also been identified in fish fed with OP diet (Fig. 5B). A PG derivative with 820 m/z has been identified as PG (18:2, 22:5) in OP sample (Fig. 3B) whereas a variety of PG derivatives with longer carbon chains have been identified in sea bream fed with OP diet (Fig. 5B).

Conclusions

The implications of this work are two-fold: firstly, a by-product of olive industry (namely olive pomace) can be valorized in the production of aquafeeds and secondly the use of OP can improve the cardioprotective properties of the final produce (sea bream) by enriching the fish lipid profile with specific cardioprotective lipid compounds of plant origin. The valorization of OP has also two positive consequences: an environmental impact since OP in olive oil producing countries is a major pollutant and also, from a food security point of view, the use of OP in the production of aquafeeds could in the future reduce aquaculture's heavy dependence on FO and therefore increase the sustainability of the aquaculture sector.

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Author Contributions

E. Sioriki and T.K. Smith collected data, C.A. Demopoulos and I. Zabetakis designed the study; all authors have contributed to the writing up of the manuscript.

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List of Table and Fig. Legends

Table 1.

Total lipid (TL), total polar lipid (TPL), total neutral lipid (TNL) content (%) and IC_{50} values expressed as μg of TPL of OP, OP diet, FO diet and fillets of aquacultured gilthead sea bream (*Sparus aurata*) fed with OP and FO diet.

Table 2.

Levels of fatty acids of TPL of OP, OP diet, FO diet and fillets of gilthead sea bream fed with OP and FO diet obtained by GC-FID, expressed in percentage of each fatty acid.

Fig. 1. Total polar fatty acid analysis by gas chromatography–mass spectrometry. (A): Chromatogram of FAMEs obtained from OP, (B): Chromatogram of FAMEs obtained from OP diet, (C): Chromatogram of FAMEs obtained from FO diet, (inserts show percentage of each fatty acid).

Fig. 2. Total polar fatty acid analysis by gas chromatography–mass spectrometry. (A): Chromatogram of FAMEs obtained from gilthead sea bream fed with OP diet, (B): Chromatogram of FAMEs obtained from gilthead sea bream fed with FO diet, (inserts show percentage of each fatty acid).

Fig. 3. Lipidomic analysis of OP. (A): Positive ion ES-MS survey scans (600-1000m/z), (B): Negative ion ES-MS survey scans (600-1000m/z). Annotation of the major lipid species, based upon daughter ion fragmentation analysis by ES-MS-MS.

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Fig. 4. Lipidomic analysis of OP diet. (A): Positive ion ES-MS survey scans (600-1000m/z), (B): Negative ion ES-MS survey scans (600-1000m/z). Annotation of the major lipid species, based upon daughter ion fragmentation analysis by ES-MS-MS.

Fig. 5. Lipidomic analysis of gilthead sea bream fed with OP diet. (A): Positive ion ES-MS survey scans (600-1000m/z), (B): Negative ion ES-MS survey scans (600-1000m/z). Annotation of the major lipid species, based upon daughter ion fragmentation analysis by ES-MS-MS.

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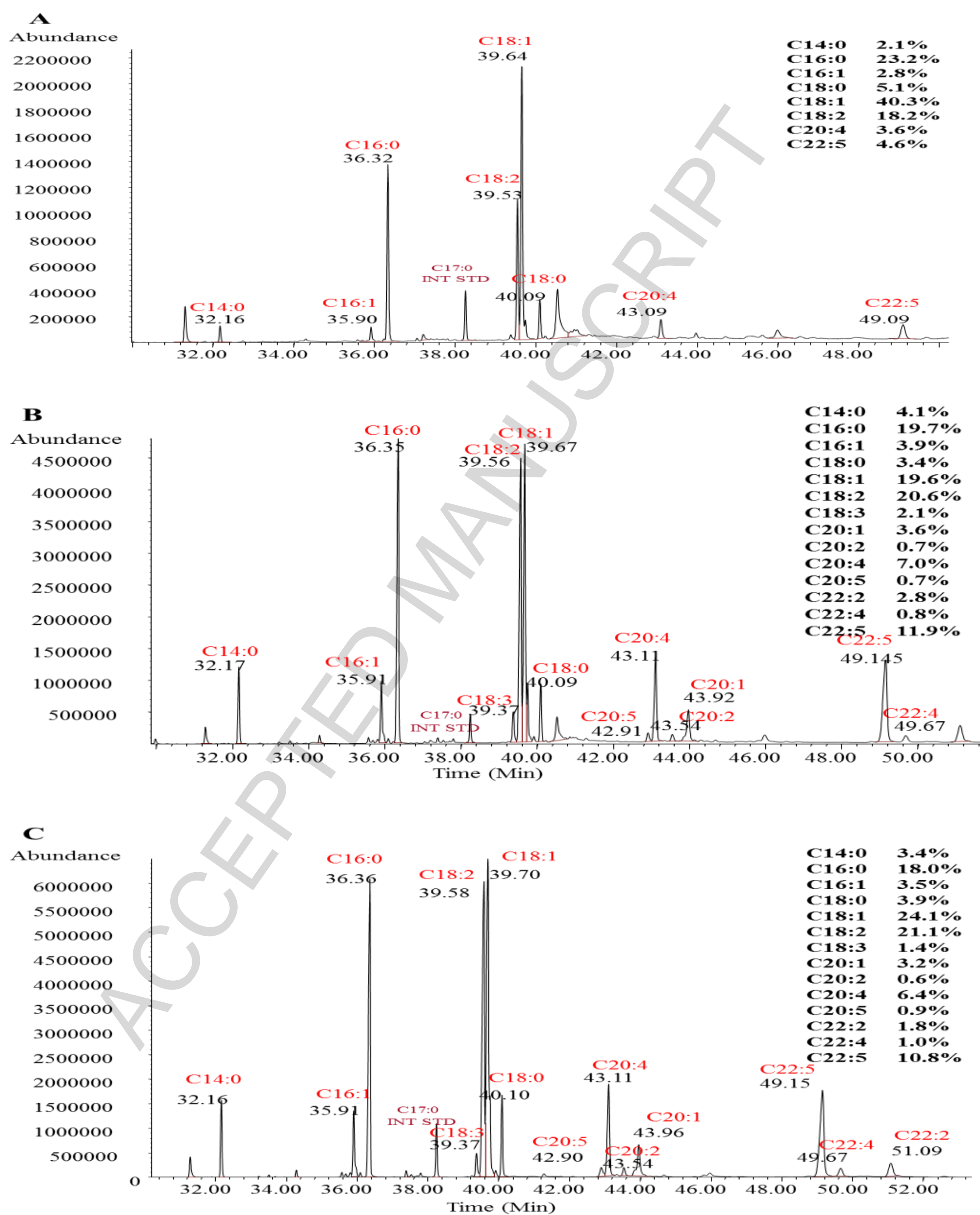

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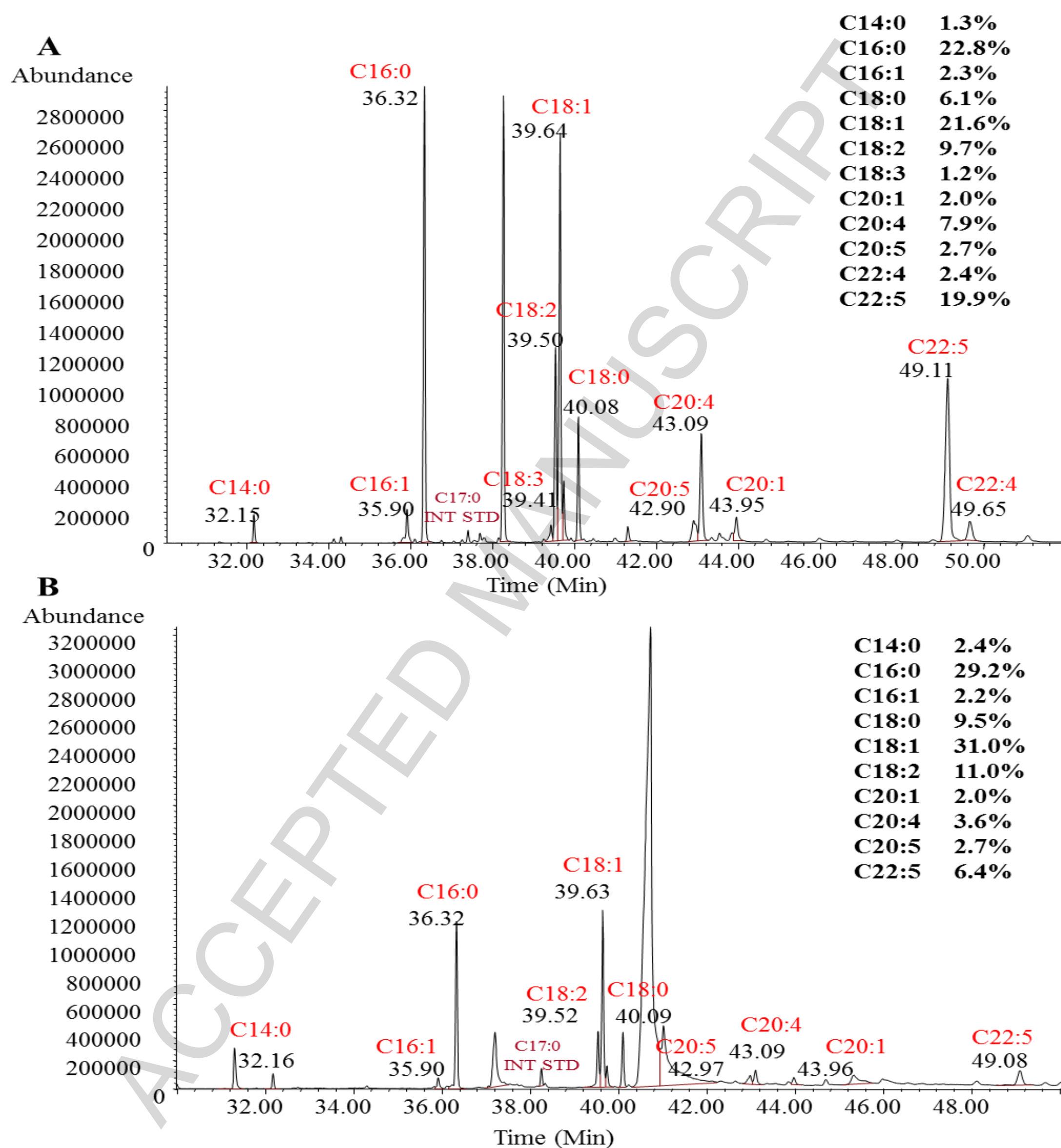

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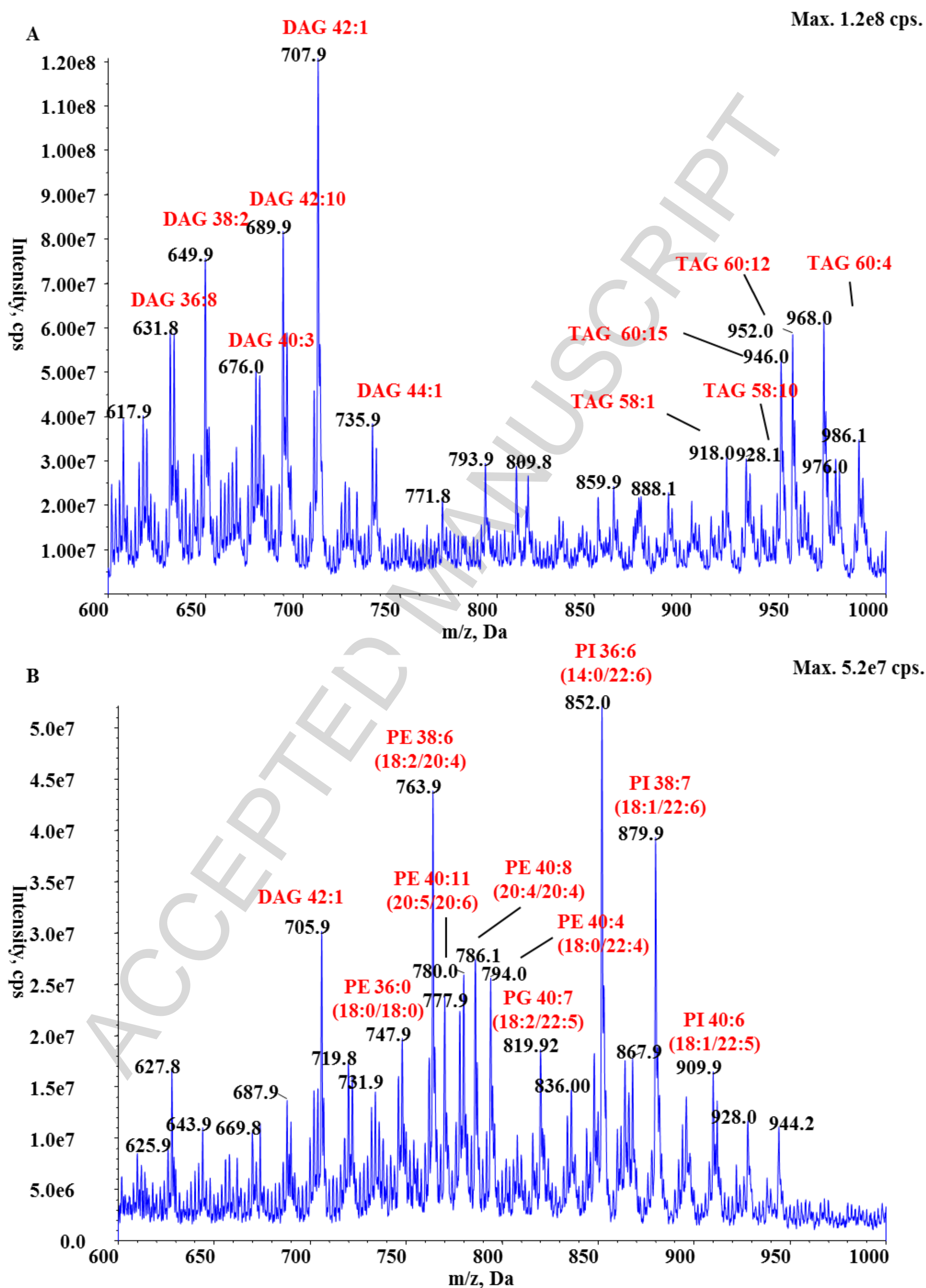

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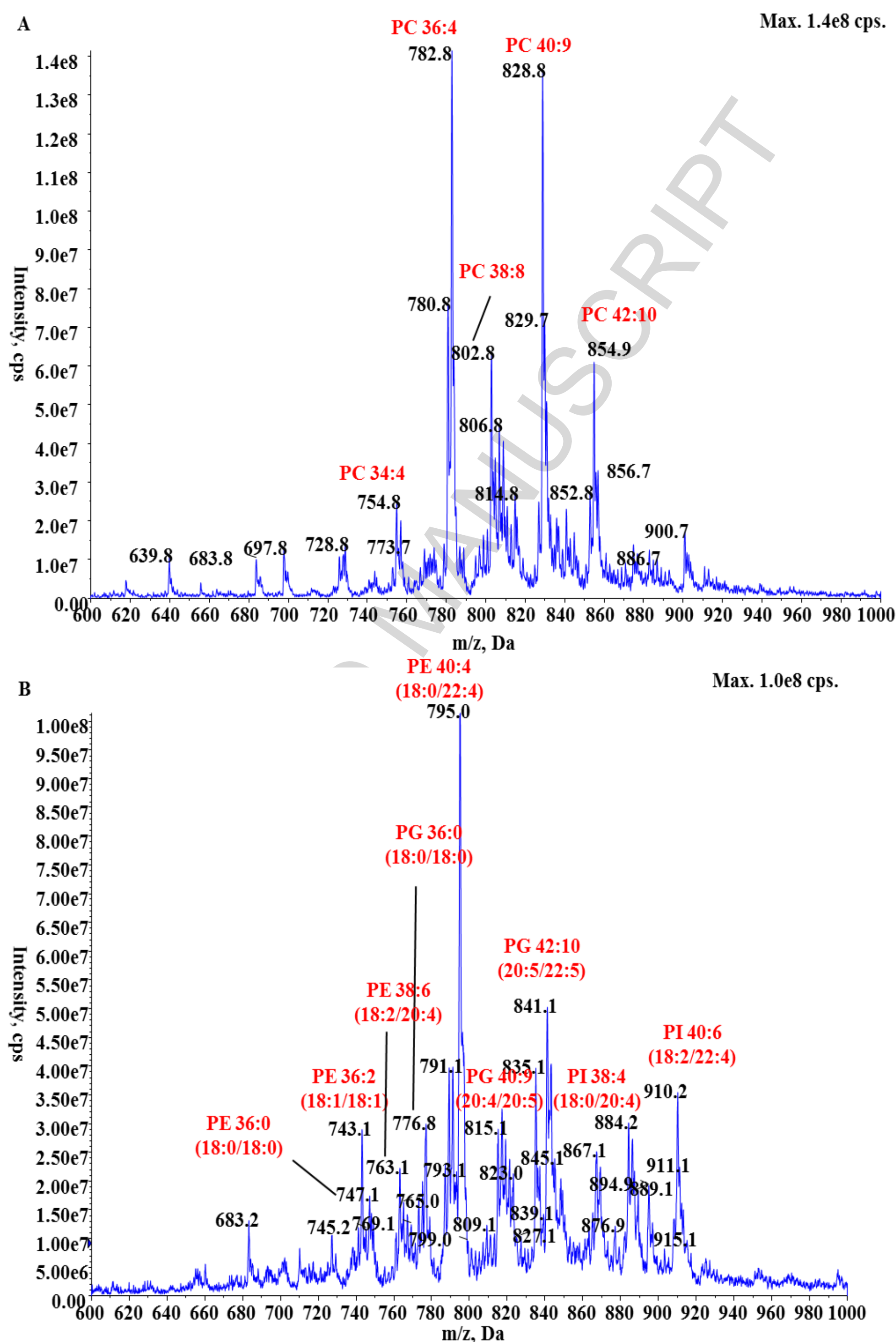

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Sample	TL (%)	TPL (%)	TNL (%)	IC_{50} (μg TPL)
OP	9.01±0.21	7.41±0.23	1.52±0.23	13.1±0.22
OP diet	13.6±2.11	2.70±0.53	10.5±1.51	40.0±2.54
FO diet	11.0±1.32	2.30±0.51	8.43±0.92	47.1±3.10
Gilthead sea bream + OP diet	7.31±0.50	0.62±0.10	6.41±0.40	57.7±2.20†
Gilthead sea bream + FO diet	7.81±0.42	0.51±0.10	6.91±0.50	179±2.81†

[1]Results are expressed as mean ± SD, data are mean values of three individual measurements.

† Indicates statistical significance between gilthead sea bream fed with OP and FO diet

($p < 0.05$) according to the Wilcoxon test.

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Table 2. Levels of fatty acids of TPL of OP, OP diet, FO diet and fillets of gilthead sea bream fed with OP and FO diet obtained by GC-FID, each fatty acid is expressed as a percentage of the total.

Fatty acids	OP	OP diet	FO diet	Gilthead sea bream + OP diet	Gilthead sea bream + FO diet
14:0	0.21±0.06	5.41±0.28	2.72±0.22	2.63±0.35	0.61±0.05
16:0	13.8±0.98	20.2±1.07	19.3±0.89	16.0±1.28	16.9±1.01
18:0	2.71±0.25	10.1±1.02	3.62±1.01	4.41±0.86	6.01±1.03
16:1	1.08±0.12	1.41±0.11	1.51±0.21	3.53±0.21	1.04±0.04
18:1 Cis (ω-9)	60.2±3.22	14.5±0.59	21.8±1.97	17.0±1.04	18.6±0.82
18:1 Trans (ω-9)	1.62±0.11	1.61±0.07	1.73±0.15	2.20±0.28	1.91±0.29
18:2 (ω-6)	11.9±0.86	25.2±1.98	24.0±1.89	8.71±0.65	15.3±1.70
18:3 (ω-3)	8.51±0.37	3.42±0.97	4.11±1.01	1.20±0.08	3.21±0.56
20:4 (ω-6)	n.d.	1.10±0.05	0.91±0.02	1.82±0.07	3.40±0.69
20:5 (ω-3)	n.d.	4.90±0.28	5.10±0.32	7.51±1.03	6.14±0.89
22:5 (ω-3)	n.d.	n.d.	0.81±0.01	4.07±0.58	3.91±0.15
22:6 (ω-3)	n.d.	12.2±0.09	14.5±0.29	30.9±1.97	23.1±2.20
Total SFA	16.7±0.23	35.7±1.96	25.6±1.08	23.0±1.89	23.5±1.89
Total MUFA	62.8±2.52	15.9±1.23	25.0±1.29	22.7±2.01	21.5±1.74
Total ω-3 PUFA	20.4±1.01	46.8±2.01	49.4±1.36	43.6±2.03	36.3±1.96
Total ω-6 PUFA	11.9±0.64	26.3±1.01	24.9±2.01	10.5±1.08	18.7±1.09
Total UFA	83.2±2.67	62.7±2.09	74.4±3.02	76.8±2.04	76.5±3.02
UFA/SFA	4.98±0.21	1.76±0.02	2.90±0.03	3.34±0.13	3.25±0.27

[1]Data are mean values of three individual measurements.

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; UFA: unsaturated fatty acid; n.d.: not detected

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Research highlights

- Gilthead sea bream was aquacultured using 8% olive pomace in fish finishing diet.
- Polar lipids of all samples were assessed *in vitro* towards platelet aggregation.
- These polar lipid fractions were also studied by ES-MS/MS and GC-MS.
- Major classes of diacyl-glycerophospholipids, (PC, PE, PI, PG, PA) were identified.
- Two PE species were identified in OP, OP-diet and fish fed with OP-diet.

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