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Designing Novel Functional Food Using Gas Chromatography

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1. Introduction

Fish and fish oils are well known to possess many protective properties against cardiovascular diseases (Kris-Etherton et al., 2003) due to high content in the long-chain ω-3 polyunsuturated fatty acids (PUFA); eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) which are thought to have anti-thrombotic (Din, Newby & Flapan, 2004) and anti-inflammatory properties (Rennie et al., 2003) and also due to micro constituents with anti-thrombotic properties (Nasopoulou et al., 2007; Nomikos et al., 2006; Kristensen et al., 2001; Panayiotou et al., 2000; Mori et al., 1997) making seafood an important component of human's diet.

In addition, the population growth and rising consumer demand result in a continuously increasing demand for fish supply and aquaculture seems to satisfy these requirements at present. Regarding aquacultured fish, one of the basic dietary ingredients of the compounded feeds is fish oil, because of its high digestibility and sufficient content of essential fatty acids, in particular ω-3 PUFA. At the moment, the aquaculture industry uses an estimate of approximately 40% and 60% of the global production of fish meal and fish oil, respectively (Tacon, 2005) while the production of fish meal has remained more or less stable from the late 1980s at about 6 million metric tons/annum (Food and Agriculture Organization (FAO), 2004) suggesting that food grade fisheries providing fish oil and fish meal may reach their limit of sustainability within the next few years (Pike & Barlow, 2003; Shepherd et al., 2005).

Fish oil substitution in compounded fish feeds by plant oils could be a promising solution in order to limit fish oil demand for fish feed formulation as well as to reduce costs since plant oils have steadily increasing production, high availability and better economic value. Thus several studies have been carried out to clarify whether certain plant oils such as soybean, linseed, rapeseed, sunflower, palm oil, olive oil and mixtures of them can be used as partial substitutes for fish oils in compounded fish feeds (Alexis, 1997; Benedito-Palos et al., 2008; Caballero et al., 2002, 2004; El-Kerdawy & Salama, 1997; Figueiredo-Silva et al., 2005; Fountoulaki et al., 2009; Izquierdo et al., 2003, 2005; Kalogeropoulos et al., 1992; Montero et al., 2003, 2005; Mourente et al., 2005, 2006; Rosenlund, 2001; Torstensen et al., 2000, 2004; Wassef et al., 2009).

Soybean oil appears to be the preferred plant lipid source regarding fish growth while considerable savings in feed costs could be achieved if used as a partial dietary substitute for fish oil within compound feeds. The same goes to linseed and rapeseed oil but to a lesser extent (Wassef et al., 2009; El-Kerdawy & Salama, 1997). Furthermore, the use of palm oil and olive oil in fish feeds has given growth and feed utilization efficiency comparable to fish fed with equivalent levels of fish oil (Caballero et al., 2002; Mourente et al., 2005; Rosenlund, 2001; Torstensen et al., 2000, 2004).

However, replacement of marine fish oils with alternate oils of plant origin in the farmed fish feeds should occur not only to provide the sufficient quantities of lipids that meet fish essential fatty acid requirements for optimum growth, but also to maintain proper immune function in fish (Montero et al., 2003). Thus, the use of vegetable oils as a sole lipid source is limited.

Many researchers seem to believe that 60% fish oil substitution in compounded fish feeds by plant oils is the preferable percentage in order not to compromise growth performance or feed utilization efficiency of fish (Alexis, 1997; Caballero et al., 2004; Izquierdo et al., 2003, 2005; Montero et al., 2005; Mourente et al., 2005, 2006; Wassef et al., 2009).

Olive pomace and olive pomace oil are natural by-products of olive oil production, which contain micro constituents with antithrombotic properties (Karantonis et al., 2008) and phenolic/polyphenolic molecules with antioxidant and other pleiotropic actions. Recent data from our research team reported for the first time that partial replacement of fish oil in gilthead sea bream grow-out diet by lipids obtained from olive pomace resulted in sufficient fish growth similar to the one of fish fed with 100% fish oil diet combined with improved antithrobotic properties of fish (Nasopoulou et al., 2011).

2. Research goals

Our survey over the last years focus both on the *in vitro* and *in vivo* study of the nutritional value - in terms of cardioprotection - of foodstuffs containing micro constituents that block (either inhibit or antagonise) Platelet Activating Factor's activity. Platelet activating factor (PAF) (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine) (Demopoulos et al., 1979) is a potent inflammatory phospholipids mediator that is implicated in the mechanism of atherogenesis (Demopoulos et al., 2003). According to this mechanism, PAF is produced during LDL oxidation (Liapikos et al., 1994) and causes in situ inflammation. It is also known that PAF is a compound of atheromatic plaque and is essential for the activation of leukocytes and their binding in the endothelial cells (Mueller et al., 1995). Furthermore, evidence of the implication of PAF in atherogenesis is provided by studies in animals, which indicates that constituents that blocks PAF's action have protective effect on atherosclerosis (Feliste et al., 1989; Subbanagounder et al., 1999), while PAF-acetylhydrolases (PAF-AHs), the main enzymes responsible for the degradation of PAF, are active in native LDL but converted inactive forms in ox-LDL, leading to higher levels of PAF (Liapikos et al., 1994). Therefore, the presence of PAF-antagonists thus PAF-inhibitors or PAF-agonists (molecules that prevent PAF binding to PAF's receptor causing platelet aggregating far less potent than the one that PAF induces) in various foodstuffs is very important in terms of cardioprotection.

European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) were the core of our survey and considering a) the fact that one of the most common cause of mortality in industrialized countries is heart disease, b) the problem of fish oil dependence

for farmed fish feed formulation and c) of environmental degradation by the olive oil production by-products we tried to design a novel fish by partial replacement of fish oil by olive pomace in the fish diet. By this attempt we aim to reinforce the anti-thrombotic properties of fish – by using olive pomace which contain proven anti-thrombotic properties in the fish diet – to contribute in the resolution of fish oil dependence for farmed fish feed production and to exploit a natural by-product.

2.1 *In vitro* studies

Regarding the *in vitro* studies lipids of many traditional foods of the Mediterranean diet, such as fish (Nasopoulou et al., 2007; Nomikos et al., 2006; Panayiotou et al., 2000; Rementzis et al., 1997), olive oil (Koussissis et al., 1993), honey (Koussissis et al., 1994), milk and yogurt (Antonopoulou et al., 1996) and red wine (Fragopoulou et al., 2000) were studied for their inhibitory and /or agonistic properties against PAF.

Total lipids of the aforementioned foodstuffs were extracted according to the Bligh-Dyer method (Bligh & Dyer, 1959). In brief, an appropriate amount of chloroform/methanol/water 1:2:0.8 (v/v/v) solution was added to each sample and mixtures were shaken well and filtered. Phase separation of mixtures in the separatory funnels was achieved by adding chloroform and water to obtain a final chloroform/methanol/water ratio of 1/1/0.9 (v/v/v). Total lipids were obtained by the chloroform phase (lower phase) that was evaporated to dryness under nitrogen's stream and lipids were weighed and redissolved in 1 ml chloroform:methanol 1:1 (v/v). One tenth of the total lipids were stored under nitrogen in sealed vials at -20 ^{o}C until used - after a short period of time - for biological assay, while the rest of it was further separated into polar lipids and neutral lipids using the counter-current distribution method (Galanos &

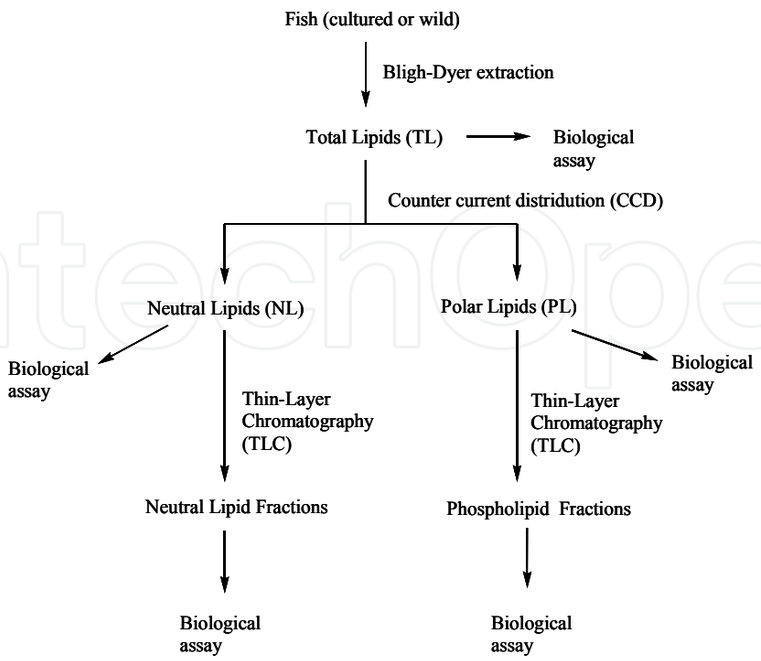


Fig. 1. Schematic diagram of the extraction and purification procedure.

Kapoulas, 1962). In brief, this method is based on polar and neutral lipids different solubility in pre-equilibrated petroleum ether and ethanol (87%). Polar lipids were soluble in ethanol while neutral lipids were soluble in petroleum ether. The obtained lipid fractions were weighed and stored under nitrogen in sealed vials at -20 ºC until used - after a short period of time - for the biological assay.

Regarding european sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) the polar and neutral lipids of farmed and wild fish specimens were further separated by preparative thin layer chromatography (Nasopoulou et al., 2007). The lipid fractions obtained were stored under nitrogen in sealed vials at -20 ºC until used - after a short period of time - for the biological assay (Figure 1).

The biological activity of total, polar and neutral lipids, as well as purified fractions of each lipid class after thin layer chromatography separations (Nasopoulou et al., 2007) was studied against washed rabbit platelets according to the method of Demopoulos et al. (1979) as shown in Figure 2.

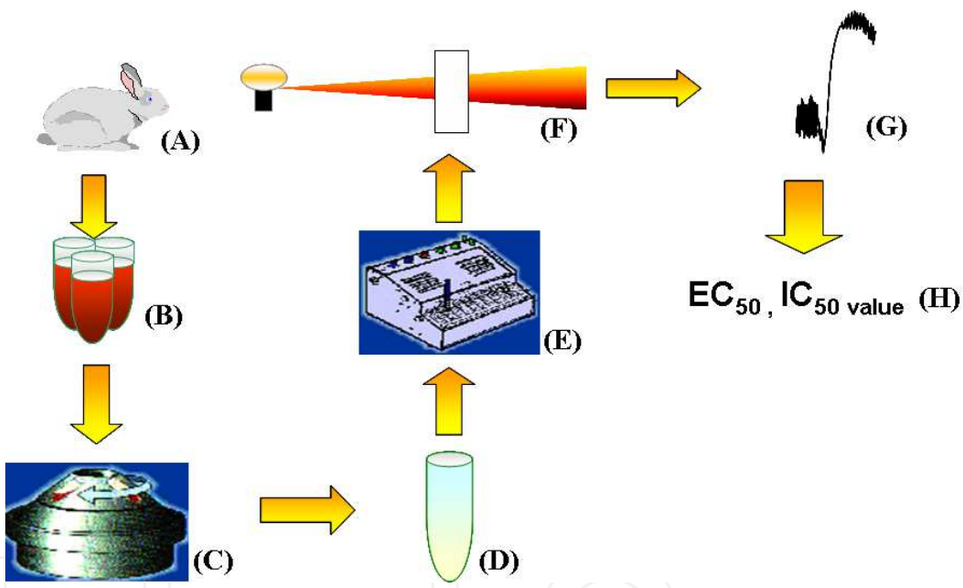


Fig. 2. Biological assay procedure.

Briefly, blood was collected through the main ear artery of the rabbit and was placed in polyethylene tubes containing a ratio of blood/anti-coagulant of 9:1 (v/v) (Figure 2 (A and B) and after adding the appropriate buffer solution and a sequence of centrifugations (Figure 2 (C)) according to the method of Demopoulos et al. (1979) washed rabbit platelets were collected (Figure 2 (D)). Following that the samples being examined and the PAF were dissolved in 2.5 mg of bovine serum albumin (BSA) per ml of saline. Various amounts of the sample being examined were added into the aggregometer cuvette - containing the washed rabbit platelets - in the aggregometer (fig 2 (E)), their absorbance was measured (fig 2 (F)) and thus, the permeability was recorded (fig 2 (G)) and their ability to aggregate washed rabbit platelets or to inhibit PAF-induced aggregation was determined (fig 2 (H)). In order to determine the aggregatory efficiency of either PAF or the samples being examined, the maximum reversible aggregation was evaluated and the 100% aggregation was determined.

The plot of the percentage of the maximum reversible aggregation (ranging from 20% to 80%) versus different concentrations of the aggregatory agent was linear.

From this curve, the concentration of the aggregatory agent, which induces 50% of the maximum reversible aggregation, was calculated. This value is defined as EC_{50}, namely equivalent concentration for 50% aggregation.

In order to determine the inhibitory properties of the samples, various amounts of the sample being examined, ranging from 0.0012 to 0.16 mg, were added into the aggregometer cuvette and their ability to inhibit PAF-induced aggregation was determined. The platelet aggregation induced by PAF (2.5×10^{-11}M, final concentration in the cuvette) was measured as PAF-induced aggregation, in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various amounts of the sample being examined. Consequently, the plot of % inhibition (ranging from 20% to 80%) versus different concentrations of the sample is linear. From this curve, the concentration of the sample, which inhibited 50% PAF-induced aggregation, was calculated. This value is defined as IC_{50} namely, inhibitory concentration for 50% inhibition.

Furthermore part of our study was to clarify the impact of the seasonal and geographical variation of european sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) on fatty acid content of fish fillets and on the anti-PAF activity of fish fillet lipids.

Briefly farmed sea bass (*Dicentrarchus labrax*) obtained from marine farms situated in Chios Island during winter (January) and summer time (June) and gilthead sea bream (*Sparus aurata*) obtained from marine farm situated in sea region of Nafpaktos during both seasons and from marine farm situated in Chios Island during summer time.

In January the average seawater temperatures of marine farm situated in Chios Island and in sea region of Nafpaktos were 13 and 15°C, respectively, while in June the average seawater temperature of marine farm situated in Chios Island and in sea region of Nafpaktos were 23 and 21°C, respectively. During the experiment, water salinity was 3.5 – 3.8% at both farms. Feeding ratio ranges from 0.8 - 1.1% of body weight per day. Fish of the same species were fed with the same commercial feed, during both winter and summer. Five specimens of each species, each season and each marine farm were analyzed. Their diets were also analysed.

Fatty acid methyl esters of fish and commercial feed total lipids prepared using a solution 0.5N KOH in CH_3OH 90% and extracted with n-hexane.

The fatty acid analysis was carried out using the internal standard method (Nasopoulou et al. 2011). A five point calibration curve was prepared using five solutions of heptadecanoic (17:0) acid methyl ester and heneicosanoic (21:0) acid methyl ester in ratios of 500:1000 (v/v), 500:500 (v/v), 500:200 (v/v), 500:100 (v/v) and 500:50 (v/v), respectively.

Five injects of 1 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 ionisation detector. The ratio of the mean area of (21:0) to that of the internal standard (17:0) is used as the y-axis variable of the calibration curve, while the concentration (mg kg^{-1}) of 21:0 is used as the x-axis variable of the calibration curve. The equation that described the calibration curve was:

$$y = 0.0012x + 0.0210, \; r = 0.996.$$

The ratio of the area of the analyte peak to that of the internal standard represents the y value at the above equation and subsequently x value represents the analyte concentration of the fatty acid in the unknown mixture.

Separation of fatty acid methyl esters was achieved on an Agilent J&W DB-23 fused silica capillary column (60 mx0.251 mm i.d., 0.25 μm; Agilent, Santa Clara California, USA). The oven temperature program was: initially 120 ^{o}C for 5 min, raised to 180 ^{o}C at 10 ^{o}C min^{-1}, then to 220 ^{o}C at 20 ^{o}C min^{-1} and finally isothermal at 220 ^{o}C for 30 min. The injector and detector temperatures were maintained at 220 and 225 ^{o}C, respectively. The carrier gas was high purity helium with a linear flow rate of 1 ml min^{-1} 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 (Nasopoulou et al., 2011).

2.2 *In vivo* studies

As mentioned before evidence of PAF implication in atherogenesis is provided by studies in animals, which indicates that PAF antagonists have protective action against atherosclerosis (Feliste et al., 1989; Subbanagounder et al., 1999), while PAF-acetylhydrolases (PAF-AHs), the main enzymes responsible for the degradation of PAF, are active in native LDL but converted inactive forms in ox-LDL, leading to higher levels of PAF (Liapikos et al., 1994). Recent *in vivo* study in animals is performed by our research team (Nasopoulou et al., 2010). More specific twelve healthy male New Zealand rabbits of specific weight 3129 ± 216 g and age 2.7 ± 0.2 months were purchased from a commercial breeder and were individually housed in atomic stain-less steel cages in constant conditions of temperature (19 + 1 ^{o}C), relative moisture (55 + 5%), and air conditioning (12 full changes of air per 1 h). The light/darkness ratio was 12 h/12 h. Rabbits were acclimatised for 5 days before the beginning of the study. Living conditions and animal handling were according to the European Regulation 609/86. The local veterinary authorities and animal ethics committee approved the study. Rabbits were randomly divided into two groups of six animals each and were given specific diets for 45 days. Group A was given atherogenic diet 1% cholesterol, while group B was given atherogenic diet enriched with gilthead sea bream polar lipids GSBPL (0.06% w/w) (Figure 3) (Nasopoulou et al., 2010).

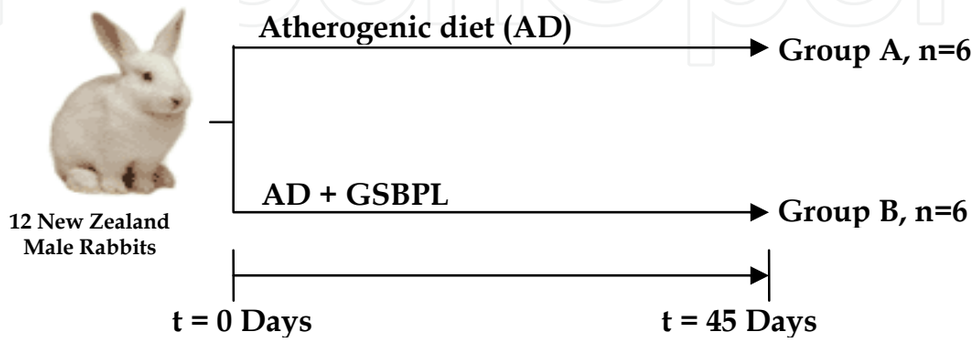


Fig. 3. Experimental design of the *in vivo* trial.

The experimental diet of group A was prepared by dissolving 30 g of cholesterol in 1000 ml of diethyl ether, whereas the experimental diet of group B was prepared by dissolving 30 g of cholesterol and 2 g of GSBPL in 1000 ml of diethyl ether. Each mixture was added to 3 kg of commercial available food for rabbits, placed on large plates, and the solvent was evaporated. This procedure was carried out every three days and food consumption was recorded every three days by measuring the weight of the required food that was added. In this series of experiments, each rabbit consumed 200 g/day, on average, so this resulted to a consumption of 133 mg of GSBPL/rabbit/day. On the 45th day, rabbits were anesthetised by intramuscular injection of 5 mg/kg body weight of xylazine (Rompun, Bayer, Leverkusen, Germany) and 25 mg/kg body weight of cetamine (Fort Dodge Laboratories Inc., Fort Dodge, Iowa, USA). Afterwards euthanasia took place by intravenous injection of 20 mg/kg body weight of pentothal (Hospital Products Division, Abbott Laboratories Abbott Park, IL, USA). The thoracic and peritoneal cavities were opened through a median longitudinal incision and the aorta was dissected from the aortic valve down to the aortic bifurcation (Nasopoulou et al., 2010).

The biochemical measurements that took place were the followings: at the beginning (0 days) and at the end of the experimental time (45 days), blood was collected from all rabbits through the main ear artery and was placed in polyethylene tubes containing a ratio of blood/anti-coagulant of 9:1 (v/v). Plasma cholesterol concentrations – total cholesterol, LDL-cholesterol and HDL-cholesterol – were determined enzymatically by CHOD-PAP, using commercial enzymatic kit (Elitech Diagnostics, Sees, France) and triglycerides concentration was determined enzymatically by GPOPAP, using a commercial enzymatic kit (Roche Diagnostics GmbH, Mannheim, Deutschland). Analyses were performed on a RA-XT autoanalyzer (Technicon Instruments, NY, USA).

In vitro $CuSO_4$-induced plasma oxidation (Schnitzer et al., 1998) was assessed by a Helios b (Spectronic Unicam, Cambridge, UK) spectrophotometer equipped with a 7 position automated sample changer. In quartz cuvettes, 20 μl of plasma were added to 880 μl of phosphate buffer solution, pH 7.4, with 146 mM NaCl at 37 ^oC for 5 min. The oxidation reaction was started by the addition of 100 μl $CuSO_4$ 1mM, and the absorbance of resulting conjugated dienes, at 245 nm, was continuously recorded for 3 h at 37 ^oC.

Platelet-rich plasma (PRP) was obtained by centrifugation of blood samples at 562g for 13 min, while platelet–poor plasma (PPP) was obtained by further centrifuging the specimens at 1750g for 20 min. The centrifugation was performed on a Heraeus Labofuge 400R (Hanau, Germany) at 24 ^oC. PRP concentration was adjusted to 300,000 platelets/ml using the respective PPP. PRP was used to test the aggregation induced by various concentrations of PAF in 2.5 mg of BSA/ml saline. The maximum reversible (or the minimum irreversible) PAF-induced platelet aggregation was determined as the 100% aggregation, and then various PAF concentrations were added, so as to achieve aggregations between 20% and 80%. These PAF-induced aggregations were of linear response to the respective PAF concentration; therefore, the EC_{50} value was calculated. EC_{50} accounts for the PAF concentration inducing 50% of the maximum aggregation. These studies were performed using a Chronolog aggregometer (model 400) coupled to a Chronolog recorder at 37 ^oC with constant stirring at 1200 rpm.

Plasma PAF-AH activity was determined by the trichloroacetic acid (TCA) precipitation procedure (Antonopoulou et al., 1994). Plasma (diluted 1:30 v/v in Tris buffer, pH 7.4) was

examined in a final volume of 200 µl. The mixture was incubated at 37 ºC for 2 min and the reaction was initiated by adding 5 µl of 800 µM [3H]-acetyl PAF/PAF solution in BSA (10 µg/µl in Tris–HCl buffer pH 7.4). The PAF-AH assay was performed at 37 ºC for 15 min. Unreacted [3H]-acetyl PAF was bound to an excess of BSA (final concentration, 0.75 mg/ml) for 0.5 min and precipitated by addition of trichloroacetic acid (final concentration, 9.6% v/v). The samples were then placed in an ice bath for 15 min and subsequently centrifuged at 16,000g for 5 min, at 4 ºC. The [3H]-acetate released into the aqueous phase was measured on a liquid scintillation counter (1209 Rackbeta, Pharmacia, Wallac, Finland). Blank assay was performed with no added plasma. The enzyme activity was expressed as pmol of PAF degraded per minute per µl of plasma.

Histopathological examination of the atherosclerotic lesions performed after comparable areas of the thoracic aortas were sectioned into 1 cm segments, fixed in 10% buffered formaldehyde, embedded in paraffin blocks, and stored at room temperature. For the histopathological examination, 5 µm thickness tissue slide sections were then cut, transferred to slides and stained with haematoxylin and eosin. The thickness of atherosclerotic lesions was measured blindly in five sections from each artery. Conventional measurements of early atherosclerosis lesions in the histopathological tissue sections of resected aortas were performed, using an automated image analysis system. The apparatus comprised a Sony-Exwave HAD Color Video Camera (Sony Corporation, Japan), fitted to a Zeiss Axiostar light microscope (Zeiss, Germany), a host computer (Pentium 90 MHz, 32 MB RAM) and Sigma Scan version 2.0 image analysis software (Jandel Scientific, Erkrath, Germany). Foam cell formation is characteristic of the early atherosclerosis lesions. In the present study, early atherosclerosis lesions were observed as foam cell layers developed inside the blood vessels (Nasopoulou et al., 2010).

2.3 Design of novel functional fish

Two plant oil sources, namely olive pomace and olive pomace oil were selected by our research team because of their content in athero-protective substances (such as PAF inhibitors) and phenolic/polyphenolic molecules (Karantonis et al., 2008), null cost since they are natural wastes of olive oil production, and fatty acid profile, which makes them likely candidates to partially substitute fish oil in compounded fish feeds. The reference diet (fish oil diet) contained 100% fish oil (cod liver oil) and its chemical composition is shown in Table 1, while the two experimental diets: olive pomace and olive pomace oil diet were compounded by substituting 8% of fish oil of reference diet, respectively (Nasopoulou et al., 2011).

All the diets were formulated at the facilities of the marine farm where the experiment took place using a twin-screw extruder creating pellets, followed by the addition of oil mixtures. The pellets were dried, sealed and kept in air-tight bags until use (Nasopoulou et al., 2011). Olive pomace and olive pomace oil originated from a local oil industry and obtained after the olive oil production procedure.

Each of the three tanks of each fish species contained fish fed with each dietary treatment (fish oil, olive pomace and olive pomace oil diet).

Two different fish species, namely gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) of initial mean body weight 340 g obtained from a commercial marine

Fillet composition (% wet weight)	Fish oil diet
Crude protein	46±4.3
Fat	21±3.2
Moisture	9.1±1.3
Dietary fiber	1.8±0.3
Ash	8.3±0.9
Energy (MJ/Kg)	23±2.6
Digestibility (%)	90±6.2
Vitamin A (IU/Kg)	20 000±410
Vitamin D(IU/Kg)	3 000±120
Vitamin E(mg/Kg)	258±19
Vitamin K3(mg/Kg)	33±7.3
Vitamin C(mg/Kg)	168±14
Cu(mg/Kg)	7±1

Table 1. Chemical composition of fish oil diet (FO diet) (% wet weight)

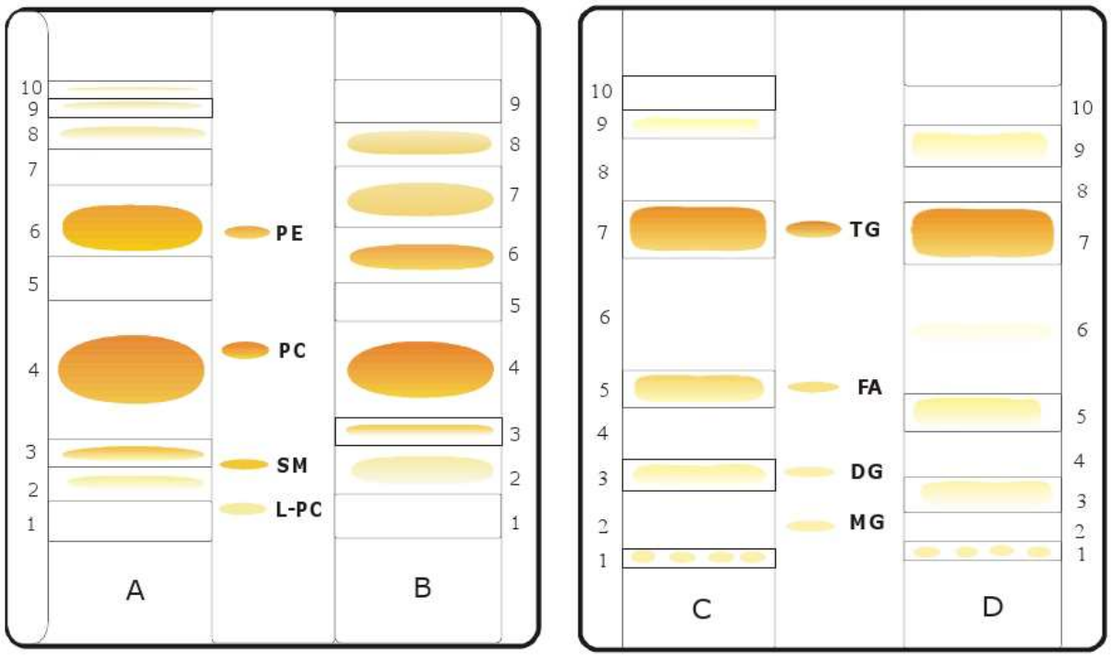
farm. The fish of each species were randomly distributed into three tanks (15 m^3 each) in groups of 600 fish per tank at the facilities of the farm. All the tanks were supplied with flow-through natural sea water system and were provided with continuous aeration and filters. The water temperature was 19–21 ^oC and oxygen content was kept close to saturation. The fish were forced to fast for three days before being transported to the tanks and then were acclimatised to the new environment for three days. The fish were fed with 1% diet of body weight per day. The fish were acclimatised for 7 days to the experimental diets, prior to feeding trial initiation. The fish were weighed under moderate anaesthesia twice throughout the experimental period (at the begging and at the end) which lasted for 90 days (May–July). The feed conversion ratio, specific growth rate, fatty acid profile of fish muscle and biological activity of fish lipids were calculated. Feed intake and morality were recorded daily. At the beginning of the on-growing period, the fish were fed only with fish oil diet (reference diet) and fifty fish samples were collected and weighed. After 60 days, 200 fish samples were collected from each fish species and dietary treatment and 10 of them provided the muscle samples to estimate the biological activity of fish lipids. At the end of the on-growing period (90 days), 200 fish samples were collected from each fish species and dietary treatment, 50 of them weighed and 10 of them provided the muscle samples to estimate the biological activity of fish lipids and the fatty acid analysis. Fish lipids obtained according to methods described above and the biological assay and fatty acid analysis performed as mentioned before (Nasopoulou et al., 2011).

2.4 Results

Regarding the *in vitro* studies lipids obtained from many traditional foods of the Mediterranean diet, such as fish (Nasopoulou et al., 2007; Nomikos et al., 2006; Panayiotou et al., 2000; Rementzis et al., 1997), olive oil (Koussissis et al., 1993), honey (Koussissis et al., 1994), milk and yogurt (Antonopoulou et al., 1996) and red wine (Fragopoulou et al., 2000) demonstrated the existence of compounds with PAF-inhibitory and/or PAF-agonistic activities in washed rabbit platelets.

More specific the content of total lipids in farmed gilthead sea bream and sea bass is increased compared to that of the respective wild species. This increase is attributed to both neutral and polar lipids' elevated levels, while the contribution of polar lipids is much higher than that of neutral lipids (Nasopoulou et al., 2007). The high amounts of total lipids in farmed fish may be attributed to the diet of the cultured fish and the confined swimming area compared to the wild fish.

The typical profile of polar and neutral lipids separation of the fish species on preparative thin layer chromatography is shown in Figure 4.



(**a**) (**b**)

Fig. 4. (a) Typical profile of the polar lipids separation of the fish species on preparative thin layer chromatography. A: wild, cultured sea bass, B: wild, cultured gilthead sea bream, L-PC: lyso phosphatidylcholine, SM: sphingomyelin, PC: phosphatidylcholine, PE: phosphatidyl ethanolamine. (b) Typical profile of the neutral lipids separation of the fish species on preparative thin layer chromatography. C: cultured gilthead sea bream, D: wild gilthead sea bream, E: wild, cultured sea bass, MG: monoglycerides, DG: diglycerides, FA: fatty acids, TG: triglycerides. The elution system used for the separation of total neutral lipids was petroleum ether:diethyl ether:acetic acid 75:30:1 (v/v/v), while the elution system used for the separation of total polar lipids was chloroform:methanol:water 65:35:6 (v/v/v).

Polar lipid fractions of farmed and wild fish of both species exhibited more potent antithrombotic activity, compared to the one of neutral lipid fractions (Nasopoulou et al., 2007), underling the more profound and beneficial effect of fish polar lipids. In addition polar lipids of both farmed and wild fish of both species demonstrated strong antithrombotic properties especially lipid fractions 4 and 6. Lipid fraction 4 corresponded to phosphatidylcholine which does not possess aggregating properties such the aforementioned lipid fraction had therefore this biological activity could be due to oxidized-

PC, which has a similar R_f value to that of phosphatidylcholine, while lipid fraction 6 exhibited potent inhibitory action against PAF activity (Nasopoulou et al., 2007). This study highlights for the first time that fish polar lipids have more potent antithrombotic effect compared to neutral lipids and that both wild and farmed fish contain strong antithrombotic properties, pointing out that both farmed and wild fish are of high nutritional value in terms of cardioprotection (Nasopoulou et al., 2007).

Regarding the impact of seasonal and geographical variation on fat, fatty acid content and the antithrombotic properties of fish lipids the study showed that total lipids obtained from gilthead sea bream during summer and winter time exhibited no statistical differences, probably due to the fact that the increased feeding intensity observed at the beginning of the summer is not sufficient enough to induce statistical changes in fat deposition in comparison with the feeding intensity at the end of the summer (Table 2), which according to the literature, induce statistical higher fat deposition (Grigorakis et al., 2002). On the other hand, total lipids obtained from sea bass during summer time were significantly reduced in comparison to total lipids obtained from sea bass during winter time (Table 2), which is in agreement with the literature (Grigorakis et al., 2004). Regarding geographical impact in gilthead sea bream fillet fat depots there was no significant difference between gilthead sea bream of Nafpaktos and Chios region (Table 2), since water temperature in both marine farms are similar during the same season.

Fish species or fish feed	Season/ Temperature °C	Fish origin	No. of individuals analyzed	TL (%)	NL (%)	PL (%)
Gilthead sea bream	December/15	Nafpaktos	5	2.91±0.11	1.39±0.05^{a,†}	1.52± 0.07††
Gilthead sea bream	June/21	Nafpaktos	5	2.72±0.08	1.19±0.02^{b,†}	1.53± 0.07^{b,††}
Gilthead sea bream	June/23	Chios	5	2.60±0.06	1.26±0.04^{a}	1.34± 0.04^{a}
Sea bass	June/23	Chios	5	2.67±0.07^{a}	1.24±0.03^{a,†}	1.53± 0.15 ††
Sea bass	December/13	Chios	5	3.68±0.30^{b}	1.88±0.10^{b}	1.80± 0.22
Gilthead sea bream feed				18.20±0.40	9.52±0.17	9.66± 0.23
Sea bass feed				18.14±0.42	9.18±0.04†	9.96± 0.33††

a, b in each column: indicates significantly different values within the same fish species and the same lipid fraction seasonally and geographically, according to the Wilcoxon test ($p < 0.05$).
†,†† in each row: indicates significantly different values within the same fish species, the same season and marine farm and different lipid fraction (NL vs PL), according to the Wilcoxon test ($p < 0.05$).

Table 2. Total lipid (TL), polar lipid (PL), neutral lipid (NL) content in farmed sea bass (*Dicentrarchus labrax)* and gilthead sea bream (*Sparus aurata*) fillets and in their commercial feed (% lipid fraction), expressed as mean±SD, (n=3)

The dominant fatty acids in the commercial feeds of sea bass and gilthead sea bream were 16:0, 18:1 ω-9, 18:2 ω-6, 20:5 ω-3 and 22:6 ω-3 (Table 3) which is in accordance with the literature (Grigorakis et al., 2002). The content of 20:4 ω-6 PUFA in the commercial feed of both farmed fish was minimal (Table 3), result which is in agreement with previous findings (Sargent et al., 1999), while 18:2 ω-6 PUFA detected in high levels in both commercial feeds (Table 3) due to the fact that this fatty acid is a constituent of plant oils used for farmed fish feed manufacture (Owen et al., 1975; Yamata et al., 1980).

Fatty acids	Gilthead sea bream feed (mg kg^{-1})	Sea bass feed (mg kg^{-1})
14:0	36.7 ± 7.33	25.8 ± 5.17
16:0	293 ± 58.5	445 ± 89.0
16:1 (ω-7)	15.8 ± 3.17	18.3 ± 3.67
18:0	20.8 ± 4.17	48.3 ± 9.67
18:1 cis (ω-9)	119 ± 23.8	145 ± 29.0
18:1 trans (ω-9)	6.67 ± 1.33	20.8 ± 4.17
18:2 (ω-6)	284 ± 56.8	335 ± 67.0
18:3 (ω-3)	48.3 ± 9.67	12.5 ± 2.50
20:4(ω-6)	-	-
20:5 (ω-3)	60.8 ± 12.2	80.8 ± 16.17
22:6 (ω-3)	117 ± 23.3	207 ± 41.3
Total SFA	350 ± 70.0	519 ± 104
Total MUFA	141 ± 28.2	184 ± 36.8
Total ω-3 PUFA	226 ± 45.2	300 ± 60.0
Total ω-6 PUFA	284 ± 56.8	335 ± 67.0
22:6 (ω-3)/20:5 (ω-3)	1.9 ± 0.3	2.6 ± 0.7

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 3. Fatty acid profiles, as determined by GC, of sea bass and gilthead sea bream commercial feeds (mg kg^{-1} of TL), expressed as mean ± SD, (n=3)

Fatty acid profiles in the fillets of the farmed gilthead sea bream and sea bass reflected fatty acid profiles of commercial feeds thus the main fatty acids in the fillets of the farmed gilthead sea bream in summer and winter time were 16:0, 18:1 (ω-9), 18:2 (ω-6), 20:5 (ω-3) and 22:6 (ω-3) (Table 4) and for sea bass were 16:0, 18:1 (ω-9), 20:5 (ω-3) and 22:6 (ω-3) (Table 5). Moreover there were no significant differences among the fatty acid profiles in seasonally obtained samples of farmed gilthead sea bream. Regarding sea bass there were a few significant differences among the fatty acid profiles in seasonally obtained samples of farmed sea bass ($P > 0.05$), such as 14:0, 16:1 and 18:1 trans fatty acids which levels where significant elevated in winter compared to summer (Table 4). The lack of significant differences in the fatty acid profile of farmed sea bass and gilthead sea bream fillets seasonally was probably because of the fact that fish were fed the same diet throughout the experimental period of our study. Same observations made other researchers (Yildiz et al., 2008), indicating that different seasons did not cause differences in the fatty acid profile of farmed fish fillets. Literature referring to seasonal impact on fatty acid content in sea bass and gilthead sea bream fillets is limited, thus it is difficult to conclude to a clear result (Grigorakis, 2007), however it can be said that fish of both species were good source of 20:5 (ω-3) and 22:6 (ω-3) (Tables 4 and 5).

Fatty acid	Summer (mg kg^{-1})	Winter (mg kg^{-1})
14:0	10.0 ± 2.00	15.0 ± 3.00
16:0	416 ± 83.2	396 ± 79.3
16:1 (ω-7)	17.3 ± 3.47	25.7 ± 5.13
18:0	89.5 ± 17.9	97.3 ± 19.5
18:1 cis (ω-9)	138 ± 27.7	193 ± 38.6
18:1 trans (ω-9)	21.3 ± 4.25	28.6 ± 5.72
18:2 (ω-6)	95.7 ± 21.8	150 ± 33.7
18:3 (ω-3)	-	-
20:4 (ω-6)	9.67 ± 1.93	11.3 ± 2.25
20:5 (ω-3)	98.7 ± 19.7	126 ± 25.1
22:5 (ω-3)	16.5 ± 3.30	27.3 ± 5.45
22:6 (ω-3)	393 ± 78.6	380 ± 75.9
Total SFA	515 ± 103	509 ± 102
Total MUFA	177 ± 35.4	247 ± 49.4
Total ω-3 PUFA	508 ± 102	532 ± 106
Total ω-6 PUFA	105 ± 21.1	161 ± 32.2
22:6 (ω-3)/20:5 (ω-3)	4.0 ± 1.2	3.0 ± 0.8

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

Table 4. Fatty acid profiles (mg kg^{-1} of TL), as determined by GC, of farmed gilthead sea bream (*Sparus aurata*) fillets during summer and winter, expressed as mean ± SD, (n=3).

Fatty acids	Summer (mg kg^{-1})	Winter (mg kg^{-1})
14:0	0.25 ± 0.05^{a}	22.1 ± 4.42 ^{b}
16:0	282 ± 56.5	229 ± 45.8
16:1 (ω-7)	3.83 ± 0.77 ^{a}	28.8 ± 5.77 ^{b}
18:0	68.2 ± 13.6	48.8 ± 9.75
18:1 cis (ω-9)	74.3 ± 14.9	121 ± 24.2
18:1 trans (ω-9)	5.08 ± 1.02 ^{a}	9.67 ± 1.93 ^{b}
18:2 (ω-6)	12.9 ± 4.98	25.3 ± 7.85
20:4 (ω-6)	2.75 ± 0.55	-
20:5 (ω-3)	92.9 ± 18.6	103 ± 20.6
22:6 (ω-3)	272 ± 54.4	222 ± 44.5
Total SFA	351 ± 70.1	300 ± 60.0
Total MUFA	83.2 ± 16.7 ^{a}	160 ± 31.9 ^{b}
Total ω-3 PUFA	365 ± 73.0	326 ± 65.1
Total ω-6 PUFA	15.7 ± 3.13	25.3 ± 5.05
22:6 (ω-3)/20:5 (ω-3)	2.9 ± 0.8	2.2 ± 0.4

a, b in each row: indicates significantly different values within the same fish species according to the Wilcoxon test ($p < 0.05$).
Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 5. Fatty acid profiles (mg kg^{-1} of TL), as determined by GC, of farmed sea bass (*Dicentrarchus labrax*) fillets during summer and winter, expressed as mean ± SD, (n=3).

The dominant fatty acids in the fillets of the farmed gilthead sea bream in both marine farms were 16:0, 18:0, 18:1 (ω-9), 20:5 (ω-3) and 22:6 (ω-3) and there were no significant differences among the fatty acid profiles in geographically obtained samples of farmed gilthead sea bream (Table 6).

Similarities in the fatty acid fillet content of gilthead sea bream of the same age, fed with the same diet and originated from two different marine farms could probably attributed to the fact that the two marine farms were of similar water temperature and salinity. However gilthead sea bream from both marine farms are good sources of 20:5 (ω-3) and 22:6 (ω-3) (Table 6).

Fatty acids	Gilthead sea bream Chios (mg kg^{-1})	Gilthaed sea bream Nafpaktos (mg kg^{-1})
14:0	17.0 ± 3.40	10.0 ± 2.00
16:0	419 ± 83.9	416 ± 83.2
16:1 (ω-7)	30.6 ± 10.5	17.3 ± 7.4
18:0	99.8± 20.0	89.5 ± 17.9
18:1 cis (ω-9)	155 ± 77.7	138 ± 27.7
18:1 trans (ω-9)	29.4 ± 5.88	21.3 ± 4.25
18:2 (ω-6)	62.1 ± 12.4	95.7 ± 19.1
20:4 (ω-6)	18.8 ± 5.77	9.67 ± 4.93
20:5 (ω-3)	153 ± 30.6	98.7 ± 19.7
22:5 (ω-3)	26.7 ± 5.33	16.5 ± 3.30
22:6 (ω-3)	405 ± 80.9	393 ± 78.6
Total SFA	536 ± 107	515 ± 103
Total MUFA	215 ± 43.1	177 ± 35.4
Total ω-3 PUFA	584 ± 117	508 ± 102
Total ω-6 PUFA	80.9 ± 16.2	105 ± 21.1
22:6 (ω-3)/20:5 (ω-3)	2.6 ± 1.2	4.0 ± 1.6

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

Table 6. Fatty acid profiles (mg kg^{-1} of TL), as determined by GC, of farmed gilthead sea bream (*Sparus aurata*) fillets obtained from Nafpaktos and Chios marine farms, expressed as mean ± SD, (n=3).

The experimental data showed that total lipids of gilthead sea bream and sea bass in summer contained more potent PAF-agonists – constituents with aggregatory activity less powerful than the one of PAF – and PAF-inhibitors, respectively than the total lipids of the aforementioned fish in winter. Such elevated biological activity of fish during summer time could be due to the increased water temperature. These data show that both fish species posses more potent antithrombotic properties during summer time.

Total lipids of gilthead sea bream from Nafpaktos marine farm exhibited more intense biological activity than total lipids of gilthead sea bream from Chios marine farm possibly due to the elevated polar lipids content of gilthead sea bream from Nafpaktos marine farm (Table 2), which are responsible for the biosynthesis of PAF antagonist.

Regarding the *in vivo* studies gilthead sea bream polar lipids (GSBPL) exhibits an anti-atherogenic effect by increasing HDL-cholesterol levels, despite the fact that total cholesterol, LDL-cholesterol and triglycerides were equally increased in both groups (group A: atherogenic diet and group B: atherogenic diet supplemented with GSBPL) (Nasopoulou et al., 2010).

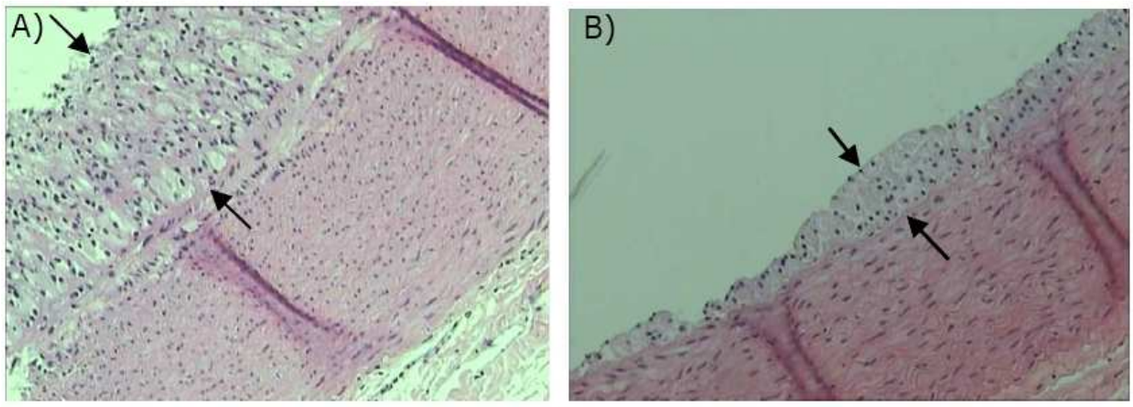
At the end of our experiment (45 days), plasma PAF-acetylhydrolase activity was significantly elevated in group B (atherogenic diet supplemented with GSBPL) compared to group A (atherogenic diet), which may be attributed to the increased HDL-cholesterol levels, since this enzyme is partially located on the HDL particles.

Rabbits of group B (atherogenic diet supplemented with GSBPL) developed early atherosclerotic lesions, of significantly lower degree compared to group A (atherogenic diet) (Figure 5) (Table 7).

	Early atherosclerosis lesions evaluation Thickness (mm) Surface area (mm^2/mm)x1000	
Group A	0.44±0.15[a]	0.45±0.16[a]
Group B	0.11±0.08[a]	0.12±0.09[a]

Results were expressed as mean ± SD.
A: atherogenic diet; B: atherogenic diet enriched with GSBPL.
[a]Donates statistical significance between groups A and B ($p < 0.05$), according to Mann-Whitney *U*-test.

Table 7. Assessment of early atherosclerosis lesions observed in rabbit aortas.



A: Group A (atherogenic diet);
B: Group B (atherogenic diet enriched with GSBPL)

Fig. 5. Representative optic micrographs x 100 of aortic wall sections stained with hematoxylin and eosin from the two experimental groups, where atherosclerotic lesions appear as foam cells (↑).

This result could be explained on the basis of the elevated PAF-acetylhydrolase activity in the plasma of rabbits of group B. This study shows that polar lipids of cultured gilthead sea bream (*Sparus aurata*) contain bioactive micro-constituents, PAF inhibitors, that inhibit PAF activity both in vitro and in vivo, consequently inhibiting early atherosclerosis development. The above data reinforce the beneficial effect of cultured gilthead sea bream polar lipids against atherosclerosis development (Nasopoulou et al., 2010).

Finally, regarding the results of the novel fish designing the growth performance factors of gilthead sea bream fed with fish oil diet in comparison with gilthead sea bream fed with olive pomace and olive pomace oil diet exhibited no statistical differences, indicating similar feed conversion ratio and specific growth rate (Nasopoulou et al., 2011). However, sea bass fed with olive pomace and olive pomace oil diet showed statistical decreased specific growth rate and statistical increased mortality in comparison with sea bass fed with fish oil diet (Nasopoulou et al., 2011), suggesting that gilthead sea bream fed with the experimental diets exhibited satisfactory growth performance – similar to gilthead sea bream fed with fish diet – and better than the one of sea bass.

Comparing gilthead sea bream fed with olive pomace to fish fed with olive pomace oil, fish fed with olive pomace diet showed significant lower ($p < 0.05$) mortality in comparison to olive pomace oil diet, while the feed intake, feed conversion ratio and specific growth rate did not exhibit significant differences (Nasopoulou et al., 2011). Therefore, olive pomace could be used as 8% dietary fish oil substitute, in gilthead sea bream without compromising the growth performance and due to significant decreased mortality it is preferable than olive pomace oil.

All classes of fatty acids, such as saturated (especially 16:0 and 18:0), monoenes (especially 18:1 cis), x-3 (20:5 and 22:6) and ω-6 (18:2) of the fish fed with olive pomace diet were found to statistically decrease compared to the fish fed with fish oil diet (Nasopoulou et al., 2011). Such alterations of the fatty acid content in the fish fillets, especially the reduction of ω-3 HUFA levels, particularly 20:5 ω-3, are in accordance with the literature (Izquierdo et al., 2003, 2005; Montero et al., 2005; Mourente et al., 2005).

The main constraint for the use of plant oils in fish feeds is the lack of ω-3 HUFA, particularly 20:5 ω-3 and 22:6 ω-3. According to the literature, the biological demand of gilthead sea bream and sea bass is at least 0.9% and 0.7% of the diet, respectively (Ibeas et al., 1994; Kalogeropoulos et al., 1992, Skalli & Robin, 2004). In the present study, the ω-3 HUFA content of experimental diets meet the theoretical values of the examined species requirements in these fatty acids (Nasopoulou et al., 2011).

The biological activity of gilthead sea bream total lipids fed with the olive pomace diet was significantly increased in comparison with the gilthead sea bream fed with the fish oil diet at the end of the experimental period (90 days) (Nasopoulou et al., 2011). The experimental data indicate that olive pomace 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). In addition, these anti-PAF lipid components possess antibacterial properties (Nasopoulou et al., 2008), protecting the fish from bacteria and prolonging shelf-life.

Gilthead sea bream is the fish species that absorbed and metabolized better the experimental diets, in particular the olive pomace diet, exhibiting improved biological activity in comparison with sea bass fed with olive pomace diet and with gilthead sea bream fed with fish oil diet, indicating that olive pomace reinforced the anti-PAF biological activity of gilthead sea bream. Moreover gilthead sea bream fed with olive pomace diet had a statistically decreased content ($p < 0.05$) of 20:5 ω-3 and 22:6 ω-3 in comparison with the ones of fish fed with the fish oil. Therefore, the more potent anti-PAF properties and subsequent cardio protective ability of the gilthead sea bream fed with the olive pomace diet

cannot be attributed to the fatty acid content since the amounts of these fatty acids are decreased.

The current work focused for the first time on improving the nutritional value of fish in terms of cardio protection by partially substituting fish oil in fish feed with olive oil industry by-products rich in phenolic compounds and PAF inhibitors, making the management of these by-products cost-effective and producing a new ''variety'' of gilthead sea bream with properties against cardiovascular diseases under the guidelines of E.C.1924/2006.

In order to confirm the cardioprotective and beneficial effects of this novel fish for humans, future research is required by using *in vivo* the biologically active compounds of the novel fish with experimental animals and to examine which compounds inhibit the formation of atheromatic plaque in blood arteries.

3. Conclusion

To summarize the scope of our work that has been carried by our group in the past 10 years, it should be highlighted that we work towards the improvement of fish feeds with the ultimate goal to produce aquacultured fish with higher nutritional value. To achieve this goal, the use of Gas Chromatography has been an extremely versatile tool: both the enrichment of fish feed with beneficial fatty acids from olive pomace and olive pomace oil and the enrichment of fish flesh with beneficial fatty acids from fish feed were screened and quantified by Gas Chromatography. In conclusion, Gas Chromatography has been a powerful analytical tool in our attempts to improve the quality of fish feeds and ultimately fish. Gas Chromatography offers the capacity to fine tune our enrichment experiments before carrying out expensive and time-consuming biological experiments. Under this scope, in our work towards novel food, that are described in this chapter, the application of Gas Chromatography has allowed us to perform successful "feed enrichment" experiments and also collect valuable analytical data on the nutritional value of the novel (enriched) fish feeds and fish.

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Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications

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The aim of this book is to describe the fundamental aspects and details of certain gas chromatography applications in Plant Science, Wine technology, Toxicology and the other specific disciplines that are currently being researched. The very best gas chromatography experts have been chosen as authors in each area. The individual chapter has been written to be self-contained so that readers may peruse particular topics but can pursue the other chapters in the each section to gain more insight about different gas chromatography applications in the same research field. This book will surely be useful to gas chromatography users who are desirous of perfecting themselves in one of the important branch of analytical chemistry.

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