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Lipid Oxidation in Fish and Fish Products of Interest for European Aquaculture

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Abstract

This thesis is based on three main trials. The overall aim of the present PhD thesis was to investigate the effect of some *infra vitam* and *post mortem* factors on oxidative stability of fish muscle. Killing procedures, such as percussion, asphyxia in air, and asphyxia by carbon monoxide (CO) were tested. Moreover, the effect of the utilization of mechanical separation technique (MSM) on oxidative stability of fish muscle was also investigated.

The first research was conducted in order to investigate the effect of slaughtering procedures on oxidative stress and oxidative stability of muscle of farmed rainbow trout (*Oncorhynchuss mykiss*). Specifically, asphyxia in air was utilized as stressful method whilst percussion as a no-stress one. Stress at slaughter was assessed both by nucleotides analysis of muscle and lipid mediators detection in fish plasma. Moreover, the overall aim was to verify if stress during slaughter is reflected by the presence of some oxidative biomarkers in plasma, and if such as stress may affect the oxidative stability of fish muscle during a long term frozen storage (-10 °C, 165 days). Results revealed that stress during slaughter can greatly influence oxidative stress and oxidative stability of rainbow trout fillets. In fact, asphyxia, which was the most stressful method, induced a higher production of some lipid mediators such as hydroperoxides and EPA-derived prostaglandins, such as 12-HpHEPE/15-HpHEPE and PGD3/PGE3. As a consequence, fillets derived from asphyxiated fish were less stable in terms of oxidative stability and showed lower shelf-life.

In the second research, Atlantic salmon was utilized as target species in order to evaluate the effect of different stunning/killing procedures on lipid and cholesterol oxidation during 14 days of refrigerated storage. Carbon monoxide (CO) has been recently utilized as a new stunning/killing procedure for Atlantic salmon (*Salmo salar*),

however no studies on its effect on fillets quality were previously performed. For this reason, lipid and cholesterol oxidation of fillets were evaluated at two times of refrigerated (2.5 °C) storage, T0 (64 h after death) and T14 (14 days from T0). The use of CO was compared with the percussion (P) method, considered as control. Results revealed that fatty acid profile, primary (conjugated dienes) and secondary (TBARS) oxidation products, cholesterol oxidation products (COPs) and carotenoids were unaffected by the killing method. Despite the low oxidative status of lipids (0.66 and 0.60 mg malondialdehyde kg^{-1} muscle in P and CO fish, respectively), cholesterol was found to be highly oxidized (0.17 and 0.13 mg COPs kg^{-1}). Moreover, storage was found to significantly affect the oxidative stability of fish muscle by increasing oxidation products. Interestingly, TBARS content doubled while the increase for COPs was not homogeneous: α- and β-epoxycholesterol increased by 25%, whereas triol and 7-ketocholesterol increased by 48 and 62% respectively. In conclusion, the quality of salmon fillets just after slaughtering and after 14 days of refrigerated storage at 2.5 °C did not change, irrespective of the killing method adopted, suggesting that the CO method may be applied without any detrimental effect on the quality of fish fillets. Neverthless, storage time was confirmed to be a critical phase in order to maintain unaltered fish fillets quality.

In the third study instead, the effect of a *post mortem* factor such as mechanical separation process for obtaining "mechanically separated meat" (MSM) from decapitated and eviscerated fish was studied. Mechanically separated meat has been utilized in certain meat and meat products, especially from pork, beef and chicken. This process however, is not so applied in European fish industries. The overall aim of this third research was to evaluate the effect of that process on physical and chemical properties of three species farmed in the European aquaculture as well as European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), and rainbow trout (*Oncorhynkus mykiss*). Specifically, MSM-burgers were compared with minced-burgers and whole fillets by evaluating colour, dienes, TBARS, and fatty acid profiles during storage.

Riassunto

Questa tesi si basa su tre studi principali. L'obiettivo generale della presente tesi di dottorato è stato quello di studiare l'effetto di alcuni fattori *infra vitam* e *post mortem* sulla stabilità ossidativa del muscolo di pesce. Le procedure d'uccisione, come la percussione, l'asfissia in aria e l'asfissia da monossido di carbonio (CO) sono state testate su specie di grande importanza economica, come il salmone atlantico e la trota iridea. Inoltre è stato studiato l'effetto dell'impiego della tecnica di separazione meccanica delle carni (CSM) impiegata su specie di pesci di interesse per l'acquacoltura europea.

La prima ricerca ha esaminato gli effetti delle procedure di macellazione sia sullo stress ossidativo sia sulla stabilità ossidativa del muscolo di trota iridea (*Oncorhynchuss mykiss*). In particolare, l'asfissia in aria è stata utilizzata come metodo di uccisione fortemente stressante, mentre la percussione come un metodo no-stress. Lo stress al momento della macellazione è stato valutato attraverso la quantificazione dei nucleotidi nel muscolo e attraverso la determinazione di mediatori lipidici nel plasma. Inoltre, gli obiettivi generali erano sia verificare se lo stress durante la macellazione si sarebbe potuto discriminare attraverso la presenza di alcuni biomarker ossidativi nel plasma, sia valutare come lo stress può compromettere la stabilità ossidativa del muscolo di pesce durante un lungo periodo di congelamento (-10 °C, 165 giorni). I risultati hanno rivelato che lo stress durante la macellazione può influenzare notevolmente lo stress ossidativo e la stabilità ossidativa di filetti di trota iridea. In realtà l'asfissia, che si è confermato il metodo più stressante, ha indotto una maggiore produzione di alcuni mediatori lipidici, come idroperossidi e prostaglandine EPA-derivate, come ad esempio 12-HpHEPE/15-HpHEPE e PGD3/PGE3. Di conseguenza, i filetti ottenuti da pesci asfissiati hanno mostrato sia una minor stabilità ossidativa sia una minore shelf-life.

Nella seconda ricerca, il salmone atlantico (*Salmo salar*) è stato utilizzato come specie bersaglio per valutare l'effetto di differenti procedure di stordimento/uccisione sull'ossidazione lipidica e del colesterolo durante 14 giorni di conservazione refrigerata. Il monossido di carbonio (CO) è stato recentemente impiegato come una nuova procedura di stordimento/uccisione del salmone atlantico, tuttavia non sono stati condotti studi sui suoi possibili effetti sulla qualità dei filetti. Per questo motivo l'ossidazione lipidica e del colesterolo nei filetti è stata valutata in due tempi di stoccaggio refrigerato (2.5 °C): T0 (64 h dopo la morte) e T14 (14 giorni da T0). L'uso di CO è stato confrontato con la percussione, metodo utilizzato per l'uccisione di questa specie. I risultati hanno rivelato che il profilo degli acidi grassi, i prodotti di ossidazione primari (dieni coniugati) e secondari (TBARS), i prodotti di ossidazione del colesterolo (COPs) e i carotenoidi sono stati influenzati dal metodo di uccisione. Nonostante il basso stato ossidativo dei lipidi (0,66 e 0,60 mg di malondialdeide kg^{-1} muscolo nei gruppi sottoposti a percussione e monossido di carbonio, rispettivamente), il colesterolo è risultato essere altamente ossidato (0,17 e 0,13 mg COP kg^{-1}). Inoltre la conservazione ha influenzato in modo significativo la stabilità ossidativa del muscolo di pesce, determinando un aumento dei prodotti di ossidazione. È interessante notare che durante la conservazione il contenuto di TBARS è raddoppiato, mentre l'incremento dei COPs non è risultato omogeneo: α- e β –epoxycolesterolo infatti sono aumentati del 25%, mentre triolo e 7-ketocolesterolo sono aumentati rispettivamente del 48 e del 62%. In conclusione, la qualità dei filetti di salmone subito dopo la macellazione e dopo 14 giorni di conservazione in frigorifero a 2,5 °C non è risultata alterata, indipendentemente dal metodo di uccisione adottato, suggerendo così che l'impiego di CO può essere applicato senza alcun effetto negativo sulla qualità del pesce. Ciononostante è stato confermato che la conservazione rappresenta una fase critica al fine di mantenere inalterata la qualità dei filetti.

Nel terzo studio invece, è stato valutato l'effetto di un fattore *post mortem* come l'applicazione del processo di separazione meccanica per l'ottenimento di "carni separate meccanicamente" (CSM) da pesci decapitati ed eviscerati. Le carni separate meccanicamente sono ormai utilizzate da anni per l'ottenimento di prodotti a base soprattutto di carne di maiale, manzo o pollo. Questo processo tuttavia a livello europeo non è altrettanto sfruttato nelle industrie di prodotti ittici. Per questo, l'obiettivo generale di questa terza ricerca è stato quello di valutare l'effetto del processo di separazione meccanica sulle proprietà fisiche e chimiche di tre specie d'interesse per l'acqucoltura europea, come spigola (*Dicentrarchus labrax*), orata (*Sparus aurata*) e trota iridea (*Oncorhynkus mykiss*). In particolare, sono stati confrontati burger ottenuti da CSM con burger ottenuti da carne macinata e con filetti interi in termini di colore, dieni, TBARS e profilo in acidi grassi, valutandone l'evoluzione durante la conservazione.

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PART I

1. INTRODUCTION

1.1 LIPID FOREWORD

Nomenclature Committee (ILCNC) developed a dynamic "Comprehensive Classification System for Lipids" that was published in 2005 (Fahy et al., 2009). For the purpose of classification, lipids are defined as hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides) and/or by carbocation-based condensations of isoprene units (prenol lipids and sterol lipids). The comprehensive classification system organises lipids into these eight well-defined categories (Table 1) that cover eukaryotic and prokaryotic sources. It has been internationally adopted and widely accepted by the lipidomics community. The system is also available online on the LIPID MAPS (website http://www.lipidmaps.org). The comprehensive classification system has been under the guidance of the ILCNC which meets periodically to propose changes and updates the classification, nomenclature, and structural representation due to the global interest for nonmammalin sources, such as plants, bacteria, fungi, algae and marine organism (Fahy et al., 2009).

Lipids are formed from structural units with a pronounced hydrophobicity. This solubility characteristic, rather than other common structural feature, is unique for this class of compounds. Lipids are soluble in organic solvents but not in water. Water insolubility is the analytical property used at the basis for their easy separation from proteins and carbohydrates (Folch et al., 1957; Bligh & Dyer, 1959).

Two general approaches have been generally accepted for lipid classification: one according to "acyl residue" characteristic, and the other according to the characteristic "neutral-polar". The first divides lipid into simple lipid (not saponifiable), those are free fatty acids, isoprenoid and tocopherols, acyl lipids (saponifiable), which are mono-, di-, triacylglycerols, phospholipids, glycolipids, diols, waxes and sterol esters. The second category divides lipids in neutral lipids (fatty acids (C>12), mono-, di-, tri-acylglycerols, waxes, sterols and sterol esters, carotenoids), and polar lipids (glycerophospholipids, glyceroglycolipids, sphingophospholipids, sphingoglycolipids).

Table 1. Lipid categories of the comprehensive classification system and the number of structures in the lipid maps database.

Category	Abbreviation	Structure in database
Fatty acyls	FA	2678
Glycerolipids	GL	3009
Glycerophospholipids	GP	1970
Prenol Lipids	PR	610
Polyketides	PK	132
Saccharolipids	SL	11
Sphingolipids	SP	620
Sterol Lipids	ST	1744

As reported by many authors, lipids have more than an important role. They work as membrane constituents (phospholipids), as fuel molecules (37 kJ/g or 9 kcal/g triacylglycerol) and as a source of essential fatty acids (linoleic C18:2n6, LA; α-linolenic C18:3n3, ALA), vitamins (sterols), and their oxidation product seemed to be involved in inflammatory response (Funk, 2001). Apart from these roles, lipids strongly influence, positively or negatively, food organoleptic quality. Indeed, some lipid compounds are indispensable as food emulsifier, colorants, food aroma substances or as their precursors.

Meat lipids are mainly composed of phospholipids and triglycerides. According to Stryer (1995), phospholipids are structural lipids in which, for most, glycerol is esterified to two fatty acids and a phosphate group. They form cell and organelle membrane, and although the content is relatively constant in the muscle (1% of the tissue weight regardless of fat content; Decker et al., 2005), their fatty acids can be altered in order to maintain the function and fluidity of the cell (McMurchie, 1988). As noted by Mapiye et al. (2012) and Luciano et al. (2013), typically glycerol-based phospholipids contain a saturated fatty acid (SFA) in the *sn*1 position and a polyunsaturated fatty acid (PUFA) in the *sn*2 position. As reported by Catalá (2010), linoleic acid, C20:4 n6 (eicosapentaenoic acid, EPA) and C22:6 n3 (docosahexanoic acid, DHA), are well known in cellular phospholipids.

Regarding triglycerides fraction, Mapiye et al. (2012) underline that they are storage lipids and are composed by three fatty acids esterified to glycerol. They represent the major lipid class in adipose tissue (Wood et al., 2008). They are accumulated when energy intake is in excess of expenditure, and are mobilised for oxidation when dietary energy is limiting. Despite their abundance in muscle tissues, they have 100 time less surface area than phospholipids on a weight basis and they are more saturated than the other (Decker et al., 2005).

Some authors, as Gandemer (1997) pointed out the attention on the susceptibility to oxidation of phospholipids comparing with triacylglycerols. That is a matter of fact, that the polyunsaturated lipid fraction is predominantly susceptible to lipid oxidation, due to the presence of double bonds (Catalá, 2010). The mechanisms of lipid oxidation and their effects on food quality will be discussed later. However, it is important to note that when lipid peroxidation affects phospholipids more than triacylglycerols can perturb the assembly of the membrane. That can lead membrane chemical and physical changes, for example in permeability and fluidity, altering ion transport and inhibiting metabolic process (Nigam & Schewe, 2000).

On the other hand, meat lipids are one of the few components of meat that can be modified in content and composition (Mapiye et al., 2012). In all vertebrates, the *in vivo* n3 LC-PUFA biosynthetic pathway is conceptually identical (Sprecher, 2000), and it involves a series of enzymatic steps catalysed by many enzymes (Emery et al., 2013). The pathway that converts the two essential polyunsaturated fatty acids (PUFA) C18:2n6 and C18:3n3 into n6 LC-PUFA (such as arachidonic acid, ARA, C20:4n6), and n3 LC-PUFA (such as EPA and DHA), respectively, is commonly named ‘‘Sprecher pathway’’ (Emery et al., 2013). Because of evolution and adaptation to the environment, each species has a different capacity for PUFA bioconversion into LC-PUFA, depending on the presence, abundance and activity of the specific enzymes in the metabolic pathway.

It can be argued that the ability of biosynthesising n3 LC-PUFA is greater in fish compared to other animals and mammals, that makes them extremely interesting for human health. As reported by Catalá (2010), linoleic and linolenic acids cannot be synthesized *de novo* by mammals, hence they are essential in the diet. The n3 fatty acids, especially EPA and DHA can be obtained from high-fat fish and marine mammals, while the n6 fatty acids are concentrated in meats and vegetable oils. High amount of n3 fatty acids was reported for different fish species when comparing with terrestrial animals. For example, in fish it is possible to find value of EPA reach from 4.74% of red scorpion fish (*Scorpaena scrofa*) to 11.7% of sardine (*Sardinella aurita*) (Özogul & Özogul, 2007) while in pigs, sheep and cattle, Wood et al. (2008) reported values around 0.31, 0.45 and 0.28%, respectively. Moreover, the content of DHA varied from a lower value of 13.3% in sardine (Özogul & Özogul, 2007) to 36% in scad muscle

(*Trachurus mediterraneous*), values higher than 0.43% found in female pig (Enser et al., 2000).

During the last decades, a large number of studies around fish were conducted in order to better understand if the nature and quantity of lipids and fatty acids might be affected by species (Haliloğlu et al., 2004; Özogul & Özogul, 2007; Prato & Biandolino, 2012), habitat (Haliloğlu et al., 2004), diet (Chen et al., 2007; Baron et al., 2009; Masiha et al., 2013) and handling operations, such as slaughter methods (Sigholt et al., 1997; Lefèvre et al., 2008).

Regarding the influence of the species on fatty acids composition of fillets, many studies were conducted with the aim to evaluate different commercial fish species. Özogul & Özogul (2007) quantified the lipid content and the fatty acid composition of a range of species from Turkish Seas, such as bogue (*Boops boops*), mullet (*Mugil cephalus*), sardine (*Sardinella aurita*); Özogul et al. (2009) examined 34 different Mediterranean fish species; Prato & Biandolino (2012) reported the data for Mediterranean species like bogue (*Boops boops*), seabream (*Dicentrarchus labrax*), two-banded seabream (*Diplodus vulgaris*), and Huynh & Kitts (2009) focused on Pacific species. The data obtained for some of those species are summarised in Table 2 and Table 3, that illustrate the total lipid content, percentage of the main fatty acids and the sum of the three categories of fatty acids, saturated, monounsaturated and polyunsaturated.

All the authors reported value obtained from species caught or purchased from the Mediterranean, Aegean, Black Sea (Özogul & Özogul, 2007; Özogul et al., 2009; Prato & Biandolino, 2012), and Pacific Ocean (Huynh & Kitts, 2009). Within the 11 fish species reported, 5 came from the Mediterranean Sea, 4 from the Ionian Sea, and 2 from the Pacific Ocean. The choice of the species reported was mainly due to the high commercial value in Mediterranean countries and Pacific North-West countries.

The lipid content (Table 3), expressed on a wet weight basis, ranged from as low as 0.73 g/100 g for *Merluccius productus* (Huynh & Kitts, 2009) to the highest amount of 8.12 g/100 g for *Mullus barbatus* (Prato & Biandolino, 2012). According to the lipid content (Ackman, 1989), it is possible to classify fish in four categoriesy: lean fish (less than 2% of lipids), low-fat fish (2-4%), medium-fat fish (4-8%) and high-fat fish (more than 8%). Of the species summarised, only the *Mullus barbatus* belongs to the high-fat category, three to the lean fish (*Merluccius* spp. and *Diplodus vulgaris*) with less than 1% of fat, and the other belongs to the low and medium categories. Globally, the authors underlined that the lipid contents presented in their work were in agreement with the average lipid contents reported for the same species in literature (Prato & Biandolino, 2012).

However, it could be interesting to note that different authors found differences in the lipid content of the same species, for example *Boops boops*, *Liza*

aurata and *Diplodus vulgaris.* Prato & Biandolino (2012) reported for *Boops boops* a lipid content of 5.2 g/100 g while Özogul et al. (2009) found a content of 3.64 g/100g. Moreover, the same authors found different values for lipid content of both *Liza aurata* (3 *vs* 1%) and *Diplodus vulgaris* (6 *vs* 2.3%). As observed by Prato & Biandolino (2012), there are several factors which might explained that marked differences, such as season, geographical origin, age and reproductive status. Unfortunately it is quite difficult to find details about the life of caught fish, especially if they are not directly caught but purchased in a local market. In their research, Prato & Biandolino (2012) reported that the species studied were caught during the spring season, while Özogul et al. (2009) did not specify the time or season of the catch. Finally, it would be useful to report the season even if it is not any attempt to make seasonal comparison both between and within the different fish species.

As mentioned before, diet is one of the main *infra-vitam* factors affecting lipid composition, and its stability as a results.

Table 2. Fatty acid composition (weight % of total fatty acids) in the total lipid of different fish species.

	Reference	Lipid %	Cat.	C16:0	C18:1	C18:2n6	C18:3n3	C20:4n6	C20:5n6	C22:6n3
Boops boops	Prato &d Biandolino (2012)	5.2	Medium-fat	29.9	12.98	1.23	0.74	0.83	6.43	15.87
Boops boops	Özogul et al. (2009)	3.64	Low-fat	20.05	20.8	0.93	0.39	0.10	5.09	18.7
Dicentrarchus labrax	Prato & Biandolino (2012)	2.33	Low-fat	29.92	10.86	3.77	1.44	2.80	6.91	13.83
Diplodus vulgaris	Prato & Biandolino (2012)	3.6	Low-fat	27.48	11.88	2.28	1.17	2.73	7.45	17.18
Diplodus vulgaris	Özogul et al. (2009)	1.04	Lean	19.45	9.72	0.89	0.18	6.80	5.30	21.93
Liza aurata	Prato & Biandolino (2012)	5.96	Medium-fat	28.12	13.23	2.04	2.83	2.33	6.12	10.81
Liza aurata	Özogul et al. (2009)	2.29	Low-fat	25.87	4.21	1.69	0.07	5.96	10.00	21.71
Merluccius productus	Huynh & Kitts (2009)	0.73	Lean	21.7	9.65	0.92	0.30	1.95	1.25	22.08
Merluccius merluccius	Özogul et al. (2009)	0.76	Lean	21.93	12.42	1.05	3.68	3.68	5.81	26.83
Mullus barbatus	Prato & Biandolino (2012)	8.12	High-fat	29.17	13.87	1.42	1.24	3.01	7.16	12.05
Mugil cephalus	Özogul et al. (2007)	2.09	Low-fat	21.5	9-73	1.40	0.34	0.12	5.39	36.2
Sardinella aurita	Özogul et al. (2007)	3.47	Low-fat	20.5	5.57	2.05	0.35	0.61	11.7	13.3
Sardinops sagax	Huynh & Kitts (2009)	6.43	Medium-fat	3.95	4.16	1.49	0.93	1.02	2.01	32.65

Table 3. Total sum of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acids (PUFA), and ratio n3/n6 of different fish species.

	Reference	ΣSFA	ΣMUFA	ΣPUFA	n3/n6
Boops boops	Prato & Biandolino (2012)	41.8	28.2	29.9	4.7
Boops boops	Özogul et al. (2007)	32.3	27.0	27.5	20.9
Dicentrarchus labrax	Prato & Biandolino (2012)	42.9	24.6	32.4	2.5
Diplodus vulgaris	Prato & Biandolino (2012)	38.1	27.9	34.0	3.9
Diplodus vulgaris	Özogul et al. (2009)	34.3	16.3	36.1	3.5
Liza aurata	Prato & Biandolino (2012)	39.7	31.4	28.9	3.1
Liza aurata	Özogul et al. (2009)	42.2	22.5	21.7	1.8
Merluccius productus	Huynh & Kitts (2009)	32.4	21.88	43.5	8.4
Merluccius merluccius	Özogul et al. (2009)	33	18.5	41.7	7.6
Mullus barbatus	Prato & Biandolino (2012)	39.9	32.4	27.7	3.3
Mugil cephalus	Özogul et al. (2007)	32.8	25.8	24.8	8.2
Sardinella aurita	Özogul et al. (2007)	38.7	17.6	31.0	8.9
Sardinops sagax	Huynh & Kitts (2009)	25.2	14.2	56.8	11.3

Fish lipid differs from mammalian lipid. The main difference is that fish lipids include up to 40% of long-chain fatty acids (14-22 carbon atoms) which are highly unsaturated. Mammalian fat will rarely contain more than two double bonds per fatty acid molecule while the depot fats of fish contain several fatty acids with five or six double bonds. In human nutrition, fatty acids such as linoleic and linolenic acids are regarded as essential since they can not be synthesized by the organism. In marine fish, these fatty acids constitute only around 2% of the total lipids, which is a small percentage compared with many vegetable oils. However, fish oils contain other polyunsaturated fatty acids which are "essential" to prevent skin diseases in the same way as linoleic and arachidonic acids. As members of the linolenic acid family (first double bond in the third position, n3 counted from the terminal methyl group), they will also have neurological benefits in growing children. One of these fatty acids, EPA, has attracted considerable attention. Danish scientists have found this acid high in the diet of a group of Greenland Eskimos virtually free from arteriosclerosis, indeed. Investigations in the United Kingdom and elsewhere have documented that EPA in the blood is an extremely potent antithrombotic factor (Simopoulos, 1991).

As well, DHA has grown of importance. Intervention studies have demonstrated beneficial effects of preformed n-3 long-chain polyunsaturated fatty acids on recognised cardiovascular risk factors, such as a reduction of plasma triacylglycerol concentrations, platelet aggregation, and blood pressure. These effects were observed at intakes of 1 g per day, well above levels that were associated with lower cardiovascular disease (CVD) risk. Respect to the latter, EFSA (2010) reported that studies indicate that oily fish consumption or dietary n-3 long-chain polyunsaturated fatty acid supplements (equivalent to a range of 250 to 500 mg of eicosapentaenoic acid plus docosahexaenoic acid daily) decrease the risk of mortality from coronary heart disease (CHD) and sudden cardiac death. However, EFSA (2010) suggested 250 mg per day of EPA+DHA as the adequate intake (for adults) in order primary prevent CHD diseases, but other international organizations proposed very different doses (Aranceta & Pérez-Rodrigo, 2012).

Unfortunately, long-chain FAs are as important as their high susceptibility to degradation, such as oxidation. It has been proved (German & Kinsella, 1985; Richards et al., 2002; Azhara & Nisa, 2006; Maqsood & Benjakul, 2011; Maqsood et al., 2012) that the lipid oxidation of food, especially of PUFA contained in fish, is strictly linked to the formation of off-flavour components, less of quality during different storage conditions, loss of nutritional value and even formation of anti-nutritional molecules. For this reason, in order to prevent possible waste of nutrient value it is important to briefly summarised the main factors affecting lipid oxidation in fish.

1.2 Lipid Oxidation in Fish

Lipid oxidation has been deeply studyiedduring several decades, and its complex mechanisms, kinetics and products are now sufficiently established. As reported by Niki et al. (2005) in 1955, the oxygenise enzyme was discovered and since then lipid peroxidation by enzymatic ways has been studied hardly. Niki et al. (2005) underline that lipid are oxidized by three distinct mechanisms:

1) enzymatic oxidation;
2) non enzymatic, free radical-mediated oxidation;
3) non-enzymatic, non radical oxidation.

The complexity of this phenomenon is confirmed by the large number of studies conducted in order to better understand lipid oxidation and to find out the best way to contrast it (Ramanathan & Das, 1992; Niki et al., 2005; Azhar & Nisa, 2006).

Lipid oxidation indeed is a very important event leading to the loss of quality of foods especially of those containing highly unsaturated fats. Quality loss, production of unpalatable flavour and odour, shortening of shelf life, losses of nutritional values (e.g. loss of PUFA) and possible production of unhealthy molecules are some of the extensive consequences of lipid oxidation in foods.

Unfortunately, lipid oxidation processes in foods or biological tissues may be more complicated than the simpler via indicated during these years. As confirmed by Schaich (2005), frequently lipid oxidation mechanisms have been proposed based on kinetics, usually of oxygen consumption or appearance of specific products (e.g., LOOH) or carbonyls (e.g., malondialdehyde), assuming standard radical chain reaction sequences. However, when side reactions are ignored or reactions proceed by a pathway different from that being measured, erroneous conclusions can easily be drawn. The same argument holds for catalytic mechanisms. Thus, multiple pathways and reaction tracks need to be evaluated simultaneously to develop an accurate picture of lipid oxidation in model systems, foods, and biological tissues.

CLASSIC FREE RADICAL CHAIN REACTION MECHANISM OF LIPID OXIDATION

Initiation (*formation of ab initio lipid free radical*)

$$L_1H \xrightarrow{k_i} L_1^{\bullet}$$

Propagation

Free radical chain reaction established

$$L_1^{\bullet} + O_2 \underset{k_\beta}{\overset{k_o}{\rightleftharpoons}} L_1OO^{\bullet}$$

$$L_1OO^{\bullet} + L_2H \xrightarrow{k_{p1}} L_1OOH + L_2^{\bullet}$$

$$L_2OO^{\bullet} + L_3H \xrightarrow{k_{p1}} L_2OOH + L_3^{\bullet} \quad \text{etc.} \quad \dashrightarrow L_nOOH$$

Free radical chain branching (*initiation of new chains*)

$$L_nOOH \xrightarrow{k_{d1}} L_nO^{\bullet} + OH^{-} \quad \text{(reducing metals)}$$

$$L_nOOH \xrightarrow{k_{d2}} L_nOO^{\bullet} + H^{+} \quad \text{(oxidizing metals)}$$

$$L_nOOH \xrightarrow{k_{d3}} L_nO^{\bullet} + {}^{\bullet}OH \quad \text{(heat and uv)}$$

$$\left.\begin{matrix} L_nO^{\bullet} \\ L_nOO^{\bullet} \\ HO^{\bullet} \end{matrix}\right\} + L_4H \xrightarrow[k_{p3}]{k_{p2}\ k_{p1}} \left.\begin{matrix} L_nOH \\ L_nOOH \\ HOH \end{matrix}\right\} + L_4^{\bullet}$$

$$L_1OO^{\bullet} + L_nOOH \xrightarrow{k_{p4}} L_1OOH + L_nOO^{\bullet}$$

$$L_1O^{\bullet} + L_nOOH \xrightarrow{k_{p5}} L_1OH + L_nOO^{\bullet}$$

Termination (*formation of non-radical products*)

Radical recombinations

$$\left.\begin{matrix} L_n^{\bullet} \\ L_nO^{\bullet} \\ L_nOO^{\bullet} \end{matrix}\right\} + \left.\begin{matrix} L_n^{\bullet} \\ L_nO^{\bullet} \\ L_nOO^{\bullet} \end{matrix}\right\} \xrightarrow[k_{t3}]{k_{t1}\ k_{t2}} \text{polymers, non-radical monomer products (ketones, ethers, alkanes, aldehydes, etc.)}$$

Radical scissions

$$\left.\begin{matrix} LOO^{\bullet} \\ LO^{\bullet} \end{matrix}\right\} \xrightarrow[k_{ts2}]{k_{ts1}} \text{non-radical products (aldehydes, ketones, alcohols, alkanes, etc.)}$$

i - initiation; o - oxygenation; β - O_2 scission; p - propagation; d - dissociation; t - termination; ts - termination/scission

Figure 1. Lipid peroxidation mechanism. Figure retrived from Schaich (2005).

Lipid oxidation has long been recognized as a free radical chain reaction (Ramanathan & Das, 1992), and the classic chain reaction scheme with three phases has been repeated in many forms. **Figure 1** is the version reported by Schaich (2005). Azhara & Nisa (2006) noted that the two major components involved in lipid oxidation are unsaturated fatty acids and oxygen. In this process, oxygen from the atmosphere is added to certain fatty acids creating unstable intermediates that eventually break down to form unpleasant odour and aroma compound. This type of oxidation is even called autoxidation, which involves primary autoxidative reactions which are further accompanied by both oxidative or non-oxidative secondary reactions. It is commonly accepted that the most important process by which PUFA and oxygen interact is the free radical chain reaction mentioned before.
This way is composed by 3 steps:

1) Initiation: *formation of ab initio lipid free radical;*

2) Propagation: *free radical chain reaction established;*

3) Termination: *formation of non-radical products.*

Initiation of lipid oxidation produces the *ab initio* lipid free radicals, $L^{\bullet}$. The initiation process is not well understood. Lipid oxidation is a very easy reaction that is nearly ubiquitous in foods and biological systems, so it is often treated as an instantaneous reaction. It is not true so that some initiators or catalysts are required to start the lipid oxidation process. In fact, only trace amounts of catalysts are needed, many situations that appear to be spontaneous or uncatalyzed are actually driven by contaminants or conditions that have gone undetected or unconsidered.

Indeed, in most foods and biological systems it is fair to say that multiple catalysts and initiators are always operative. To achieve full protection against lipid oxidation and attain long-term stability of any material, control strategies must include elimination, or at least inhibition, of initial alkyl radical production in lipids (Schaich, 2005). Light, oxygen, metal, and high temperature are common free radical initiators.

As reported by Schaich (2005), sometimes secondary abstraction reactions of lipid alkoxyl radicals ($LO^{\bullet}$) and peroxyl radicals ($LOO^{\bullet}$) are presented as initiation reactions because they form $L^{\bullet}$ radicals. That is true when lipid oxyl radicals are from outside sources, e.g., lipoxygenase reactions followed by Fe^{2+} and Fe^{3+} reactions with LOOH. However $LO^{\bullet}$ and $LOO^{\bullet}$ deriving from the initial $L^{\bullet}$ or its subsequent reactions are considered to mediate propagation or chain branching (initiation of secondary chains) rather than *ab initio* initiation.

The driving force in the chain reaction is the repeated abstraction of hydrogens by $LOO^{\bullet}$ to form hydroperoxides, generally called peroxides or primary products of oxidation (Azhar & Nisa, 2006), plus free radicals on a new fatty acid. At this point, it is

possible to determine conjugated dienes and trienes (CD) and peroxide value (PV). Concerning to this, Azhar & Nisa (2006) reported that the primary products in linoleate autoxidation are *cis-trans* and *trans-trans* conjugated diene-hydroperoxides.

As said before, lipid peroxides are very unstable and break down easily or tend to rearrangement or to cyclise. The reaction cyclisation as mentioned by Niki et al. (2005) is important only for PUFA having more than three double bonds, and it does not take place during oxidation of linoleates. Interestingly, Medina et al. (1999) reported that the mechanism necessary to the formation of 2-ethilfuran starts with the decomposition of 12-hydroperoxide of linolenate(C18:3 n3), the 14-hydroperoxide of eicosapentaenoate (C20:5n3), and the 16-hydroperoxide of docosahexaenoate (C22:6n3). They can undergo β–cleavage to produce a conjugated diene radical, which can react with oxygen to produce a vynil hydroperoxide. The cleavage of the vynil hydroperoxide by loss of a hydroxyl radical forms an alkoxyl radical, that undergoes cyclasation, thus producing 2-methylfuran. Confirming that previous study, Maqsood & Benjakul (2011) found traces of 2-methylfuran in some samples of Asian seabass (*Lates calcarifer*).

Hydroperoxide decomposition proceeds by a free radical mechanism, and a large amount of molecules could be formed as a consequence. They can be detected as secondary oxidation products or “malondialdheyde-similar” thanks to chemical analyses as the well known TBARS method, presented later. However, no all the molecules can be detected, as in case of volatile, with TBARS method, so a more sophisticated method, as gas-cromatography (GC) is commonly utilised. Products like carbonyl compounds like alchohols, acids, hydrocarbons, lactones and esters are formed, which are strictly linked to sensory profile of fish (Maqsood & Benjakul, 2011). Add some information, Catalá (2010) asserts that some of the aldheydes formed during lipid peroxidation are highly reactive and may be considered as second toxic messengers. It is the case of 4-hydroxy-2-nonenal (HNE) that is formed during lipid peroxidation of n6 PUFA, such as linoleic acid (C18:2n6) and arachidonic acid (C20:4n6). Furthermore, Maqsood & Benjakul (2011) found heptanal, octanal and hexanal as the major aldehydes in Asian seabass slices.

Maqsood & Benjakul (2011) underlined that many authors have been using aldehydes as indicators of lipid oxidation in a number of foods, including fish, because these compounds possess a low threshold values and are the major contributors to the development of off-flavour and odour (Boyd et al., 1992; Ross & Smith, 2006). Specifically, some authors seem to have tested that propanal and heptanal can serve as a reliable indicator of flavour deterioration for fish products, while hexanal contributes to the rancidity in meat (Ross & Smith, 2006).

The process continues indefinitely until no hydrogen source is available or the chain is intercepted. In summary, the radical chain reaction imparts several unique characteristics to lipid oxidation:

1. lipid oxidation is autocatalytic-once started, the reaction is self-propagating and self-accelerating (Azhar & Nisa, 2006)

2. many more than one LOOH is formed and more than one lipid molecule is oxidized per initiating event. This points out one reason why it has been so difficult to study initiation processes—initiators become the proverbial needle in a haystack once oxidation chains become established.

3. Very small amounts of pro- or antioxidants causes large rate changes.

4. The reaction produces multiple intermediates and products that change with reaction conditions and time.

These features present distinct challenges in measuring and controlling lipid oxidation, and are part of the reason why lipid oxidation is a major problem in storage stability of foods. Firstly, more than one parameter (CD, PV, TBARS, volatile) are necessary in order to define the oxidative profile of a sample. In some cases, it would be useful to introduce a sensory evaluation of the sample. Moreover, lipid oxidation can be promoted by many factors thus a specific knowledge is needed in order to maintain fillets quality as longer as possible.

1.3 Factors Affecting Lipid Oxidation In Fish

Many studies have been conducted during these years in order to better understand the role of different factors on lipid oxidation of fish. Both *infra vitam* and *post mortem* factors were deeply investigated. Feeding, stress during pre-slaughter activities, handling and storage are only few of that factors. For this reason, it has been considered useful to insert in the present thesis a comprehensive review of the main causes which affect lipid oxidation. Finally, we would like to specify that part of the contents of this introduction behaves to the Review titled "From farm to fork: lipid oxidation in fish products. A review", reported in Part II of the present PhD thesis as accepted paper.

1.3.1 Feeding Antioxidant

Fish lipids are rich in n-3 fatty acids that are essential to human health. Lipid oxidation is a major concern during processing and storage of fish because it contributes to quality deterioration and decreases marketability of fish products. Fillet accumulation of antioxidants, e.g. vitamin E (vit. E) or astaxanthin, during feeding may prevent quality deterioration associated with lipid oxidation following processing and storage.

Table 4 reports the results of some studies concerning the effect of dietary antioxidants on lipid oxidation. The role of vit. E is clearly discerned from Stéphan et al. (1995) who in turbot (*Scophthahus maximus*) demonstrated that TBARS level of low tocopherol diet is almost 100 times more than that of the highest tocopherol level diet. In addition, looking at the results immediately after death (data not shown) is possible to find slightly higher TBARS level (0.029 mg MDA/kg) in fish fed low dietary α-tocopherol (20 mg/kg feed), than in fish fed high antioxidant (320 mg/kg feed) for which 0.016 mg MDA/kg was measured. Hence, the antioxidant properties of tocopherol seem accentuated by long term frozen storage (6 months storage at -20 °C). Interestingly, the same authors performed in parallel a *in vitro* study on antioxidant ability of α-tocopherol. Uncertain patterns might be discerned. On one hand, α-tocopherol antioxidant activity is increased by increasing concentrations. On the other hand, the extent of antioxidant effect seemed to be strictly dependent on lipid content and composition of the matrix, especially PUFA n3 content. So the higher lipid and PUFAn3 content, the higher Vit. E antioxidant activity. That fact seemed to be confirmed by Chaiyapechara et al. (2003) who found that antioxidant activity of Vit. E was higher in fat rainbow trout (9.60% fat) than in fish containing 8.4% lipid. Furthermore, the action was expressed preferentially in long term frozen storage (24 weeks at -30 °C) than in a short refrigerated one (7 days at 4 °C). Unfortunately,

authors did not analyse the α-tocopherol content during the storage, so it is not possible to unevenly establish the antioxidant role of α-tocopherol.

Table 4. Effect of antioxidant supplementation in diet on secondary lipid oxidation products of different species.

Antioxidant	Quantity (mg/kg)	Feeding length	Species	Storage length	Temperature (°C)	TBARS (mg MDA/kg fillet)	References
α-tocopherol	20	34 weeks	Turbot	6 months	-20	0.259	Stéphan et al. (1995)
α-tocopherol	70	34 weeks	Turbot	6 months	-20	0.063	Stéphan et al. (1995)
α-tocopherol	320	34 weeks	Turbot	6 months	-20	0.029	Stéphan et al. (1995)
α-tocopherol/ astaxanthin	100/40	6 months	R. trout	12 months	-28	0.39	Jensen et al. (1998)
α-tocopherol/ astaxanthin	100/40	6 months	R. trout	18 months	-28	0.47	Jensen et al. (1998)
α-tocopherol/ astaxanthin	600/40	6 months	R. trout	12 months	-28	0.39	Jensen et al. (1998)
α-tocopherol/ astaxanthin	600/40	6 months	R. trout	18 months	-28	0.51	Jensen et al. (1998)
α-tocopherol	65	8 months	Hybrid tilapia	7 days	4	2.88	Huang et al. (2003)
α-tocopherol	200-300	8 months	Hybrid tilapia	7 days	4	1.08	Huang et al. (2003)
α-tocopherol	65	8 months	Hybrid tilapia	8 weeks	-40	5.76	Huang et al. (2003)
α-tocopherol	200-300	8 months	Hybrid tilapia	8 weeks	-40	4.32	Huang et al. (2003)

Confirming the antioxidant action of α-tocopherol, Huang et al. (2003) found that different diet vit. E supplementation levels influenced lipid oxidation by increasing the lag phase (initiation phase) of lipid peroxidation during either refrigerated (7 days at 4 °C) and frozen storage (8 weeks at -40 °C) of hybrid tilapia (*Oreochromis niloticus* × *O. aureus*). Results show that TBARS are affected by the dietary vit. E supplementation, and fish fed with high vit. E levels show to be less prone to be oxidised than the low level fed ones. Similar results were found by Zhang et al. (2007) in *Sparus macrocephalus* fillets. Even in this case, fillets of fish fed with high tocopherol levels (553 or 1069 mg/kg) for 8 weeks exhibited significantly low (1.44 g/kg fillet) levels of oxidation products during 9 days of ice storage thanks to their high tocopherol muscle content.

In summary, it is possible to assert that α-tocopherol antioxidant activity is increased by increasing concentration levels in feed; α-tocopherol performs better in high fat substrates; α-tocopherol acts preferentially in long term frozen storage.

Jensen et al. (1998) fed rainbow trout (*Oncorhynchus mykiss*) with different astaxanthin and α-tocopherol levels for 6 months in order to understand the role of feeding antioxidant on lipid stability of the raw fish during frozen storage (-28 °C, 12 or 18 months). Globally, storage reduced both astaxanthin and tocopherol content in fish fillet, although the highest decrease was observed for astaxanthin content. Thus, results suggest that astaxanthin might protect against lipid oxidation during the early stages of oxidative deterioration, where α-tocopherol has little effects, thanks to carotenoids' role as scavengers of free radicals during the initiation of lipid oxidation. Such an ability is confirmed by the funding of Choubert et al. (2011), that found that carotenoid supplemented diets (100 mg astaxanthin/kg feed or 80 mg canthaxanthin/kg feed) did not significantly reduce TBARS content in rainbow trout during long term (18 months) frozen storage (- 20 °C).

Recently, new natural antioxidants (as thymol, carvacrol, and lycopene) have been utilising in feed supplementation. Based on Giannenas et al. (2012) funding, not a unique pattern for carvacrol and thymol might be discerned. Indeed, the authors found that feeding rainbow trout with thymol (6 mg/kg) for 8 week improved oxidative stability during a short refrigerated storage (5 days at 4 °C) more than carvacrol supplementation (12 mg/kg). TBARS content of fish treated with thymol remains unaltered for the entire trial, at 2.25 μg /g protein, while the carvacrol group raised up 2.78 μg/g protein. Interestingly, Girao et al. (2012) tested the antioxidant ability of feed supplementation with lycopene (600 mg/kg) on Nile tilapia (*Oreochromis niloticus*) undergone stress confinement. Two main effects of lycopene may be discerned. Firstly, no alteration of TBARS content accompanied by unaltered enzymatic antioxidant activity (catalase, glutathione reductase, lactate dehydrogenase) in not

stressed fish fed lycopene suggests that lycopene should have an antioxidant role by removing reactive oxygen species (ROS) generated by cellular metabolism. Latter, lycopene abolished the effect of stress during confinement, remaining unchanged both enzymatic activities and TBARS (stick at 0.32 µg/kg), thus confirming it played an important role during initiation phase of lipid oxidation. More recently, Sahin et al. (2014) studied the effect of lycopene supplementation at different concentration (0, 200, or 400 mg/kg) on stressed rainbow trout quality performance. They found that dietary supplementation of lycopene reduces the detrimental effects of stress (high stocking density) on growth performance of fish and modulates the oxidative status via activating host defence system at cellular level. It appears that lycopene can be added up to 400 mg/kg to rainbow trout diets to improve flesh quality.

Rosemary extract has been utilising in fish feeding during the last years, however contrasting results were found. Data from Hernández et al. (2014) show that animals fed diets containing high dose of rosemary extract (1200, 1800, and 2400 mg/kg) have a significantly lower TBARS index than the control group (no addicted group) or the group fed with low dose (600 mg/kg) over the first 7 days of storage (average 0.11, 0.13, and 0.22 mg MDA/kg, respectively). However, on day 21st, a certain tendency emerged towards an increase in the TBARS index as the dose increased, possibly due to a pro-oxidant effect of the rosemary extract at high doses. The lowest rosemary dose raised up to 0.49 mg/kg, while the other groups achieved at maximum 0.71 mg/kg, even if any statistical differences emerged. While comparing rosemary, thymol, carvacrol, or synthetic antioxidant as BHT feed supplementation for their capability of prevent lipid oxidation, Álvarez et al. (2012) found the following increasing stability order: carvacrol > rosemary=BHT>thymol. Thus, during 14 days of refrigerated storage, fillets from fish fed diet with carvacrol (500 mg/kg, 18 weeks) showed the lowest TBARS content (0.2 mg MDA/kg fillet), while the maximum was reached by thymol group with 0.4 mg MDA/kg.

1.3.2 Lipoxygenase And Cycloxigenase Activity: Lipid Mediators In Fish

Lipoxygenases are a family of iron-containing enzymes that catalyze the aerobic oxidation of fatty acids with cis-nonconjugated pentadiene structures to generate optically active conjugated LOOH without releasing a lipid free radical (Schaich, 2005). German & Kinsella (1985) proposed that a potential source of initiating species in fish, as hydroperoxides (Hp) for example, could be generated by endogenous enzymes liberated from the tissue itself. Kanner & Kinsella, as noted by German & Kinsella (1985), had before demonstrated that tissue peroxidase could initiate lipid peroxidation: they reported the activity of lipoxygenases as catalyst for the insertion of oxygen into an unsaturated fatty acid forming a highly reactive hydroperoxide as product.

Schaich (2005) summarised the enzymatic reaction saying that hydroperoxides are synthesized in a cage reaction involving electron transfer to the lipid from the ferrous iron atom in the enzyme's active site and removal of the basallylic hydrogen as the rate determining step. Oxygen bound to a separate site on the enzyme is activated to react with the free radical, then Hp donation from the enzyme completes the LOOH before it is released. As the oxygen always adds against to the hydrogen removal, the resulting conjugated dienes are always trans-, cis-relative to the hydroperoxide.

Perhaps just as important, LOOH produced by lipoxygenase can accumulate to relatively high levels under appropriate conditions (e.g., cold and dark, as in frozen materials), then lead to a cascade of rapid oxidation when LOOH decomposes. It should be noted that lipoxygenase acivity and heme autoxidation are strictly connected as demonstrated by the mechanism for initiation of tissue lipid peroxidation reported below (Figure 22).

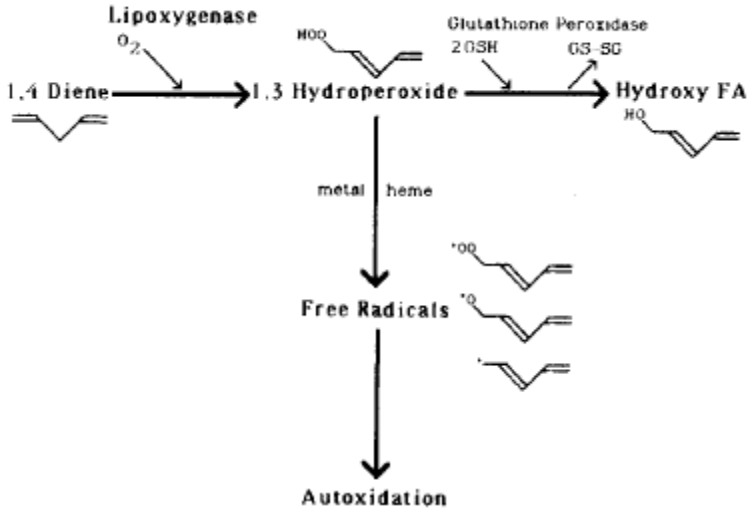

Figure 2. Mechanism for initiation of tissue lipid peroxidation via lipoxygenase activity as proposed by German & Kinsella (1985).

Cyclooxygenase (COX) is another enzyme which plays a central role in the biosynthetic pathway to prostaglandins from arachidonic acid (ARA). Indeed, COX is also known as prostaglandin-endoperoxide synthase (PTGS). It converts free ARA, released from membrane phospholipids at the sn-2 ester binding site by the enzymatic activity of phospholipase A2, to prostaglandin (PG) H_2. The reaction involves both cyclooxygenase (dioxygenase) and hydroperoxidase (peroxidase) activity. The cyclooxygenase activity incorporates two oxygen molecules into ARA or alternateve polyunsaturated fatty acid substrates, such as linoleic acid and eicosapentaenoic acid (EPA). Metabolism of ARA forms a labile intermediate peroxide, PGG_2, which is reduced to the corresponding alcohol (PGH_2) by the hydroperoxidase enzyme's activity. There are two isozymes of COX encoded by distinct gene products: a constitutive COX-1 (this enzyme) and an inducible COX-2, which differ in their regulation of expression and tissue distribution. This gene encodes COX-1, which regulates angiogenesis in endothelial cells. COX-1 is also involved in cell signalling and maintaining tissue homeostasis.

It has been well documented that an important role in immune and inflammatory responses in fish is played by a series of derived oxidation metabolites from PUFAs (Rowley et al., 1995; Arts & Kohler, 2008; Rowley et al., 2012). C20 long chain fatty acids such as ARA, EPA, and dihomo-gamma-linolenic (DGLA, C20:3n6) are precursors of many eicosanoids by both enzymatic and/or non-enzymatic pathways. Particularly, 4-series leukotrienes (LT), lipoxine (LX), 12-hydroxy-eicosatetraenoic acid (12-HETE), and 12-hydroxy-eicosapentaenoic acid (12-HEPE) generated through the

action of lipoxygenases (LOX) were found to be produced *in vitro* by head kidney leukocytes extracted from rainbow trout under biological stimuli (Pettitts et al., 1991; Knight et al., 1993; Rowley et al., 2012). Moreover, COX products, such as 2-series prostaglandins (PG) and tromboxanes (TX), were found in leucocytes (Knight et al., 1993) and thrombocytes (Lloyd-Evans et al., 1994) of rainbow trout, underlining the role of these lipids in immunomodulation and other pro-inflammatory responses.

As noted by Wendelaar Bonga (1997), stress responses in fish concern complex mechanisms involving stimulation of oxygen uptake and transfer, mobilization of energy substrates, reallocation of energy away from growth and reproduction, and mainly suppressive effects on immune functions. These stress responses may in many ways resembles inflammatory processes.

As a consequence, eicosanoids have been recently employed as lipid stress conditions markers thought their role as oxidative stress biomarkers in fish is still unclear, especially concerning EPA, and DHA-derivatives. Considering their complex metabolism, it is likely that multiple compounds are involved into inflammation at the same time, but how inflammatory stimulus, such as slaughter, modify the plasma lipid profile has not been described in detail. Previous studies reported COX products, such as prostaglandins, to be the major stress biomarkers, although no clear trends for PG synthesis in response to stress could be uniquely discerned (Oxley et al., 2010; Olsen et al., 2012) due to different behaviours related to tissues, diet, and time after stress. According to Balvers et al. (2012), under stress stimuli prostaglandins levels in plasma were down-regulated, especially PGE_2. Confirming previous results, Secci et al. (2016) found that stress condition at slaughter decreased PGE_2 levels, although PGE_3/PGD_3 was only found in stressed group (killed by asphyxia). Interestingly, authors found the opposite trend between PGE_2 (from ARA) and PGE_3/PGD_3 (from EPA) reflected an ARA and EPA competition as substrates of COX. Oxley et al. (2010), focusing on PGE_2, $PGF_{2\alpha}$, 8-keto-$PGF_{2\alpha}$ production in gut tissues as markers of acute stress (chase in a net for 15 minutes), found an up-regulation of COX1 and COX2 1h post stress even though a different extent was reported both for site and diet. Similarly, Olsen et al. (2012), subjecting the fish to the same acute stress previously proposed by Oxley et al. (2010), found a striking effect on the eicosanoids content in midgut, but only marginal in hindgut. For the major eicosanoids PGE_2, PGE_3, PGD_2, $PGF_{2\alpha}$ and $PGF_{3\alpha}$, stress was reported to cause a significant reduction only in the midgut until 24 hours post stress.

Even if many authors agree with PGE_2 as the main product of COX activity, no clear trends for PG synthesis in response to stress could be uniquely discerned. In fact, both Oxley et al. (2010) and Olsen et al. (2012) found out that site, diet, and time affected the eicosanoids production because of a different up-regulation of COX isophorms. According to this, Rowley et al. (2012) found no conclusive evidence that increase in PGE generation was caused by changes in the expression of

cyclooxygenases. Hence, the tendency to decrease of PGE_2 revealed for asphyxiated rainbow trout (Secci et al., 2016) is in agreement both to Olsen et al. (2012) and Balvers et al. (2011), who found a fast general decrease of PGE_2 in midgut section of rainbow trout 1 hour post stress and in mice plasma after stress stimulus, respectively.

Despite the tendency to use prostaglandins as biomarker, the study of Secci et al. (2016) revealed that 12-HpEPE/15-HpEPE (detected together) could be good markers of stress because they were highly produced under stress condition while they were not detected in no-stressed fish (rainbow trout killed by percussion). Interestingly, even if the biosynthesis of some eicosanoids was previously found to involve the formation of hydroperoxide intermediates (Rowley et al., 1994), it was the first time that HpEPEs were found in stressed trout plasma. Hydroperoxy derivatives are the primary products of LOX that are easily reduced into hydroxides by glutathione peroxidase (GPX) (Guichardant et al., 2011). This enzyme has been deeply characterized in fish during last years in order to associate its expression with stress during life. Particularly, increasing stress time was related with a down-regulation of GPX. Briefly, Pacitti et al. (2013) found four GPX isoforms called GPX1b1, GPX1b2, GPX4a1, and GPX4a2 differently distributed in fish tissues. According to the authors, the main isoforms contained in blood were GPX1b2 and GPX4a1 where they are present in order to limit the high oxidative stress that occurred in erythrocyte and lysosomal membranes. The same isoforms were detected by Malandrakis et al. (2014) following the GPX expression in different tissue of gilthead sea bream exposed to physical stress (confinement) for increasing time. That study pointed out a time-dependent regulation of glutathione peroxidase. Particularly, inducing stress for one minute caused an up-regulation of the enzyme while increasing time changed in a down-regulation of GPX even if a high variability was reported. Inhibition of GPX together with an increase of the activity of LOX caused by stress during slaughter might explain the presence of HpEPE in asphyxiated fish whilst the absence in the percussion-killed ones (Secci et al., 2016). Moreover, the same trend in enzymes modulation seemed to be confirmed by the significant decrease of other eicosanoids such as 5-HEPE, and 17HDoHE from EPA and DHA, respectively, obtained by Secci et al. (2016).

The 5-lipoxigenase pathway is complex, yielding a range of monohydroxy fatty acids (e.g. 5-HEPE), leukotriene, and lipoxins extensively characterized in the previous century due to the role of that subclasses of eicosanoids in antinflammatory system. Rowley et al. (1994) suggested that macrophage from *O. mykiss* was able to generate both leukotriene and lipoxins under calcium ionophore stimulation, even if that ability was greatly affected by the presence of a FLAP (5-Lipoxygenase Activating Protein). More recently, Rowely et al. (2012), deepened the interaction between eicosanoids and the immune system in salmonid fish, demonstrating that the incubation of zymosan with trout macrophages resulted in a greater amount of LTB and LXA derived

from EPA than from ARA. Secci et al. (2016) partially confirmed this pattern, by detecting only LTB_4 (from ARA). Nevertheless, it seemed that 5-lipoxygenase rather directed on EPA than on ARA, probably as a consequence of the higher concentration of the first as free fatty acid. In fact, as shown by Ashton et al. (1994), a direct relation between substrates availability and products of lipoxygenase exists. Particularly, the authors found that leukocytes from fish maintained for more that 8 weeks on a vegetable oil (rich in ARA precursor) containing diet produced lower percentage of lipoxygenase products derived from eicosapentaenoic acid compared with those cells from fish fed with fish oil (rich in EPA).

Concerning 15-lipoxygenase activity, it was discovered in the gill tissue of teleost fish during purification of the previously recognized and more preponderant 12-lipoxygenase enzyme by German & Creveling (1990). The Authors reported the enzyme was active toward polyunsaturated fatty acids present in the tissue producing hydroxylated metabolites from fatty acids with 18-, 20-, and 22-carbon chain lengths at carbons 13, 15, and 17, respectively. This means that, in theory, the main product of 15-LOX with EPA as substrate should be 15-HEPE. Contrarily, Secci et al. (2016) revealed a smaller amount of that compound comparing with LOX-12 products, showing that 15-lipoxygenase was less active than 12-LOX. However, that result could be explained thanks to German & Creveling (1990) who found that the total activity of this enzyme following purification using hydroxylapatite was significantly greater than in the crude tissue preparation, suggesting that an inhibition was present in intact cells. Supporting this founding, Rowley et al. (1994) showed that the range of products from ARA and EPA synthesized by rainbow trout macrophages incubated with calcium ionophore or zymosan was dominantly due to the presence of 5 and 12-LOX activity in intact cells, whereas lysate cells revealed a further 15-lipoxygenase activity. Data obtained by Secci et al. (2016) showed 6 and 3 times greater activity for 12- and 5-LOX, respectively in percussion-killed rainbow trout (no stress).

More recently, DHA was found to be a fairly good substrate of LOX in human to produce various hydroxylated end-products after reduction of the hydroperoxide intermediates by GPX. They are 4-HDoHE, 7-HDoHE, and 11-HDoHE (Lagarde et al., 2013) and all of them were found by Secci et al. (2016) in plasma of rainbow trout. In addition to this, those derivatives of DHA have been described as precursor of a bioactive family, called resolvins and protectins, a class of compounds with active anti-inflammatory and inflammation resolving properties in mammal (Masoodi et al., 2008). In Secci's et al. (2016) research, RvD1 and PD1 were searched though they were not detected, according to Olsen et al. (2012). Hong et al. (2005) identified neuroprotectin D1, resolvin D5, resolvin D1 and resolvin D2 from trout brain cells challenged *in vitro*. Moreover, Chung et al. (2013) reported the presence of RvD1 (low concentration 0.29 ± 0.09 ng/g muscle) after exposition to increasing levels of H_2O_2

during life of medaka fish (*Oryzias latipes*). Particularly, they found that RvD1 increased until 2 hours of exposition, regardless oxidant concentration levels. However, the cited authors did not explain the exact mechanisms and the functional role as lipid mediator of this class of molecules still need to be identified. Although resolvins nor protectins were detected in other researches (Secci et al., 2016), the presence of their precursor 4-HDoHE, 7-HDoHE, and 11-HDoHE provided a first evidence of LOX activity on DHA in fish plasma (Secci et al., 2016). Moreover, the trend on their decreasing level caused by stressed slaughtering method could be a first evidence of 4-HDoHE, 7-HDoHE, and 11-HDoHE degradation to RvD1 and/or PD1 in order to reduce/resolve stress conditions.

1.3.3 Preslaughter Procedure: Starvation And Crowding

Food quality is perceived as a global concept. Food should be primarily safe, tasty and healthy. However, food safety and ethics are increasingly of global interest. In this context, commonly pre-slaughter practices that may be responsible for animal stress are starvation and crowding. Starving the fish for some days prior to slaughter is a common practice in the case of farmed fish, with the scope to delay spoilage by reducing the amount of faeces in the intestine. During the last decades, many authors have investigated the influence of starvation on flesh quality in different fish species such as *Sparus aurata* (Ginés et al., 2002; Álvarez et al., 2008), *Dentex dentex* (Suárez & Cervera, 2010), *Onchorynchus mykiss* and *Salmo trutta* (Bayir et al., 2014) but only a few of them focused on the induced oxidative stress.

Álvarez et al. (2008) exposed *S. aurata* to 24, 48, or 72 hours of starvation and among others parameters they evaluated TBARS on fillets. Although no significant differences between starvation periods emerged, a trend may be discerned. Particularly, it seemed that longer the starvation time higher the TBARS values. Indeed, 2.50±0.90, 3.63±1.62, and 4.57±1.75 mg MDA/kg were found in *S. aurata* starved for 24, 48, and 72 hours, respectively.

Interestingly, Bayir et al. (2014) measured oxidative stress indicators, such as reactive oxygen species (ROS) in liver and muscle samples from *Onchorynchus mykiss* and *Salmo trutta* exposed to a 45-day starvation period at low water temperature. They found that in both species lipid peroxidation increased with starvation length, even if the metabolic response to food deprivation in the muscle of each species was different.

Crowding is a temporary status immediately before killing when fish can be collected in very high density. As reported by Pérez-Sánchez et al. (2013), crowding

causes a complex stress by affecting hepatic gene expression, antioxidant defence system, cell–tissue repair mechanism, xenobiotic metabolism and stress transcriptional regulation. This response, similar to the one described by Bayir et al. (2014) for starvation, may explain the fundings of Bagni et al. (2007), that monitored the effect of crowding (density >70 kg/m^3) on the oxidative stress of two common Mediterranean species (gilthead sea bream, *Sparus aurata*, and European sea bass, *Dicentrarchus labrax*). Oxidative stress was determined in terms of increment of the reactive oxygen metabolites (ROMs) and of anti-oxidant power (AOP). From the data emerged that in the case of stress conditions, the ROMs production can be counteracted by an adaptive response, such as the activation of the AOP mechanism. However, the stress extent may greatly affect this response, by shifting from a positive response (high AOP, low ROMs) to a negative one (low AOP, high ROMs). The former is the case of uncrowded fish, the latter of crowed fish. Furthermore, stress response seemed to strictly depend on species. Indeed, gilthead sea bream showed to be less affected by the application of stress than European sea bass (no significant differences between two stress groups were found for AOP and ROMs). Gilthead sea bream as well showed a lower survival time than European sea bass. Nathanailides et al. (2011) supported the hypothesis that increased levels of stress can lead to increased lipid oxidation in European sea bass fillets. In details, fish were processed with a high stress method (the water was lowered and the fish were captured using a net, then killed by immersion in an ice cold bath) or with a lower stressful one (the level of water was lowered and fish were anaesthetized moderately by immersion in a 30 mg/L clove oil bath for 5 minutes, then slaughtered by immersion in ice cold sea water). Results showed that the handling stress prior to slaughtering affects significantly TBARS contents, which were 1.04 and 1.16 mg MDA/kg in no stressed and stressed fish, respectively.

In conclusion, from the cited studies emerged that pre-slaughter stress may induce complex metabolic responses: rapid ATP depletion may generate various pro-oxidant substances, which in turn may induce an activation of AOP mechanism for ROS and ROMs depletion. Unfortunately, high stressful conditions or stress length may cause the adaptive response to be useless resulting in an increase of lipid oxidation.

1.3.4 KILLING

Stunning/killing procedures applied in aquaculture are different and fish species vary in their response to the different methods utilised. Mediterranean aquaculture species are usually killed by asphyxiation in air, immersion in ice/water slurry or by percussive stunning. Ice killing is usually used in selective fisheries. Recent alternative stunning/killing processes have been experimentally investigated for Mediterranean fish species in an effort to develop and optimize commercial methods by assuring both high standards of fish welfare and product quality (EFSA, 2008; Poli, 2009). It has been widely reviewed that pre-slaughter (as anaesthesia) and slaughter stressful practices could have an important effect on the flesh quality in fish (Poli et al., 2005). A clear effect emerged mostly on the physical properties of flesh, because severe stress at slaughter time exhausts muscular energies, produces more lactic acid, reduces muscular pH, and increases the rate of *rigor mortis* onset. In this way this practices could have significant negative effects on fish technological traits and in their flesh quality.

According to Hultin (1992), anaerobiosis influences the conversion of xanthine dehydrogenase to xanthine oxidase. The latter enzyme transfers electrons directly to molecular oxygen producing superoxide and hydrogen peroxide, which can produce hydroxyl radicals in the presence of redox iron. These compounds have been proposed as among the principal initiators of lipid oxidation in biological tissues. Thus, the rapid conversion of ATP to hypoxanthine and of xanthine dehydrogenase to xanthine oxidase could influence lipid oxidation time of fresh and semi-preserved fish, especially when molecular oxygen is reintroduced during *post mortem* processing.

Tejada & Huidobro (2002) found out that slaughter method (percussion, ice salt-water slurry bath, and asphyxia) has no clear influence on the oxidative stability of gilthead sea bream (*Sparus aurata*), probably due to the interaction of many factors such as stress, handling speed after death, and lipid content of flesh.

Morzel & van de Vis (2003) studied the effect of killing methods on eel (*Anguilla anguilla* L.) lipid oxidation. Particularly, electricity and oxygen removal (new killing method) lead to the higher quality of eels in comparison with the dry-salt technique, by reducing stress and improving freshness. Furthermore, less stressful practices seemed to reduce the extent of lipid oxidation. In details, authors pointed out that enhanced lipid oxidation in salt-bath eels can be partially explained by the physical damage to the muscle, thereby increasing the cell ruptures and the consequent accessibility to the catalytic enzymes. In addition, the presence of salt may be considered in some extent a slight pro-oxidant.

Results from Giuffrida et al. (2007) were in agreement with this explanation. Particularly, ice slurry slaughtered gilthead sea bream (*Sparus aurata*) showed better

ATP/IMP levels (an indicator of less stressed fish) and to be less prone to lipid oxidation, as revealed by the MDA values, 0.158 mg MDA/kg flesh against 0.227 mg MDA/kg flesh in CO_2-slaughtered fish. The same pattern was found in electrical stunned rainbow trout (*O. mykiss*) whilst compared electricity with anoxia and bleeding as killing methods. TBARS values for these groups were 0.68, 1.09, and 1.03 mg MDA/kg flesh, respectively.

Sakai & Tereyama (2008) studied the effect of bleeding as killing method on chub mackerel (*Scomber japonicus*) lipid oxidation. Struggling death in iced sea water was utilised as control. The MDA content in the muscles of the bleeding samples were significantly higher than those of the control after 119 hours of storage at 0 °C, with 0.367 and 0.184 mg/kg, respectively. On the contrary, no differences were found in 4-hydroxyhexanal content of the samples. These results confirmed that fish subjected to stressful conditions were more prone to be oxidised and suggested that bleeding can be considered as a stressing killing method.

On the contrary, Duran et al. (2008) found that slaughter method (asphyxia or percussion) had no effect on the MDA values of carp (*Cyprinus carpio*). However, when considering rainbow trout (*O. mykiss*) the MDA content of flesh from fish slaughtered by asphyxiation was significantly higher than that of specimens slaughtered by percussion (4 and 3 mg MDA/kg flesh, respectively). It is important to note that the fat level of trout was higher than that of carp (5% against 1%), which led to an observed difference in the MDA contents of trout slaughtered by different methods.

The effects of different stunning/killing procedures (anaesthesia with clove oil, anaesthesia with 2-phenoxyethanol, percussive stunning, immersion in ice/water slurry, chilling on ice, and anaesthesia with clove oil followed by immersion in ice/water slurry) on flesh quality of European sea bass (*Dicentrarchus labrax*) were investigated by Simitzis et al. (2014). Globally, MDA ranged between 29.9 and 95 mg/kg flesh in chilling on ice and percussion slaughtered sea bass. Despite such large range of values, authors did not find any significant difference among the tested killing methods, suggesting no killing effects on lipid oxidation.

Interestingly, in contrast to the results previously seen, in a recent study Secci et al. (2016) found out the link between stress during slaughter and lipid oxidation. Their results revealed the presence of very high level of reactive molecules, such as hydroperoxides, in stressed rainbow trout whilst they were not detected in not-stressed group. Thus, probably as a consequence of the greater enzymatic activity under stress condition, the presence of lipid oxygenated products affected the development of lipid oxidation during *post mortem* storage.

1.3.5 METALS

Redox-active metals are the initiators of perhaps greatest importance for lipid oxidation in oils, foods, and biological systems (Schaich, 2005) because they are ubiquitous and active in many forms, and trace quantities (<<micromolar) are sufficient for effective catalysis (Schaich, 1992). Only metals undergoing one-electron transfers appear to be active catalysts; these include cobalt, iron, copper, vanadium, manganese and magnesium.

Azhar & Nisa (2006) assert that generally Cu^{2+} and Fe^{2+} are the most active catalysts of both lean and fat fish, as well as crustaceans and shellfish. Cadmium, cobalt and zinc instead seem to induce rancidity in fat fish and not in lean fish.

As reported by Schaich (2005), the mechanisms and rates of metal-catalyzed initiation operative in individual reaction systems are determined by a complex mixture of factors: the metal and type of complexes it forms (inner sphere or outer sphere), the chelator or complexing agent, redox potential of the metal and its complexes, solvents, phase localization of the metal, and availability of oxygen or preformed hydroperoxides.

Metal autoxidation and hydroperoxide decomposition are both very active processes in foods, oils, and biological tissues where metals are always present. A particular case is that concerning the autoxidation of hemoglobins (Hb) where the conversion of ferrous heme protein to met ($^{+3}$) heme protein (metHP) appears to be a critical step, enhancing lipid oxidation (Everse & Hsia, 1997 as referred by Maqsood & Benjakul, 2011). Heme catalysis of lipid oxidation was first reported in 1924 as noted by Schaich (2005) and its role has been investigating for a long time. In a review dedicated to hemoglobin-mediated lipid oxidation in the fish muscle, Maqsood et al. (2012) revealed that heme pigments such Hb and myoglobin (Mb) are believed to be the most important endogenous promoters of lipid oxidation in fish muscle and for the development of fishy odours, along with microbial growth.

Hemoglobin is a protein consisting in a globin portion plus a porphyrin heme, the latter containing an iron atom (Fe, charged atom) coordinated inside the heme ring. Hemoglobin is made up of four polypeptide chains and each chain contains one heme group (FIGURE 3). The porphyrin ring consists of four pyrrole molecules cyclically linked together (by methene bridges) with the iron ion bound in the centre. The iron ion may be either in the Fe^{2+} (ferrous) or in the Fe^{3+} (ferric) state (Maqsood et al., 2012).

Just after death, nearly all the heme iron exists in the ferrous valence state. Oxygen can be bound to the ferrous iron (oxyhemoglobin) or the iron binding site can be vacant (deoxyhemoglobin) (Richards et al., 2002).

The process by which ferrous Hb is converted to ferric metHb is called autoxidation and it occurs when oxygen is released from oxyhemoglobin to form ferric methemoglobin and the superoxide anion radical ($O_2^{\bullet -}$) (Richards et al., 2002); as reported by Maqsood et al. (2012) autoxidation could generate even other different radical as $OOH^{\bullet}$ depending on whether deoxy or oxy heme protein undergoes autoxidation. Figure 4 reported the process of Hb autoxidation as proposed by Maqsood et al. (2012).

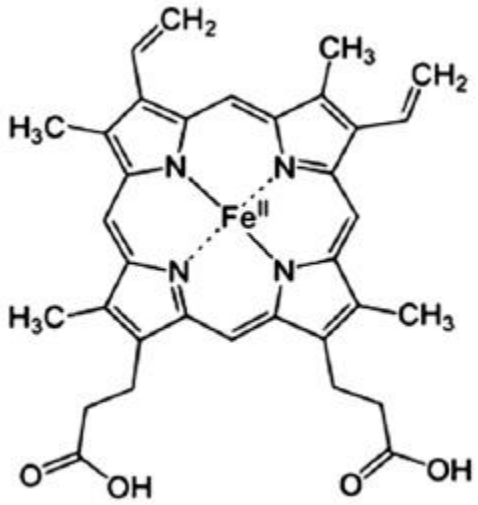

Figure 3. Hemoglobin structure.

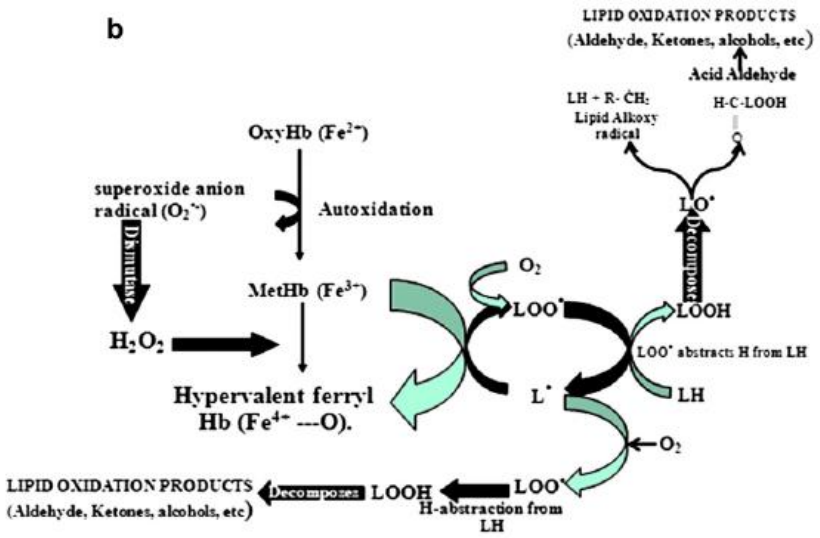

Figure 4. Hb autoxidation mechanism and lipid oxidation initiation. LH: lipid; $L^{\bullet}$: lipid radical; $LO^{\bullet}$: lipid alkoxy radical; $LOO^{\bullet}$: lipid peroxy radical; LOOH: lipid hydroperoxide (Maqsood et al., 2012).

Both of the radical forms can readily be converted to hydrogen peroxide (H_2O_2), which enhances the ability of heme proteins to promote lipid oxidation (Maqsood et al., 2012) thanks to the subsequent formation of a ferryl protein radical, an initiator of lipid oxidation, as shown by Kanner & Harel (1985).

That heme compounds catalyze lipid oxidation in food and biological systems has been extensively documented (Richards et al., 2002; Maqsood & Benjakul, 2011; Maqsood et al., 2012), but how this occurs is still not clear. Several investigations have reported varying efficiencies in promoting lipid oxidation among hemoglobins from different species, e.g. pollock Hb is more oxidant in washed fish muscle than Hb from mackerel, following in decreasing order by menhaden Hb and flounder Hb (Undeland et al., 2004). Pollock Hb has also been found to have higher activity in promoting lipid oxidation compared to that of horse mackerel Hb, which was also found to be more effective than seabass Hb (Maestre et al., 2009). Furthermore, trout Hb exhibits likewise greater pro-oxidant ability that Hb from tilapia, whilst haemoglobin from mackerel and haemoglobin from herring are more active than that from trout (Richards & Hultin, 2003; Richards et al., 2007). According to this, Pazos et al. (2009) verified that trout Hb shows lower ability to promote free radicals in the presence of preformed hydroperoxides (cumene hydroperoxide, 1140 and 11400 μM) than cod and herring Hbs. That reduced capacity of generating free radicals seemed to display a direct correlation with the haemoglobin vulnerability to undergo oxidative alterations

either in spontaneous or hydroperoxide-forced conditions. Particularly, cod and herring Hbs exhibited greater formation of metHb (derived from autoxidative processes) during 5 days of incubation at 4 °C, and the observed increase of metHb was equal to 4.0-9.0 μM. Conversely, trout Hb showed an accumulation pattern which raised metHb from 0.5 up to 5 μM. In summary, trout accumulated lower metHb values than cod and herring.

Moreover, cod and herring Hbs showed to be less stable even in presence of preformed hydroperoxides. Taking into consideration that metHb has been demonstrated to have stronger capacity than Hb to activate lipid oxidation (Grunwald & Richards, 2006; Maestre et al., 2009; Pazos et al., 2009) and considering that cod and herring Hbs showed to be more prone to be oxidised than trout Hb, it is possible to correlate the ability to promote free radicals with Hb vulnerability to oxidative alteration.

The promotion of lipid oxidation by Hb and Mb has been proposed to involve a ferrylHb radical, as said before, that initiates the oxidation (Everse & Hsia, 1997 as cited by Maqsood et al., 2012). Another pathway of lipid oxidation mediated by Hb includes the action of iron released from the heme protein (Gray et al., 1996; Morrissey et al., 1998 as cited by Maqsood et al., 2012), as it catalyses the breakdown of preformed lipid hydroperoxides, thereby initiating the production of alkoxyl radicals. These molecules are capable of abstracting a hydrogen atom from polyunsaturated fatty acids with the subsequent propagation of lipid oxidation processes (Maqsood et al., 2012).

1.3.6 HANDLING

It is widely reviewed that any process causing disruption of the muscle membrane system (such as grinding, freezing, and cooking) results in exposure the lipid fraction to oxygen, and thus accelerates the development of the oxidative damage. However, one of the first processes after stunning and killing procedures in fish industry is the blood removal. Although it is not a kind of handling altering the lipid structure, blood removal is strictly linked to the quality deterioration of fish muscle, especially to the lipid oxidation. Richards & Hultin (2002) studied the contribution of blood and blood components to lipid oxidation in rainbow trout (*O. mykiss*) and Atlantic mackerel (*Scomber scombrus*). They performed a complex project, finding out three main points: bleeding significantly reduced the probability of rancidity (expressed both as sensory score and TBARS value) development during storage; this probability strictly depended on species and type of muscle considered (trout *vs*

mackerel, light *vs* dark muscle); the extent of lipid oxidation was more pronounced in minced muscle as compared to the intact one.

The first point was confirmed by many authors. Tejada & Huidobro (2002) recognised the greater extent of lipid oxidation of ungutted gilthead sea bream (*Sparus aurata*) when comparing with gutted samples at day 11 of refrigerated storage (+2 °C), reaching as high as 8 mg MDA/kg flesh, a value commonly utilised as rancidity threshold. However, according to the same authors, such an increase seemed not to be significant.

Sakai et al. (2006) attempted to measure hemoglobin (Hb) content in bled skipjack tuna (*Katsuwonus pelamis*) flesh, and they analysed malondialdehyde contents and 4-hydroxyhexenal (HHE) in the muscle as indicators of the lipid oxidation level. Firstly, Hb content was lower in bleeding samples than in the control ones, containing 0.07 and 1.01 mg/g, respectively. Concerning lipid oxidation, Sakai et al. (2006) did not find significant differences in MDA content in samples while bleeding fish showed lower level of HHE than the control samples, both immediately after death (not detected *vs* 0.20 nmol/kg) and after 2 days of storage at 0 °C (0.07 and 0.43 nmol/kg, respectively).

More recently, Maqsood & Benjakul (2011) confirmed that bleeding decreases Hb content and consequently lipid oxidation in Asian sea bass muscle (*Lates calcarifer*). Their results indicate that lipid oxidation (measured as PV, TBARS, and volatiles) was more pronounced in the un-bled samples during 15 days of refrigerated storage (2 °C). Particularly, blood contains a high amount of haemoglobin which action as pro-oxidant is still discussed. However, the extent of lipid oxidation is affected not only by Hb concentration but also by the presence of different type of Hbs in fish muscle (Richards & Hultin, 2002) and their breakdown during storage, resulting in the release of non-heme iron (Maqsood & Benjakul, 2011).

At this point it is easy to understand that different species as well as different kind of muscle may greatly differ in term of Hb content and composition, so causing a different susceptibility of the muscle to be oxidised. This is the case of muscle that contains large amount of blood, such as dark muscle, which is found to be more prone to be oxidised (Richards & Hultin, 2002). In addition, Hb concentration might explain the higher values of lipid oxidation in minced muscle than in whole/intact one (Richards & Hultin, 2002). Indeed, the mechanical action of mincing can provoke rupture blood vessels, erythrocytes, and some other cells and so cause Hb release. As stated, that release can promote lipid oxidation.

At the same time, grounding increases the exposition area of muscle to atmosphere oxygen, moving to a real pro-oxidant factor. According to them, Thiansilakul et al. (2011) confirmed that myoglobin (Mb) was able to catalyse lipid oxidation in washed Asian sea bass (*Lates calcarifer*) minced intensively. Primary and

secondary oxidation products as well as off-odour development were significantly higher in Mb addicted samples than in the control ones (no Mb addicted). At day 8 of storage (4 °C), volatiles were mainly composed by 11.54, 11, and 7.08% of 1-octen-3-ol, hexanal, and 2-pentyl furan, respectively, whilst hexanal and 2-pentyl furan applied globally for 3.7% in the control samples. These changes were more likely associated with metmyoglobin formation occurring in washed mince, as a consequence of the increase in storage time. Moreover, lipid oxidation in washed mince with added myoglobin was mainly governed by pH. Specifically, lowest the pH (6) highest the lipid oxidation extent was.

Recently, it was also found that the higher the heme affinity of Mb, the lower the myoglobin-mediated lipid oxidation was obtained (Richards et al., 2009). Therefore, low pH was not only associated with Mb oxidation, but also weakened the heme-globin complex, leading to a release of heme group, which was able to induce the lipid oxidation.

1.3.7 STORAGE

The problem of the quality deterioration during storage is well known and it is related to both temperature and storage time. As well, the quality lowering rate depends on species of fish. Nishimoto et al. (1985) found out that the highest temperature of storage the fastest deterioration of fish freshness. To date, many authors have focused on quality changes during storage by studying separately ice or chilling storage and the frozen one.

The concentration of TBARS in good quality frozen and chilled fish or in fish stored on ice is typically between 5 and 8 mg MDA/kg whereas levels of 8 mg MDA/kg are generally regarded as the limit of acceptability for most species (Schormüller, 1968). More strictly, Ke et al. (1984) proposed that TBARS values for fish products below 0.58 mg/kg were perceived as not rancid; 0.58–1.51 mg/kg as slightly rancid, but acceptable; and above 1.51 mg/kg were perceived as rancid.

Özyrut et al. (2009) studied red mullet (*Mullus barbatus*) and goldband goatfish (*Upeneus moluccensis*), both belonging to the Mullidae family, funding different shelf-life and lipid oxidation levels when stored 11 days at 2 °C. The authors analysed both primary (PV) and secondary (TBARS) lipid oxidation products. PV significantly raised from 0.64 and 0.83 meq peroxide oxygen/kg fat to 2.26 and 4.82 meq/kg at the end of the trial, in red mullet and goldband goatfish, respectively. TBARS values were found stable around 0.51 and 0.57 mg MDA/kg flesh for both species during the whole storage. Similar PV values were obtained by Timm-Heinrich et al. (2013) studying the oxidative changes of rainbow trout (*Oncorynchuss mykiss*) during ice storage (12 days

at 2 °C) by following PV and volatile contents. PV were below 0.5 meq O_2/kg flesh during the first 5 days of storage. After, PV had a slightly increase up to 0.56 meq O_2/kg on day 7, whilst they started raising significantly up to 6 meq O_2/kg on day 12. Similarly, the volatile fraction started to increase significantly from day 5 onwards, especially 1-penten-3-ol, 1-penten-3-one, and 2-pentenal. Globally, volatiles were found in low concentration (ng/kg), confirming a little oxidation during 12 days of storage on ice. Concerning TBARS level, Etemadian & Shabanpour (2014) found their increase from an initial value of 0.56 mg MDA/kg of muscle to 2.92 mg MDA/kg of *Rutilus frisii kutum* slices during 15 days of iced storage.

Probably related to bacterial growth at positive temperature, the 7th day appeared to be critical even in other papers. For example, Hernández et al. (2009) studied lipid oxidation of aquacultured meagre (*Argyrosomus regius*) fillets during 18 days of storage at 4 °C, finding significant differences from day 7 onwards. Particularly, TBARS gradually increased from 0.10 mg MDA/kg flesh to 2.55 mg MDA/kg. In agreement, Simitzis et al. (2014) found that, in general, positive temperature (4 °C) increased MDA levels around 3.6 times in 7 days of storage. In the same paper, Simitzis et al. (2014) looked for TBARS level even in frozen (-20 °C) samples, finding that freezing raised up TBARS level around 2.7 times after 90 days. An interesting connection between refrigerated and frozen storage was found some years before by Huang et al. (2003) who measured the same MDA value (7.2 μg) in hybrid tilapia fillets (*Oreochromis niloticus* × *O. aureus*) stored for 7 days at 4 °C or for 8 weeks at -40 °C.

When meat and meat products are stored under frozen conditions, microbial spoilage may be delayed, but fat deterioration occurs and the meat constituents may be oxidized (Ojagh et al., 2014). Interestingly, the main cause of lipid oxidation during frozen storage seemed to be due to the enzymatic lipolysis activity. Indeed, Karlsdottir et al. (2014a) indicated that enzymatic lipolysis was the driving factor influencing the fillets quality over storage and it mostly affects long chain polyunsaturated lipids in the light muscles.

However, previous studies had confirmed that low storage temperatures were optimal for preserving fish from oxidative deterioration. Refsgaard et al. (1998) compared lipid oxidation of Atlantic salmon (*Salmo salar*) fillets stored at -10 or -20 °C for 34 weeks. The content of lipid hydroperoxides and free fatty acids increased during storage as affected by a significant time-temperature interaction, and the changes were fastest in salmon stored at -10 °C. Specifically, hydroperoxides raised from 0 to 10 meq O_2/kg, while FFA increased from 1 to 8.7% in 34 weeks of storage. Such as oxidative products increase was associated with a decrease in highly unsaturated fatty acid content (C20:5n3, C22:5n3, and C22:6n3) in Atlantic salmon stored at -10 or -20 °C. Also for the polyunsaturated fatty acids, significant time-temperature interaction effects were found, confirming the fastest decrease at -10 °C. Concerning to volatile

products, aldehydes and ketones were identified. For hexanal, heptanal, (E)-2-hexenal, (E,E)-2,4-heptadienal, and nonanal significant time effects were found with increasing concentrations during storage, independently of storage temperature, while temperature influenced significantly hexanal and 2-hexanal. A small increase in the amount of secondary lipid oxidation products was also observed by Jensen et al. (1998). The significant and preservative action of negative storage temperature was confirmed by Choubert et al. (2011), that determined lipid oxidation (TBARS) in packed rainbow trout (*Oncorhynchus mykiss*) stored for 18 months at -20 °C. Results showed that TBARS significantly increased after the first month of storage, but not other changes occurred during the 5 later months.

Recently, the study of Baron et al. (2007) aimed at investigating protein and lipid oxidation during frozen storage of rainbow trout fillets, stored for 13 months at -20, -30, or -80 °C. Lipid oxidation was followed by measuring lipid hydroperoxides (PV), as well as secondary oxidation products (volatiles). There was a significant increase in the level of lipid hydroperoxides after 8 months of frozen storage for fish stored at -20 °C, which was even more pronounced after 13 months, reaching 6.6 meq/kg of fat indicating on-going oxidation. In contrast, samples stored at -80 and -30 °C did not show any significant increase in peroxides during the entire storage period (with $p = 0.26$ and $p= 0.07$, respectively). Measurement of secondary oxidation products was followed for 13 months together with the development of hexanal (an oxidation product of linoleic acid), and 1-penten-3-one and t,t-2,4-heptadienal, both oxidation products of n-3 fatty acids. Other volatiles were also measured during storage (1-penten-3-ol, heptanal, 1-octen-3-ol, t-2-octenal, nonanal, t,c-2,6-nonadienal, decanal), and their development was generally in agreement with what is reported for hexanal, 1-penten-3-one, and t,t-2,4-heptadienal. Volatile patterns indicating that fish stored at -20 °C was the most oxidised and that little difference was observed between -80 and -30 °C. On the basis of their observations the ranking order -20 °C > -30 °C > -80 °C was obtained for the development of oxidation products in fish stored at freezing temperatures. Likewise, both Indergård et al. (2014) and Karlsdottir et al. (2014a) recently confirmed that pattern. The first authors examined lipid oxidation, by PV and TBARS, in Atlantic salmon during a long-term frozen storage at –25, –45 and –60 °C. After 1 year of storage at –25 °C, the concentration of PV in fish red and white muscles increased from 1.26 to 1.82, and from 1.08 to 1.76 meq O_2/kg fat, respectively. Formation of TBARS was higher in the red muscles than in the white, and reached a value of 14.04 mg MDA/kg fish after 1 year of storage at –25 °C. Decreasing the temperature to –45 °C inhibited PV and TBARS formation. In the latter paper, the authors studied the lipid deterioration of two lean fish species, i.e. saithe (*Pollachius virens*) and hoki (*Macruronus novaezelandiae*), during frozen storage at -20 and -30 °C (up to 18 months). As even in the previous case, Karlsdottir et al. (2014a) analysed

both light and dark muscles. Results showed significant lipid deterioration with extended storage time, but lower storage temperature showed significantly more preservative effects. The formation of hydroperoxides as well appeared to be strongly influenced by species. Saithe was very stable during the first 12 months of storage regardless to storage temperature. After 18 months of frozen storage, however, a slight, yet significant, increase of peroxide (up to 50 mmol/kg muscle) was observed in light and dark muscle types. On the other hand, hydroperoxide formation in hoki showed a much more pronounced and more progressive peroxide formation over time at both storage temperatures (Karlsdottir et al., 2014b). However, as previously shown for handling, the extent of oxidation rate was showed to be strictly connected with the type of muscle considered. Indeed, dark muscle showed to be the most prone to be oxidised regardless to storage temperature, by ranging up to 325 and 250 mmol/kg at -20 °C and -30 °C, respectively. The light muscle presented a pattern similar to saithe, by remaining almost unaltered for the first 12 months. As for saithe, at the end of storage a significant increase of peroxide was observed (50, and 100 mmol/kg muscle at -30 and -20 °C, respectively). It has to be noted that the marked difference in peroxide content between hoki dark and light muscle is likely due to the considerably higher lipid content in dark muscle than in the light one (7.6% *vs* 0.6%). The difference in fat content might explain either the difference between oxidation susceptibility of saithe when compare with hoki, since saithe fat content ranged from 0.6 to 1.1%, whilst hoki has from 0.6 to 7.6% in light and dark muscle, respectively. TBARS results for both the saithe dark and light muscle showed low/no formation of secondary oxidation products up to month 6, followed by a sharp increase up to month 12, after which only the dark muscle values continued to increase. As well as peroxide values, low temperature protected against oxidation as revealed by the significantly higher increase of TBARS in -20 °C stored samples than in the -30 °C stored ones.

2. THE CONSIDERED SPECIES

The present PhD thesis considered four species of interest for European aquaculture. Specifically, rainbow trout (*Oncorhynchus mykiss*), atlantic salmon (*Salmo salar*), sea bass (*Dicentrarchus labrax*), and sea bream (*Sparus aurata*) have been studied. In the following chapters, a brief description of the cited species is presented. Specifically, rainbow trout and atlantic salmon characteristics are discussed together (2.1) because both own to salmonid group, and sea bass and sea bream are presented in 2.2. These two species indeed are both carnivorous marine finfish that have quite similar biology and life histories.

2.1 RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND ATLANTIC SALMON (*SALMO SALAR*)

Rainbow trout (*Oncorhynchus mykiss*), whose name refers to the rainbow-coloured line on its skin, is a species of salmonid. Native in the Pacific coastal area of the United States of America, it was introduced into Europe at the end of the 19th century (O'Neill, 2006). Rainbow trout have been cultured for hundred years, and are the most widely farmed trout in the world. Rainbow trout are farmed today in nearly all European countries, especially in the coastal countries with a temperate climate. Italy leads freshwater cultured species in Europe, by providing more than 30000 ton. Rainbow trout can tolerate a wide range of water temperatures and other environmental variables, but they required high oxygenated water (Hardy, 2002).

Farming of Atlantic salmon (*Salmo salar*) began in Norway in the late 1960s. During the following decades the production has spread to other countries, for example Chile, Scotland, and Canada due to biological constraints and seawater temperature requirements (8-14 °C). In 2013 the world production was 1.84 million tonnes representing the main species of salmonids produced. It is a versatile ra material which can be utilised for a variety of products such as smoked, fresh, sushi, as well as ready-made meals justifying the high economic value of this specie, which reach more than 9 billion of EUR in 2013 (www.marineharvest.com).

Both of these two species have the ability to store pigments, generally red, by which their flesh results coloured. The main pigment occurring naturally in salmonids is astaxanthin (3,3'-diidrossi-β-carotene-4,4'-dione), while canthaxanthin (β,β-carotene-4,4'-dione) is the main artificial one. They belong to carotenoid group and they are considered as red-coloured pigments. The main repository of fish carotenoids is the

skin; considerable amounts are also encountered in the ovaries, in the liver, and in the flesh (Hardy, 2002; Storebbaken, 2002). Whilst the firsts are probably associate with the carotenoid role on reproduction, the latter site is the most important for the appeal on consumers. Indeed, colour is recognized as an important characteristic and selection criterion for food choice by consumers (Koteng, 1992).

However, fish do not possess the power to synthesize carotenoid *de novo* so their presence in fish tissues is strictly associated with alimentary carotenoids or diet supplementation. In the European Union, canthaxanthin is currently authorised for use as a colouring agent up to a level of 80 mg per kg in complete feed stuffs for salmonids. When combined with astaxanthin, there is a maximum permitted level of 100 mg total canthaxanthin plus astaxanthin per kg even if the limit of 80 mg per kg for canthaxanthin has to be respected (EC, 2002). Nevertheless, EC (2002) scientific opinion suggested to reduce the level at 25 mg canthaxanthin/kg of feed in order to guarantee safety assessment. Canthaxantin indeed is the sole pigment for which an acceptable daily intake (ADI) has been established. It means that 0.03 mg pigment per kg body weight have been considered safety for avoid diseases, such as functional damage to the retina (SCF, 1997).

The deposition of carotenoids in fish flesh is related both to the type of carotenoids and to their concentration in the animal diet (Torrisen, 1986; Foss et al. 1987). Astaxanthin has been proved to be faster deposited than canthaxanthin in muscle, as shown in Figure Errore. L'origine riferimento non è stata trovata.5. Moreover, the retention rate of pigments in flesh decreases with increase in diet dose because of the decrease in the bioavailability of the pigments. However, many factors may be involved in the process of carotenoids deposition that explain the marked differences in their amount.

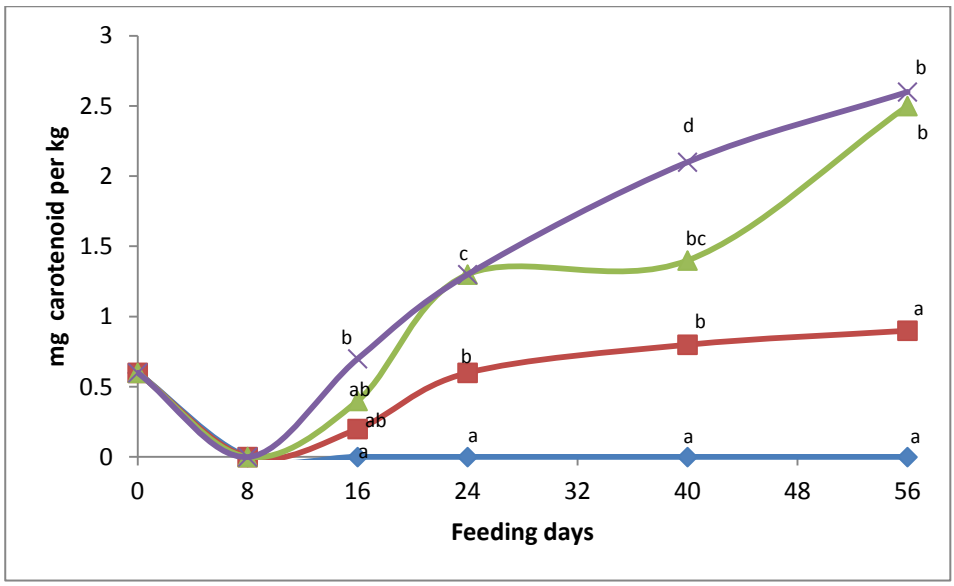

Figure 5. Carotenoid concentration in Atlantic salmon fillets derived from fish fed with 4 different carotenoids concentration (0, blue line; 30, red line; 60, green line; 90, purple line) as reported by Foss et al. (1987). a, b indicate significant differences ($p < 0.05$) between concentrations for each time.

2.2 Sea bass (Dicentrarchus labrax) and Sea bream (Sparus aurata)

European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) are both carnivorous marine finfish that have quite similar biology and life histories. Sea bass and gilthead sea bream have been historically cultured in coastal lagoons and saltwater ponds because they are euthermic (5-28 °C) and euryhaline (3‰ to full strength sea water).

These species were two of the first to be cultured on a commercial scale in Europe and to date they are cultured in similar production systems, often coexisting on the farm sites. The European sea bass (*Dicentrarchus labrax*) was the first marine non-salmonid species to be commercially cultured in Europe and at present is the most important commercial fish widely cultured in Mediterranean areas. The mass-production of juveniles sea bass started in the late 1960s. During the late 1960s, France and Italy competed to develop reliable mass-production techniques for juvenile sea bass and, by the late 1970s, these techniques were developed in most Mediterranean countries to provide hundreds of thousands of larvae. Greece, Turkey, Italy, Spain, Croatia and Egypt are the biggest producers. According to the Federation of European Aquaculture Producers (FEAP), 134,978 tons of European sea bass were produced in the Mediterranean Sea in 2012 (FEAP, 2015).

Traditionally, the intensive rearing systems for gilthead sea bream were developed during the 1980s and definitively achieved in 1988-1989 in Spain, Italy and Greece. Thanks to its high adaptability to intensive rearing conditions, both in ponds and cages, its annual production increased regularly raised 138,694 tons in the Mediterranean countries in 2012. Greece is the leading producer in the world with approximately 45% of the total production (FAO, 2014).

In Europe, the sea bass and sea bream industries have grown strongly in the last decade. Production is mostly exported, mainly to Italy and Spain. However, the farming of European sea bass and gilthead sea bream in the Mediterranean region is undergoing a transformation from being an industry of high margins and low volumes to one of low margins and high volumes. The rapid development of production in sea cages has led to declining prices and the rapid saturation of the market. These facts, together with the parallel increase of the input of fish, such as fish meal and oils (or their replacers), and a small traditional market for these species (mainly in southern Europe) compared with the Atlantic salmon market, make very difficult for farmers to increase their profits.

Another problem is the lack of diversified products, and limited market development and promotion. Indeed, compared to many other species of farmed fish, such as salmon or trout, European sea bass and gilthead sea bream have so far been

mainly marketed as whole and fresh, with only limited volumes undergoing any form of processing or value-addition (Stirling University, 2004). In any case, product development in aquaculture sector has been very limited. One major reason is the conservatism of Mediterranean consumers, who are used to seeing the fish whole when sold retail.

Thus, at the moment, market conditions seem very far from those that pertained in the first half of the 1990s, but there are a few marketing strategies for improve and/or increase the sea bass and sea bream profitably. One of these is the production of low quantities of higher quality fish (e.g. organic fish) or by producing unconventional fish sizes. Product development is now under way, for example the use of modified atmosphere packaging (MAP) for giving the product a longer shelf-life. However, more product development is certainly necessary if additional quantities of bass and bream want to be absorbed in the current markets and expand existing ones by opening new markets and attracting new consumers.

3. AIM OF THE STUDY

As emerged from the previous chapters, lipid oxidation is a very important event leading to the quality of foods, especially of those containing highly unsaturated fats. Quality losses, production of unpalatable flavour and odour, shortening of shelf life, losses of nutritional values (eg. loss of PUFAs) and possible production of unhealthy molecules are some of the extensive consequences of lipid oxidation in foods.

Fish lipids are highly unsaturated and contain other polyunsaturated fatty acids which are considered as "essential" for human health such as eicosapentaenoic (EPA, C20:5n3) and docosahexaenoic (DHA, C22:6n3) acids. Nonetheless, long-chain fatty acids are as important as their high susceptibility to degradation, such as oxidation. It has been proved (Azhar & Nisa, 2006; Maqsood & Benjakul, 2011; Maqsood et al., 2012) that lipids of fish are highly prone to be oxidised and many factors during fish supply chian can affect the pattern of this reaction.

For this reason, the overall aim of this study was to assess the effects of different extrinsic factors (killing method, storage, mechanical separation process) on lipid oxidative stability of fillets from different farmed species.

The specific goals were to study:

- ✓ The effect of killing method both on plasma and muscle oxidative stability of rainbow trout during long term frozen storage (**Research I**). Specifically, the effects of asphyxia in air and percussion were considered both in terms of animal welfare and fish quality in order to understand the role of stress on quality loss during storage.

- ✓ The effect of killing method on lipid and cholesterol oxidation of farmed atlantic salmon during refrigerated storage (**Research II**). Particularly, new stunning/killing method, such as utilisation of carbon monoxide, has been evaluating as no stress alternative. However, if it can be utilised without any detrimental effect on fillets quality is still unclear.

- ✓ The effect of mechanical separation treatment (MSM) of fillets from European sea bass, Gilthead sea bream, and rainbow trout on oxidative stability of their derived-products (**Research III**). This technique is commonly utilised for other terrestrial species, such as poultry or pig, in order to reduce production wastes and create ready-to-eat products, whereas it is not so expoited by fish industry. Thus, it could be interest to evaluate MS as new technology in fish industry for reducing wastes and opening new market.

4. MATERIAL AND METHODS

Fish were obtained from different fish farms. Specifically, as summerised in Table 5, rainbow trout for Research I were farmed in San Michele all'Adige (Trento, Trentino Alto Adige, Italy) whilst trout for Research III were raised in Tuscany (Italy); atlantic salmon came from Matre (Norway); sea bass and sea bream finally were farmed in the south of Tuscany (Italy). Globally, 56 rainbow trout and 30 Atlantic salmon were sacrificed for testing the roles of killing method, and storage methods on oxidative stability of fish fillets. Moreover, Research III took into consideration the effects of Mechanical Separation Process on three different species farmed in Tuscany: European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), and rainbow trout (*Oncorhynchuss mykiss*) (Table 5).

Globally, the following analysis were conduced: determination of lipid content, fatty acid (FA) profiles (of both muscle and plasma), nucleotides, eicosanoids and docosanoids (lipid mediators) in fish plasma. Considering lipid oxidation measurements, conjugated dienes, thiobarbituric acid reactive substances (TBARS), cholesterol oxidation products (COPs), and carotenoid content have been taken into consideration. The different research activities were composed by some of the previously listed analysis. An overview of these assessments is given in the Table 6 and described in depth in the following chapters.

Finally, experimental set-up for each research is described in the Part II, which collects the papers that have been originated from the three researches performed during the PhD period, summerised in Table 7.

Table 5. List of fish sample origins.

	Sample	Type of Farm	Location	n° fish	Weight Range	Scope
Research I	R. trout	Not experimental Farm	San Michele all'Adige (Trento, Italy)	28	1127 ± 258 g	Evaluate the effect of killing method on oxidative stability of fillets (frozen storage)
Research II	A. salmon	Experimental Farm	Matre (Norway)	30	1104 ± 125 g	Evaluate the effect of killing method on oxidative stability of fillets (refrigerated storage)
Research III	European sea bass	Not experimental Farm	Grosseto (Italy)	18	427 ± 22	Evaluate the effect of mechanical treatment (MSM) of fish on oxidative stability of derived-products (refrigerated and frozen storage)
	Gilthead sea bream	Not experimental Farm	Grosseto (Italy)	18	432 ± 42	
	Rainbow trout	Not experimental Farm	Garfagnana (Italy)	18	357 ± 52	

Table 6. List of the analyses conducted in each research.

		Research I	Research II	Research III	Method
CHEMICAL ANALYSES	Proximate composition	●	●	●	Official AOAC (2012)
	Fatty acids analysis of muscle and plasma	●	●	●	GC-FID
	Nucleotides	●			HPLC, photodiode-fluorescence
	Lipid mediators	●			LC-MS/MS
	Carotenoids		●		HPLC, UV-DAD
CHEMICAL OXIDATION ANALYSES	Cholesterol and cholesterol oxidation products (COPs)		●		GC-FID
	Dienes		●	●	Spectrophotometer
	TBARS	●	●	●	Spectrophotometer
PHYSICAL ANALYSIS	Colour			●	Colorimeter

Table 7. List of papers derived from PhD research activities.

Kind of study	Derived	Kind of publication	Status	Journal/Congress
Bibliography analysis	Paper I	Review	In press	Italian Journal of Animal Science.
Research I	Paper II	Article	Published	Food Chemistry.
	Annex I	Oral communication		Eurofed Lipid Congress 2015.
Research II	Paper III	Article	Published	Journal of the Science of Food and Agriculture.
Research III	Paper IV	Article	Submitted	Food Control.

4.1 Lipid Content And Extraction

Quantification of lipid content can be performed with different methods, changing in solvent quantity and separation time. The two most utilised methods are Bligh & Dyer (1959) and Folch et al. (1957). Both methods are based on the lipid hydrophobicity, and the separation phase derived from the addiction to samples of methanol, chloroform and water. Homogenised samples were separated by overnight rest or centrifugation followed by filtration. The chloroform phase contained the lipid extract which can be gravimetrically measured. The extract can be also utilised for fatty acids, cholesterol and cholesterol oxidation products, astaxanthin, and tocopherol analyses. The following scheme summarised the methods utilised for total lipid content analysis in the present researches.

	Sample (g)	Methanol (mL)	Chloroform (mL)	Water (mL)	Separation phase
Research I	2	6	6 *	3	Centrifugation
Research II	10	121	60.5	37.9 (KCl)	Overnight
Research III	2	30.2	15.1	11.5 (KCl)	Overnight

* Toluene was here substituted for chloroform.

4.2 Fatty Acid Profiles Of Muscle And Plasma

Fatty acid profiles were determined by gas-chromatography. Many methods have been developed or improved during the years, and different laboratory have chosen their ordinary method based on the equipment owned. In order to make the process clear, first the common steps utilised were summarised, then the details were reported. Fatty acid analysis starts from the methylation of lipid extracts. Nonadecanoic acid (C19:0) or tricosanoic acid (C23:0) were utilised as internal standard, while fatty acids were identified by comparing the FAME retention time with the standard Supelco 37 component FAMEs mix (Supelco, Bellefonte, PA, USA). For research I, lipid extracts were methylated according to the method of Lepage & Roy (1986). The fatty acid methyl esters (FAMEs) were analysed by gas chromatography and flame ionization detection (GC/FID, Clarus 500, Perkin Elmer, Shelton Alto, CT, USA). FAMEs separation was achieved on a Supelco SP-2330 fused silica capillary column (30 m × 0.25 mm i.d., 0.2 µm film; Supelco, Bellefonte, PA, USA). The oven temperature was started at 140 °C and it was increased to 205 °C at the rate of 1 °C/min. The injector and detector temperatures were set at 275 °C and 260 °C, respectively. Samples in toluene (1 µL) were injected (split ratio 1:10) into the column with the carrier gas (nitrogen) kept at a constant pressure of 10 psi. Chromatograms were recorded with the TotalChrom™ Chromatography Data System (Perkin Elmer) computing integrator software.

The second and third researches instead utilised the trans-esterification method proposed by Morrison & Smith (1964). The FA composition was determined by using a Varian GC 430 gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 capillary column (30 m × 0.32 mm i.d., 0.25 µm film and polyethylene glycol bonded phase; Supelco). The oven temperature was held at 100 °C for 2 min, increased to 160 °C over 4 min at the rate of 12 °C/min, and then increased to 220 °C over 14 min at the rate of 3 °C/min and kept at 220 °C for 25 min. The injector and the detector temperatures were set at 220 °C and 300 °C, respectively. One µL of sample in hexane was injected into the column with the carrier gas (helium) kept at a constant flow of 1.5 mL/min. The split ratio was 1:20. Chromatograms were recorded with the Galaxie Chromatography Data System 1.9.302.952 (Agilent) computing integrator software.

4.3 Nucleotides

ATP and derived nucleotides were determined as proposed by Özogul et al. (2000). Briefly, 1.5 g of muscle were homogenised with 10 mL of perchloric acid 6%

(PCA, Sigma-Aldrich, St. Luis, MO, USA). After a centrifugation at 3200 × g at 4 °C for 10 minutes (Allegra®X-12R, Beckman Coulter Inc, Brea, CA, USA) and holding at -20 °C for 20 min, the samples were filtered using Filter-Lab® 100 mm filter paper (Filtros Anoia, S.A., Barcelona, Spain). The extract was adjusted to pH 6.8-7 using 0.6 and 0.1 M potassium hydroxide (Sigma-Aldrich, Poole, Dorset, UK), filtered and finally it was brought to 25 mL volume with 50 mM phosphate buffer (pH 7). Before HPLC injection, 100 µL of sample were filtered using a 13 mm GHP 0.2 µm filter (Waters, Milford, MA, USA).

HPLC analyses were made using an Alliance® HPLC Model 2695 (Waters) apparatus equipped with a photodiode array detector model 2996 (Waters) and a multi λ fluorescence detector (Waters). The column was a ZORBAX Eclipse XDB-C8, 4.60 × 75 mm, particle diameter 3.5 µm (Agilent), used at a temperature of 35 °C.

Nucleotide standards [adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (Ino), hypoxanthine (Hx)], and tetrabutylammonium bromide were purchased from Sigma-Aldrich Chemical Company (Poole, Dorset, UK). Acetonitrile was purchased from Merck KGaA (Darmstadt, Germany) and water HPLC grade was obtained from Scharlab S.L. (Sentmenat, Spain). Separation was performed in continuous gradient elution using two mobil phases. Phase A was 50 mM phosphate buffer/10 mM tetrabutylammonium bromide dissolved in HPLC grade water and adjusted to pH 7 with 0.1 m potassium hydroxide. The solution was prepared daily and filtered through a 0.2 µm 47 mm GHP membrane (Waters). Phase B was acetonitrile. The injection volume was 10 µL and detection was monitored at 254 nm. The total separation time was 12 min with a rate flux of 1 mL/min. The results were expressed as mM nucleotides/g muscle.

4.4 Eicosanoids And Docosanoids From Plasma

PUFA derivatives together with free arachidonic (ARA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids were quantified according to the methodology previously developed by Dasilva et al. (2014) using SPE extraction prior to LC-MS/MS analysis. Briefly, plasma samples (300 µL) were diluted with 30% cold methanol (v/v), to a final volume of 1.2 mL. The internal standard 11HETE-d8 was added to each sample. The spiked samples were incubated on ice during 10 min and then centrifuged at 3200 × g for 10 min, at 4 °C, to remove any precipitated proteins which might cause interferences. The clear supernatant and washes of the resultant pellet with 30% methanol were collected in amber glass vials and subjected to SPE on Oasis-HLB cartridges (60 mg, 3 mL, Waters, MA, USA). After that, extracts were evaporated to dryness under a fine stream of nitrogen; the residue was dissolved in 100 µL ethanol and analysed by LC-MS/MS in a Waters C18-Symmetry column, 150×2.1 mm, 3.5 µm (Milford, MA, USA) using a binary eluent system of water (A) and methanol (B), both with 0.02% (v/v) of formic acid, as mobile phases. The flow rate was set at 0.2 mL/min; the column effluent was directly introduced in the ESI without splitting, and injection volume was set to 10 µL. Operating conditions of the ESI source were negative ion mode with a gas flow rate of 40 units, spray voltage of 5.5 kV, capillary temperature of 300 °C and S-lens radio-frequency level of 60%. The quantification of target compounds was made using the most intense, or selective, transition for each analyte and identification was helped comparing the MS/MS spectra, recorded in the range from 90 to 400 m/z units.

4.5 Conjugated Dienes

As peroxide values, conjugated dienes are considered primary lipid oxidation products. Conjugated dienes (CD) were measured according to Srinivasan et al. (1996). Briefly, 2 g of sample were homogenate in 6 mL water, then 0.5 mL of that extract were added to 5 mL hexane:isopropanol (3:2, v/v). Before reading the absorbance at 233 nm, samples were centrifuged 5 min at 2000 × g. The concentration of conjugated dienes was obtained by using the molar extinction coefficient of 25200 mL /($mmol^{-1}$ cm^{-1}). The results were expressed as mol hydroperoxides/kg muscle.

4.6 TBARS

The 2-thiobarbituric acid reactive substances (TBARS) were measured according to Vyncke (1970). Two g of sample were homogenised with 10 mL of 5% trichloroacetic acid (TCA) solution for 60 sec. Samples were stored at -30 °C for 10 min in order to precipitate the protein fraction. Then the samples were centrifuged and filtered. Five mL of the extracts were added with 2 mL of 0.02 M thiobarbituric acid (TBA) and incubated at 93 °C for 40 min. The absorbance was read at 560 nm and the results were expressed as mg of malonaldehyde/kg sample using a calibration curve determined with eight standard solutions of TEP (1,1,3,3,-tetra-ethoxypropane) at concentration ranging from 0.2 to 3.1 µM.

4.7 Cholesterol and Cholesterol Oxidised Products (COPs)

The content of cholesterol and COPs in fish fillets was determined in the total lipids extracted according to Folch et al. (1957). One hundred and fifty µL dihydrocholesterol in chloroform (2 mg/mL) and 25 µL of 19-hydroxycholesterol (1 mg/mL) in n-hexane/isopropanol (1 mg/mL, in 4/1) were added to 300 mg of lipid extract as internal standards for cholesterol and COPs, respectively. Three hundred mg of total lipids were dissolved in n-hexane:isopropanol (4:1, v/v) and directly cold saponified. One tenth of the unsaponifiable matter was utilised for the determination of the total cholesterol, whereas the remaining part (9/10) was purified by NH_2-SPE cartridge for COPs purification. Cholesterol and COPs were then silylated with a silylation solution composed by a pyridine solution of hexamethyldisilazane and trimethylchlorosilane (Sweely et al., 1963). After a nitrogen stream drying, the extracts were dissolved in n-hexane. Both cholesterol and COPs were identified by GC-FID (GC 2000 plus, Shimadzu, Columbia, MD, USA) equipped with a VF 1-ms apolar capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Varian, Palo Alto, CA, USA). For cholesterol and COPs determination, 2 µL of sample in hexane were injected into the column with the carrier gas (hydrogen) flux at 1 mL/min and the split ratio was 1:10. The run was carried out in constant pressure mode. The oven temperature has held at 250 °C for 1 min, increased to 260 °C over 20 min at the rate of 0.5 °C/min, and then increased to 325 °C over 13 min at the rate of 5 °C/min and kept at 325 °C for 15 min. The injector and the detector temperatures were set at 325 °C (Serra et al., 2014). Retention times were 7.5 min for 7 β-hydrocholesterol, 8.9 min for α-epoxycholesterol, 9 min for cholesterol, 11.0 min for β-epoxycholesterol, 14.8 min for triol-cholesterol,

and 19.6 min for 7-ketocholesterol. The chromatograms were recorded with the LabSolution software (Shimadzu, Columbia, MD, USA). Cholesterol and COPs were calculated by comparing the area of the samples and the internal standards and expressed as g/kg of fillets.

4.8 CAROTENOIDS

The content of carotenoids (mainly astaxanthin) and Vit. A in fish fillets was determined in the lipid extracts after addiction of 0.7 µL apocarotenal as internal standard and cold saponification (Sander et al., 1989). Unsaponifiable matter was resuspended in 200 µL of hexane/isopropanol (4:1) solution. Finally, 20 µL of each sample were quantified using a Prostar HPLC (Varian) equipment with UV-DAD and C_{18} reverse phase column (ChromeSep HPLC Columns SS 250 mm $\times$ 4.6 mm with ChroSEMp guard column Omnispher 5 C_{18}) as suggested by Maschrazak et al. (2002) and Mestre-Prates et al. (2006). The mobile phases were (A) methanol:acetonitrile:water (10:70:20) and (B) methanol:ethylacetate (70:30). The flow was 90:10 of A and B, respectively, kept at 1 mL/min for 15 min followed by 50:50 (1 mL/min) for 5 min and 0:100 (1.5 mL/min) for the last 10 minutes. Carotenoids were detected at 450 nm while Vit. A was detected at 325 nm. Analytes were quantified by using an external calibration curve, obtained from retinol at concentration range of 0.045-7 µg/mL. Carotenoids and Vit. A were finally expressed as µg/kg of fillets.

4.9 PHYSICAL ANALYSIS: COLOUR

A Dr Lange Spectro-color® colorimeter (Keison International Ltd, UK) equipped with a Spectral qc 3.6 software was utilised for colorimetric measurement. Colour was measured in triplicate on the epaxial-cranial fillet position. Colour measurements were carried out according to the CIELab system (CIE, 1976). CIELab is the second of two systems adopted by CIE (Commission Internationale de l'Éclairage) in 1976 as models that better showed uniform colour spacing in their values. CIELab is an opponent colour system based on the earlier (1942) system of Richard Hunter called L, a, b. Colour opposition correlates with discoveries in the mid-1960s that somewhere between the optical nerve and the brain, retinal colour stimuli are translated into distinctions between light and dark, red and green, and blue and yellow. CIELAB indicates these values with three axes: L*, a*, and b*. Figure 6 shows the special distribution of the colour. Specifically, the central vertical axis represents lightness

(signified as L*) whose values run from 0 (black) to 100 (white). The other two axis values run from positive to negative. On the a axis, positive values indicate amounts of red while negative values indicate amounts of green (redness index, a*). On the b axis, yellow is positive and blue is negative (yellowness, b*). For both axes, values range from -60 and +60, while zero represents neutral grey.

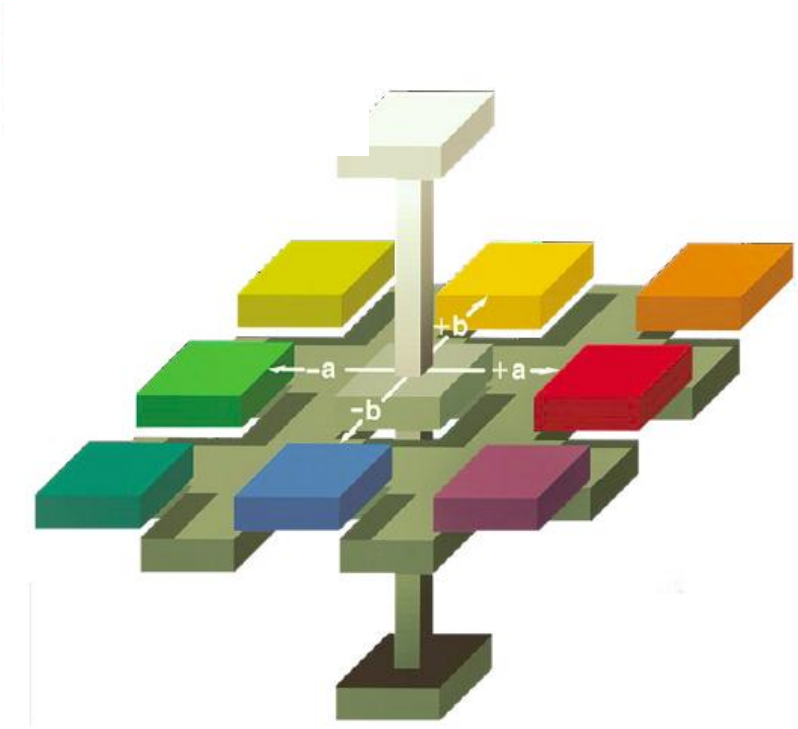

Figure 6. **Colour distribution on CIELab scale.**

5.0 STATISTIC

Data obtained from the different researches were statistically analysed. Briefly, results of Reserach I were obtained by using the General Linear Model procedures of the Statistical Analysis Software SAS 9.1 (2004) for Windows. A one-way ANOVA tested the stunning method as fixed effect.

Data related to proximate composition of atlantic salmon fillets (Research II) was submitted to ANOVA by the PROC GLM of the SAS 9.1 (2004), where Killing method (K: Percussion, CO), Storage time (S: T0, T14) and the Killing method × Storage time (K × S) interaction were included in the model as fixed effects. The other data, such as oxidation values, were analyzed as completely randomized design with repeated measures, using the MIXED procedure of SAS. The model included the fixed effects of the Killing method (K: Percussion, CO), of the Storage time (S: T0, T14) and the Killing method × Storage time (K × S) interaction, while the individual fish was included in the model as random effect nested within the killing method. The covariance structure was compound symmetry, which was selected on the basis of Akaike's information criterion of the mixed model of SAS. Statistical significance of the killing effect was tested against variance of fish nested within killing method according to repeated measures design theory. Finally, multiple comparisons among means were performed using the Tukey's test and were considered significant for p values <0.05. The coefficients of the residual (after the above model) correlations between the analysed parameters were also calculated.

The statistical analysis of data collected during Research III was performed using SPSS version 17.0 software (SPSS Inc. Illinois). Normality of data distributions was tested by the Kolmogorov-Smirnov test. Fatty acid incidences were subjected to one-way analysis of variance (ANOVA) with 'treatment' as a fixed effect, using the Bonferroni post-hoc test to check the significance of the differences among levels (WB, FB and MSM samples). The primary and secondary oxidation products and antioxidant capacity were subjected to two-way ANOVA with 'treatment' and 'storage' and their interaction as fixed effect, using Bonferroni post hoc test to check again the significance of the differences among levels (WB, FB and MSM samples), and storage (T0 and T90).

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PART II

PAPER I

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From farm to fork: lipid oxidation in fish products. A review

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Abstract

Lipid oxidation is a very complex and important event threatening the quality of foods especially of those containing highly unsaturated fats. Fish are the main source of polyunsaturated fatty acids that, unfortunately, are highly susceptible to degradation process, such as oxidation. Fish supply chain generally involves many steps and each of them together with their interaction might play a central role in muscle quality maintenance. From this review emerged that antioxidants supplementation diet can play a central role to limit the detrimental effects of stress (pre-slaughter or at killing) and storage. In this sense, lycopene shows the best antioxidant activity during stressful conditions while α-tocopherol acts preferentially in long term frozen storage. Stress just before or at slaughter can greatly threaten flesh quality both immediately and after storage by inducing numerous metabolic pathways, that often involve the production of very reactive molecular species, such as hydroperoxides. Common operation such as bleeding can significantly reduce both reactive molecules and hemoglobin, which is recognized as a great pro-oxidant. Temperature and duration are two critical points of storage phase which has to be considered even by consumers. Frozen storage at very low temperatures (-30, -40 °C) confirms to be the best storage practice. Finally, cooking can compromise aromatic profile of cooking fillets. Thus, feeding antioxidant, reducing stress both during pre-slaughter practice and at killing, good storage practices, if associate with an appropriate cooking method (low

temperature, short time) seem to be the clues for preserving the fragile lipid fraction from farm to fork.

Key words Fish, lipid oxidation, PUFA, TBARS

Introduction

Lipid oxidation has been deeply studied in the course of the past recent decades, and its complex mechanisms, kinetics and products are now to a large degree well established. As reported by Schaich (1992), mechanisms frequently proposed are based on kinetics, usually prerequisite of either oxygen consumption or appearance of peroxides (indicated as peroxide value, PV), malondialdehyde (MDA, expressed as thiobarbituric acid reactive substances, TBARS), free fatty acids (FFAs), and/or volatile compounds, therein assuming standard radical chain reaction sequences. However, when the above mentioned side reactions are either ignored or reactions proceed by a pathway different from that being measured, erroneous conclusions can be easily drawn. Thus, these various pathways and or reaction tracks need to be evaluated simultaneously to reflect a near-to-realistic picture of the most likely pathway of lipid oxidation in either of the model systems, foods, or biological tissues. The complexity of this phenomenon can be seen by the large number of studies reporting lipid oxidation and how best it either resembled or contrasted by comparisons within this subject (Ramanathan and Das, 1992; Niki *et al.*, 2005; Azhar and Nisa, 2006; Okpala *et al.*, 2014).

Lipid oxidation indeed is a very important event leading to the quality of foods, especially of those containing highly unsaturated fats. Quality losses, production of unpalatable flavour and odour, shortening of shelf life, losses of nutritional values (eg. loss of polyunsaturated fatty acids, PUFAs) and possible production of unhealthy molecules are some of the extensive consequences of lipid oxidation in foods.

Fish lipid differs from mammalian lipid. The main difference is that fish lipids include up to 40% of long-chain fatty acids (14-22 carbon atoms) which are highly unsaturated. Mammalian fat will rarely contain more than two double bonds per fatty acid molecule while the depot fats of fish contain several fatty acids with five or six double bonds. Moreover, fish oils contain other polyunsaturated fatty acids which are considered as "essential" such as eicosapentaenoic (EPA, C20:5n3) and docosahexaenoic (DHA, C22:6n3) acids. Indeed, EFSA (2010) reported that a daily intake of 250-500 mg of EPA+DHA decreases the risk of mortality from coronary heart disease and sudden cardiac death. This supports the previous funding that EPA in blood is an extremely potent antithrombotic factor (Simopoulos, 1991).

Nonetheless, long-chain fatty acids are as important as their high susceptibility to degradation, such as oxidation. It has been proved (German and Kinsella, 1985;

Richards *et al.*, 2002; Azhar and Nisa, 2006; Maqsood and Benjakul, 2011; Maqsood *et al.*, 2012) that the lipid oxidation of food, especially of PUFA contained in fish, is rather linked to the formation of off-flavour components, less of quality during different storage conditions, loss of nutritional value and even formation of anti-nutritional molecules.

Fish supply chain generally involves many steps and each of them might play a central role in the maintenance of muscle quality. Indeed, farming, killing, handling, and storage are only some of the steps between farm and consumers' kitchens and plate. For this reason, in order to prevent possible waste of nutrient value it is important to briefly review the main factors affecting lipid oxidation of fish from farm to fork. The structure of this contribution is schematically organized such that at subsequent sections, the antioxidant in feed is presented, thereafter, preslaughter procedures of starvation and crowding, then followed by the killing activity, then, handling up to cooking.

Antioxidant in feed

Fish lipids are rich in n-3 fatty acids that are essential to human health. Lipid oxidation is a major concern during processing and storage of fish because it contributes to quality deterioration and decreases marketability of fish products. Fillet accumulation of antioxidant, eg. vitamin E (vit. E) or astaxanthin, during feeding may prevent quality deterioration associated with lipid oxidation following processing and storage.

Table 1 reports the results of some studies concerning the effect of dietary antioxidant on lipid oxidation. The role of vit. E is clearly discerned from Stéphan *et al*. (1995) who demonstrated that TBARS level of low tocopherol diet is almost 100 times more than that of the highest tocopherol level diet in turbot (*Scophthahus maximus*). In addition, looking at the results immediately after death (data not shown) is possible to find slightly higher TBARS level (0.029 mg MDA/kg) in fish fed low dietary α-tocopherol (20 mg/kg feed), than in fish fed high antioxidant (320 mg/kg feed) for which 0.016 mg MDA/kg was measured. Hence, the antioxidant properties of tocopherol seem accentuated by long term frozen storage (6 months storage at -20 °C). Interestingly, the same authors performed in parallel an *in vitro* study on antioxidant ability of α-tocopherol. Uncertain patterns might be discerned. On one hand, α-tocopherol antioxidant activity is increased by increasing concentrations. On the other hand, the extent of antioxidant effect seemed to be strictly dependent on lipid content and composition of the matrix, especially PUFAn3 content. So the higher lipid and PUFAn3 content, the higher Vit. E antioxidant activity. That fact seemed to be confirmed by Chaiayapechara *et al*. (2003) who found antioxidant activity of Vit. E higher in fat rainbow trout (9.60% fat) than in fish containing 8.4% lipid. Furthermore, the action

was expressed preferentially in long term frozen storage (24 weeks at -30 °C) than in a short refrigerated one (4 °C). Unfortunately, authors did not analyse the α-tocopherol content during the storage, so it is not possible to unevenly establish the antioxidant role of α-tocopherol.

Confirming the antioxidant action of α-tocopherol, Huang *et al*. (2003) investigating hybrid tilapia (*Oreochromis niloticus* ♀ *O. aureus*) found that different diet vit. E supplementation levels could influence lag phase (initiation phase) of lipid peroxidation with apparent increase during either refrigerated (7 days at 4 °C) and frozen storage (8 weeks at -40 °C). As shown in Table 1, TBARS are affected by the dietary vit. E supplementation, and fish fed with high vit. E levels show to be less prone to be oxidised than the low fed ones. Similar results were found by Zhang *et al*. (2007) in *Sparus macrocephalus* fillets. Even in this case, fillets of fish fed with high tocopherol (553 or 1069 mg/kg) diet for 8 weeks exhibited significantly low (1.44 g/kg fillet) levels of oxidation products during 9 days of ice storage thanks to their high tocopherol muscle content.

In summary, it is possible to assert that α-tocopherol antioxidant activity is increased by increasing concentration levels in feed; α-tocopherol performs better in high fat substrates; α-tocopherol acts preferentially in long term frozen storage.

Jensen *et al*. (1998) fed rainbow trout (*Oncorhynchus mykiss*) with different astaxanthin and α-tocopherol levels for 6 months in order to understand the role of feeding antioxidant on lipid stability of the raw fish during frozen storage (-28 °C, 12 or 18 months). Globally, storage reduced both astaxanthin and tocopherol content in fish fillet, although the highest decrease was observed for astaxanthin content. Thus, results suggest that astaxanthin might protect against lipid oxidation during the early stages of oxidative deterioration, where α-tocopherol has little effects, thanks to carotenoids' role as scavengers of free radicals during the initiation of lipid oxidation. Such an ability is confirmed by the funding of Choubert *et al.* (2011), that found that carotenoid supplemented diets (100 mg astaxanthin/kg feed or 80 mg canthaxanthin/kg feed) did not significantly reduce TBARS content in rainbow trout during long term (18 months) frozen storage (- 20 °C).

Recently, new natural antioxidants (as thymol, carvacrol, and lycopene) have been utilising in feed supplementation. As reported by Giannenas *et al.* (2012) it appears that a less unique pattern for carvacrol and thymol might be discerned. Indeed, the authors found that feeding rainbow trout with thymol (6 mg/ kg) for 8 weeks improved oxidative stability during a short refrigerated storage (5 days at 4 °C) more than carvacrol supplementation (12 mg/kg). TBARS content of fish treated with thymol remains unaltered for the entire trial, at 2.25 μg /g protein, while the carvacrol group raised up 2.78 μg/g protein. Interestingly, Girao *et al.* (2012) tested the antioxidant ability of feed supplementation with lycopene (600 mg/kg) on Nile tilapia (*Oreochromis*

niloticus) undergone stress confinement. Two main effects of lycopene may be discerned. Firstly, no alteration of TBARS content accompanied by unaltered enzymatic antioxidant activity (catalase, glutathione reductase, lactate dehydrogenase) in not stressed fish fed lycopene suggests that lycopene should have an antioxidant role by removing reactive oxygen species (ROS) generated by cellular metabolism. Latter, lycopene abolished the effect of stress during confinement, remaining unchanged both enzymatic activities and TBARS (stick at 0.32 µg/kg), thus confirming it played an important role during initiation phase of lipid oxidation. More recently, Sahin *et al.* (2014) studied the effect of lycopene supplementation at different concentration (0, 200, or 400 mg/kg) on stressed rainbow trout quality performance. They found that dietary supplementation of lycopene to fish reduces the detrimental effects of stress (high stocking density) on growth performance and modulates oxidative status via activating host defence system at cellular level. It appears that lycopene can be added up to 400 mg/kg to rainbow trout diets to improve flesh quality.

During the last years, rosemary extract has been utilising in fish feeding, however contrasting results were found. Data from Hernández *et al.* (2014) show that animals fed diets containing high dose rosemary extract (1200, 1800, and 2400 mg/kg) have a significantly lower TBARS index than the control group (no added group) or the group fed with low dose (600 mg/kg) over the first 7 days of storage (average 0.11, 0.13, and 0.22 mg MDA/kg, respectively). However, on day 21st, a certain tendency emerged towards an increase in the TBARS index as the dose increased, possibly due to a pro-oxidant effect of the rosemary extract at high doses. The lowest rosemary dose raised up to 0.49 mg/kg, while the other groups achieved at maximum 0.71 mg/kg, even if any statistical differences emerged. While comparing rosemary, thymol, carvacrol, or synthetic antioxidant as BHT feed supplementation for their capability of prevent lipid oxidation, Álvarez *et al.* (2012) found the following increasing stability order: carvacrol>rosemary=BHT>thymol. Thus, during 14 days of refrigerated storage, fillets from fish fed diet with carvacrol (500 mg/kg, 18 weeks) showed the lowest TBARS content (0.2 mg MDA/kg fillet), while the maximum was reached by thymol group with 0.4 mg MDA/kg.

Preslaughter procedure: starvation and crowding

Food quality is perceived as a global concept. Food should be primarily safe, tasty and healthy. However, food safety and ethics is increasingly of global interest. In this context, commonly pre-slaughter practices that may be responsible for animal stress are starvation and crowding. Starving the fish for some days prior to slaughter is a common practice in the case of farmed fish, with the scope to delay spoilage by reducing the amount of faeces in the intestine. During the last decades, many authors

have investigated the influence of starvation on flesh quality in different fish species such as *Sparus aurata* (Ginés *et al.*, 2002; Álvarez *et al.,* 2008), *Dentex dentex* (Suárez and Cervera, 2010), *Onchorynchus mykiss* and *Salmo trutta* (Bayir *et al.*, 2014) but only a few of them focused on the induced oxidative stress.

Álvarez *et al.* (2008) exposed *S. aurata* to 24, 48, or 72 hours of starvation and among others parameters they evaluated TBARS on fillets. Although no significant differences between starvation periods emerged, a trend may be discerned. Particularly, it seemed that the TBARS values increased with starvation time. Indeed, 2.50±0.90, 3.63±1.62, and 4.57±1.75 mg MDA/kg were found in *S. aurata* starved for 24, 48, and 72 h, respectively.

Interestingly, Bayir *et al*. (2014) measured oxidative stress indicators, such as reactive oxygen species (ROS) in liver and muscle samples from *Onchorynchus mykiss* and *Salmo trutta* exposed to a 45-day starvation period at low water temperature. They found that in both species lipid peroxidation increased with starvation length, even if the metabolic response to food deprivation in the muscle of each species was different.

Crowding is a temporary status immediately before killing when fish can be collected in very high density. As reported by Pérez-Sánchez *et al.* (2013), crowding causes a complex stress by affecting hepatic gene expression, antioxidant defence system, cell–tissue repair mechanism, xenobiotic metabolism and stress transcriptional regulation. This response, similar to the one described by Bayir *et al.* (2014) for starvation, may explain the funding of Bagni *et al.* (2007), that monitored the effect of crowding (density >70 kg/m^3) on the oxidative stress of two common Mediterranean species (gilthead sea bream, *Sparus aurata*, and European sea bass, *Dicentrarchus labrax*). Oxidative stress was determined in terms of increment of the reactive oxygen metabolites (ROMs) and of anti-oxidant power (AOP). From the data emerged that in case of stress conditions, the ROMs production can be counteracted by an adaptive response, such as the activation of the AOP mechanism. However, the stress extent may greatly affect this response, by shifting from a positive response (high AOP, low ROMs) to a negative one (low AOP, high ROMs). The former is the case of uncrowded fish, the latter of crowded fish. Furthermore, stress response seemed to strictly depend on species. Indeed, gilthead sea bream showed to be less affected by the application of stress than European sea bass (no significant differences between two stress groups were found for AOP and ROMs). Gilthead sea bream as well showed a lower survival time than European sea bass. Nathanailides *et al.* (2011) supported the hypothesis that increased levels of stress can lead to increased lipid oxidation in European sea bass fillets. In details, fish were processed with a high stress method (the water was lowered and the fish were captured using a net, then killed by immersion in an ice cold bath) or with a lower stressful one (the level of water was lowered and fish

were anaesthetized moderately by immersion in a 30 mg/L clove oil bath for 5 minutes, then slaughtered by immersion in ice cold sea water). Results showed that the handling stress prior to slaughtering affects significantly TBARS contents, which were 1.04 and 1.16 mg MDA/kg in no stressed and stressed fish, respectively.
The above cited studies of this subsection let emerge that pre-slaughter stress may induce complex metabolic responses: rapid ATP depletion may generate various pro-oxidant substances, which in turn may induce an activation of AOP mechanism for ROS and ROMs depletion. Unfortunately, high stressful conditions or stress length may cause the adaptive response to be useless resulting in an increase of lipid oxidation.

Killing

Stunning/killing procedures applied in aquaculture are different and fish species vary in their response to the different methods utilised. Mediterranean aquaculture species are usually killed by asphyxiation in air, immersion in ice/water slurry or by percussive stunning. Ice killing is usually used in selective fisheries. Recent alternative stunning/killing processes have been experimentally investigated for Mediterranean fish species in an effort to develop and optimize commercial methods by assuring both high standards of fish welfare and product quality (EFSA, 2008; Poli, 2009). It has been widely reviewed that pre-slaughter (as anaesthesia) and slaughter stressful practices could have an important effect on the flesh quality in fish (Poli *et al.*, 2005). A clear effect emerged mostly on the physical properties of flesh, because severe stress at slaughter time exhausts muscular energies, produces more lactic acid, reduces muscular pH, and increases the rate of *rigor mortis* onset Poli et al. (2005). In this way these practices could have significant negative effects on fish technological traits and in their flesh quality.
According to Hultin (1992), anaerobiosis influences the conversion of xanthine dehydrogenase to xanthine oxidase. The latter enzyme transfers electrons directly to molecular oxygen producing superoxide and hydrogen peroxide, which can produce hydroxyl radicals in the presence of redox iron. These compounds have been proposed as among the principal initiators of lipid oxidation in biological tissues. Thus, the rapid conversion of ATP to hypoxanthine and of xanthine dehydrogenase to xanthine oxidase could influence lipid oxidation time of fresh and semi-preserved fish, especially when molecular oxygen is reintroduced during *post mortem* processing.
Tejada and Huidobro (2002) found out that slaughter method (percussion, ice salt-water slurry bath, and asphyxia) has no clear influence on the oxidative stability of gilthead sea bream (*Sparus aurata*), probably due to the interaction of many factors such as stress, handling speed after death, and lipid content of flesh.

Morzel and van de Vis (2003) studied the effect of killing methods on lipid oxidation of eel (*Anguilla anguilla* L.). Particularly, electricity and oxygen removal (new killing method) resulted in higher quality of eels in comparison with the dry-salt technique, by reducing stress and improving freshness. Furthermore, less stressful practice seemed to reduce the extent of lipid oxidation. In details, authors pointed out that enhanced lipid oxidation in salt-bath eels can be partially explained by the physical damage to the muscle, thereby increasing the cell ruptures and the consequent accessibility to the catalytic enzymes. In addition, the presence of salt may be considered in some extent a slight pro-oxidant.

Results from Giuffrida *et al.* (2007) were in agreement with this explanation. Particularly, ice slurry slaughtered gilthead sea bream (*Sparus aurata*) showed higher (and then better) ATP/IMP levels (an indicator of less stressed fish) and to be less prone to lipid oxidation, as revealed by the MDA values, 0.158 mg MDA/kg flesh against 0.227 mg MDA/kg flesh in CO_2-slaughtered fish. The same pattern was found in electrical stunned rainbow trout (*O. mykiss*) whilst compared electricity with anoxia and bleeding as killing methods. TBARS values for these groups were 0.68, 1.09, and 1.03 mg MDA/kg flesh, respectively. Sakai and Tereyama (2008) studied the effect of bleeding as killing method on chub mackerel (*Scomber japonicus*) lipid oxidation. Struggling death in iced sea water was utilised as control. The MDA content in the muscles of the bleeding fish samples were significantly higher than those of the control after 119 hours of storage at 0 °C, with 0.367 and 0.184 mg/kg, respectively. On the contrary, no differences were found in 4-hydroxyhexanal content of the samples. These results confirmed that fish subjected to stressful conditions were more prone to be oxidised and suggested that bleeding can be considered as a stressing killing method.

On the contrary, Duran *et al.* (2008) found that slaughter method (asphyxia or percussion) had no effect on the MDA values of carp (*Cyprinus carpio*). However, when considering rainbow trout (*O. mykiss*) the MDA content of flesh from fish slaughtered by asphyxiation was significantly higher than that of specimens slaughtered by percussion (4 and 3 mg MDA/kg flesh, respectively). It is important to note that the fat level of trout was higher than that of carp (5% against 1%), which led to an observed difference in the MDA contents of trout slaughtered by different methods.

The effects of different stunning/killing procedures (anaesthesia with clove oil, anaesthesia with 2-phenoxyethanol, percussive stunning, immersion in ice/water slurry, chilling on ice, and anaesthesia with clove oil followed by immersion in ice/water slurry) on flesh quality of European sea bass (*Dicentrarchus labrax*) were investigated by Simitzis *et al.* (2014). Globally, MDA ranged between 29.9 and 95 mg/kg flesh in chilling on ice and percussion slaughtered sea bass. Despite such large

range of values, authors did not find any significant difference among the tested killing methods, suggesting no killing effects on lipid oxidation.

Interestingly, in contrast to the results seen previously, in a recent study Secci *et al.* (2016) found out the link between stress during slaughter and lipid oxidation. Their results revealed the presence of very high level of reactive molecules, such as hydroperoxides, in stressed rainbow trout whilst they were not detected in not-stressed group. Thus, probably as a consequence of the greater enzymatic activity under stress condition, the presence of lipid oxygenated products affected the development of lipid oxidation during *post mortem* storage.

Handling

It is widely reviewed that any process causing disruption of the muscle membrane system (such as grinding, freezing, and cooking) results in exposure the lipid fraction to oxygen, and thus accelerates the development of the oxidative damage. However, one of the first processes after stunning and killing procedures in fish industry is the blood removal. Although it is not a kind of handling altering the lipid structure, blood removal is strictly linked to the quality deterioration of fish muscle, especially to the lipid oxidation. Richards and Hultin (2002) studied the contribution of blood and blood components to lipid oxidation in rainbow trout (*O. mykiss*) and Atlantic mackerel (*Scomber scombrus*). They performed a complex project, finding out three main points: bleeding significantly reduced the probability of rancidity (expressed both as sensory score and TBARS value) development during storage; this probability strictly depended on species and type of muscle considered (rainbow trout *vs* mackerel, light *vs* dark muscle); the extent of lipid oxidation was more pronounced in minced muscle as compared to the intact one.

The first point was confirmed by many authors. Tejada and Huidobro (2002) recognised the greater extent of lipid oxidation of ungutted gilthead sea bream (*Sparus aurata*) when comparing with gutted samples at day 11 of refrigerated storage (+2 °C), reaching as high as 8 mg MDA/ kg flesh, a value commonly utilised as rancidity threshold. However, according to the same authors, such an increase seemed not to be significant.

Sakai *et al.* (2006) attempted to measure haemoglobin (Hb) content in bled skipjack tuna (*Katsuwonus pelamis*) flesh, and they analysed malondialdehyde contents and 4-hydroxyhexenal (HHE) in the muscle as indicators of the lipid oxidation level. Firstly, Hb content was lower in bleeding samples than in the control ones, containing 0.07 and 1.01 mg/g, respectively. Concerning lipid oxidation, Sakai *et al*. (2006) did not find significant differences in MDA content in samples while bleeding fish showed lower level of HHE than the control samples both immediately after death (not detected *vs*

0.20 nmol/kg) and after two days of storage at 0 °C (0.07 and 0.43 nmol/kg, respectively).

More recently, Maqsood and Benjakul (2011) confirmed that bleeding decreases Hb content and consequently lipid oxidation in Asian sea bass muscle (*Lates calcifer*). Their results indicate that lipid oxidation (measured as PV, TBARS, and volatiles) was more pronounced in the un-bled samples during 15 days of refrigerated storage (2 °C). Particularly, blood contains a high amount of haemoglobin which action as pro-oxidant is still discussed. However, the extent of lipid oxidation is affected not only by Hb concentration but also by the presence of different type of Hbs in fish muscle (Richards and Hultin, 2002) and their breakdown during storage, resulting in the release of non-heme iron (Maqsood and Benjakul, 2011).

At this point it is easy to understand that different species as well as different kind of muscle may greatly differ in term of Hb content and composition, so causing a different susceptibility of the muscle to be oxidised. This is the case of muscle that contains large amount of blood, such as dark muscle, which is found to be more prone to be oxidised (Richards and Hultin, 2002). In addition, Hb concentration might explain the higher values of lipid oxidation in minced muscle than in whole/intact one (Richards and Hultin, 2002). Indeed, the mechanical action of mincing can provoke rupture blood vessels, erythrocytes, and some other cells and so cause Hb release. As stated, that release can promote lipid oxidation.

At the same time, grounding increases the exposition area of muscle to atmosphere oxygen, moving to a real pro-oxidant factor. According to them, Thiansilakul *et al.* (2011) confirmed that myoglobin (Mb) was able to catalyse lipid oxidation in washed Asian sea bass (*Lates calcarifer*) minced intensively. Primary and secondary oxidation products as well as off-odour development were significantly higher in Mb addicted samples than in the control ones (no Mb addicted). At day 8 of storage (4 °C), volatiles were mainly composed by 11.54%, 11%, and 7.08% of 1-octen-3-ol, hexanal, and 2-pentyl furan, respectively, whilst hexanal and 2-pentyl furan applied globally for 3.7% in the control samples. These changes were more likely associated with metmyoglobin formation occurring in washed mince, as a consequence of the increase in storage time. Moreover, lipid oxidation in washed mince with added myoglobin was mainly governed by pH. Specifically, lowest the pH (6) highest the lipid oxidation extent was.

Recently, it was also found that the higher the heme affinity of Mb, the lower the myoglobin-mediated lipid oxidation was obtained (Richards *et al.*, 2009). Therefore, low pH was not only associated with Mb oxidation, but also weakened the heme-globin complex, leading to a release of heme group, which was able to induce the lipid oxidation.

The problem of the quality deterioration during storage is well known and it is related to both temperature and storage time. As well, the quality lowering rate depends on species of fish. Nishimoto *et al*. (1985) found out that the highest temperature of storage the fastest deterioration of fish freshness. To date, many authors have focused on quality changes during storage by studying separately ice or chilling storage and the frozen one.

The concentration of TBARS in good quality frozen and chilled fish or in fish stored on ice is typically between 5 and 8 mg MDA/kg whereas levels of 8 mg MDA/kg are generally regarded as the limit of acceptability for most species (Schormüller, 1968). More strictly, Ke *et al*. (1984) proposed that TBARS values for fish products below 0.58 mg/kg were perceived as not rancid; 0.58–1.51 mg/kg as slightly rancid, but acceptable; and values above 1.51 mg/kg were perceived as rancid.

Özyrut *et al.* (2009) studied red mullet (*Mullus barbatus*), and goldband goatfish (*Upeneus moluccensis*), both belong to the Mullidae family, funding different shelf-life and lipid oxidation levels when stored 11 days at 2 °C. The authors analysed both primary (PV) and secondary (TBARS) lipid oxidation products. PV significantly raised from 0.64 and 0.83 meq (peroxide oxygen/kg fat) to 2.26 and 4.82 meq/kg at the end of the trial, in red mullet and goldband goatfish, respectively. TBARS values were found stable around 0.51 and 0.57 mg MDA/kg flesh for both species during the whole storage. Similar PV values were obtained by Timm-Heinrich *et al.* (2013) studying the oxidative changes of rainbow trout during ice storage (12 days at 2 °C) by following PV and volatile content. PV were below 0.5 meq O_2 /kg flesh during the first 5 days of storage. After, PV had a slightly increase up to 0.56 meq O_2/kg on day 7, whilst they started raising significantly up to 6 meq O_2/kg on day 12. Similarly, the volatile fraction started to increase significantly from day 5 onwards, especially 1-penten-3-ol, 1-penten-3-one, and 2-pentenal. Globally, volatiles were found in low concentration (ng/kg), confirming a little oxidation during 12 days of storage on ice. Concerning TBARS levels, Etemadian and Shabanpour (2014) found their increase from an initial value of 0.56 mg MDA/kg of muscle to 2.92, 2.67 and 2.31 mg MDA/kg of *Rutilus frisii kutum* slices during 15 days of iced storage.

Probably related to bacterial growth at positive temperature, the seventh day appeared to be critical even in other papers. For example, Hernández *et al.* (2009) studied lipid oxidation of aquacultured meagre (*Argyrosomus regius*) fillets during 18 days of storage at 4 °C, finding significant differences from day 7 onwards. Particularly, TBARS gradually increased from 0.10 to 2.55 mg MDA/kg flesh. In agreement, Simitzis *et al*. (2014) found that, in general, positive temperature (4 °C) increased MDA levels around 3.6 times in 7 days of storage. In the same paper, Simitzis *et al*. (2014) looked for TBARS level even in frozen (-20 °C) samples, finding that freezing raised up TBARS

level around 2.7 times after 90 days. An interesting connection between refrigerated and frozen storage was found some years before by Huang *et al*. (2003) who measured the same MDA value (7.2 μg) in hybrid tilapia fillets (*Oreochromis niloticus* × *O. aureus*) stored for 7 days at 4 °C or for 8 weeks at -40 °C.

When meat and meat products are stored under frozen conditions, microbial spoilage may be delayed, but fat deterioration occurs and the meat constituents may be oxidized (Ojagh *et al*., 2014). Interestingly, the main cause of lipid oxidation during frozen storage seemed to be due to the enzymatic lipolysis activity. Indeed, Karlsdottir *et al*. (2014a) indicated that enzymatic lipolysis was the driving factor influencing the fillet quality over storage and it mostly affects long chain polyunsaturated lipids in the light muscles.

However, previous studies had confirmed that low storage temperatures were optimal for preserving fish from oxidative deterioration. Refsgaard *et al*. (1998) compared lipid oxidation of Atlantic salmon fillets (*Salmo salar*) stored at -10 or -20 °C for 34 weeks. The content of lipid hydroperoxides and free fatty acids increased during storage as affected by a significant time-temperature interaction, and the changes were fastest in salmon stored at -10 °C. Specifically, hydroperoxides raised from 0 to 10 meq O_2/kg, while FFA increased from 1 to 8.7% in 34 weeks of storage. Such as oxidative product increase was associated with a decrease in highly unsaturated fatty acid content (C20:5n3, C22:5n3, and C22:6n3) in Atlantic salmon stored at -10 or -20 °C. Also for the polyunsaturated fatty acids, significant time-temperature interaction effects were found, confirming the fastest decrease at -10 °C. Concerning to volatile products, aldehydes and ketones were identified. For hexanal, heptanal, (E)-2-hexenal, (E,E)-2,4-heptadienal, and nonanal significant time effects were found due to increasing concentrations during storage, independently of storage temperature, while temperature influenced significantly hexanal and 2-hexanal levels. A small increase in the amount of secondary lipid oxidation products was also observed by Jensen *et al*. (1998). The significant and preservative action of negative storage temperature was confirmed by Choubert *et al*. (2011), that determined lipid oxidation (TBARS) in packed rainbow trout stored for 18 months at -20 °C. Results showed that TBARS significantly increased after the first month of storage, but not other changes occurred during the 5 later months.

Baron *et al*. (2007) studied the lipid oxidation during frozen storage of rainbow trout fillets, stored for 13 months at -20, -30, or -80 °C. Lipid oxidation was followed by measuring lipid hydroperoxides (PV), as well as secondary oxidation products (volatiles). There was a significant increase in the level of lipid hydroperoxides after 8 months of frozen storage for fish stored at -20 °C, which was even more pronounced after 13 months, reaching 6.6 meq/kg of oil, indicating on-going oxidation. In contrast, samples stored at -80 and -30 °C did not show any significant increase in peroxides

during the entire storage period (with p = 0.26 and p= 0.07, respectively). Measurement of secondary oxidation products was followed for 13 months, and the development of hexanal (an oxidation product of linoleic acid), and 1-penten-3-one and t,t-2,4-heptadienal, both oxidation products of n-3 fatty acids. Other volatiles were also measured during storage (1-penten-3-ol, heptanal, 1-octen-3-ol, t-2-octenal, nonanal, t,c-2,6-nonadienal, decanal), and their development was generally in agreement with what is reported here for hexanal, 1-penten-3-one, and t,t-2,4-heptadienal. Volatile patterns indicated that fish stored at -20 °C was the most oxidised and that little difference was observed between -80 and -30 °C. On the basis of their observations the ranking order -20 °C > -30 °C > -80 °C was obtained for the development of oxidation products in fish stored at freezing temperatures. Likewise, both Indergård *et al.* (2014) and Karlsdottir *et al*. (2014a) recently confirmed that pattern. The first authors examined lipid oxidation, by PV and TBARS, in Atlantic salmon during a long-term frozen storage at –25, –45 and –60 °C. After 1 year of storage at –25 °C, the concentration of PV in red and white fish muscles increased from 1.26 to 1.82, and from 1.08 to 1.76 meq O_2 /kg fat, respectively. Formation of TBARS was higher in the red muscles than in the white ones, and reached a value of 14.04 mg MDA/ kg fish after 1 year of storage at –25 °C. Decreasing the temperature to –45 °C inhibited PV and TBARS formation. In the latter paper, the authors studied the lipid deterioration of two lean fish species, i.e. saithe (*Pollachius virens*) and hoki (*Macruronus novaezelandiae*), during frozen storage at -20 and -30 °C (up to 18 months). As even in the previous case, Karlsdottir *et al*. (2014a) analysed both light and dark muscles. Results showed significant lipid deterioration with the extended storage time, but lower storage temperature showed significantly more preservative effects. The formation of hydroperoxides as well appeared to be strongly influenced by species. Saithe was very stable during the first 12 months of storage regardless to storage temperature. After 18 months of frozen storage, however, a slight, yet significant, increase of peroxides (up to 50 mmol/kg muscle) was observed in light and dark muscle types. On the other hand, hydroperoxide formation in hoki showed a much more pronounced and more progressive peroxide formation over time at both storage temperatures. However, as previously shown for handling, the extent of oxidation rate was showed to be strictly connected with the type of muscle considered. Indeed, dark muscle showed to be the most prone to be oxidised regardless to storage temperature, by ranging up to 325 and 250 mmol/kg at -20 °C and -30 °C, respectively. The light muscle presented a pattern similar to saithe, by remaining almost unaltered for the first 12 months. As for saithe, at the end of storage a significant increase of peroxide was observed (50, and 100 mmol/kg muscle at -30 and -20 °C, respectively) in hoki samples. It has to be noted that the marked difference in peroxide content between hoki dark and light muscle is likely due to the

considerably higher lipid content in dark muscle than in the light one (7.6% *vs* 0.6%). The difference in fat content might explain either the difference between oxidation susceptibility of saithe when compared with hoki, since saithe fat content ranged from 0.6% to 1.1%, whilst hoki has from 0.6% to 7.6% in light and dark muscle, respectively. TBARS results for both the saithe dark and light muscle showed low/no formation of secondary oxidation products up to month 6, followed by a sharp increase up to month 12, after which only the dark muscle values continued to increase. As well as peroxide values, low temperature protected against oxidation as revealed by the significantly higher increase of TBARS in -20 °C stored samples than the -30 °C stored ones.

Cooking

Prior to consumption, fresh and frozen fish usually undergo different preserving treatments or different cooking processes, while the consumption of raw fish is not considered a traditional custom in the Western society. Boiling, frying, pan-frying, grilling, roasting, baking and microwaving are the most popular cooking methods. However, despite making food safer and tastier, the temperatures reached during cooking process may affect radically the characteristics and composition of food by enhancing lipid oxidation on behalf the other processes.

In general, the cooked saithe fillets exhibited an increase in hydroperoxide levels after steaming (Karlsdottir *et al.*, 2014b) which is a characteristic sign that thermally catalyzed oxidation has taken place. TBARS were in line with peroxide content, by showing an increase after cooking. That pattern was more evident in samples previously frozen stored (6 months) in which TBARS raised from less than 0.72 to 5 mg MDA/kg muscle. Even concerning the volatile fraction, storage time seems to negatively affect aroma of cooked fillets. Particularly, Aro *et al.* (2002) noted that short chain acids, deriving from aldehydes oxidation and microbial fermentation and partially causing unpleasant odour, increased in herring when baked after storage (48 hours).

High temperature and medium of cooking may be the main responsible for the different effect on cooked meat. This fact is mostly evident for frying which seems to lead a wide variety of changes. Figure 1, as example, reports data from Al-Saghir *et al*. (2004) and Tokur (2007) for Atlantic salmon and rainbow trout, respectively. Salmon fried with olive or corn oil at 180 °C increase its PV 3 or 2 times, respectively, whilst sunflower frying doubled PV in rainbow trout. The significant differences in peroxide content in both cases seem to depend on the first oxidation state and degree of unsaturation of the frying oils. Indeed, as reported by Al-Saghir *et al*. (2004) olive oil has a high initial peroxide value (11.8±0.03 meq O_2/kg), which provides the explanation for the increased peroxide value of salmon after frying with olive oil.

Concerning the oxidative effects of frying, it has to be underlined that is very hard to follow clearly lipid oxidation, especially its primary products. In effect, high temperatures accelerate oxidation but oil dissolves oxidation products, reducing their concentration in fillet and making the oxidative damage difficult to assess (Weber *et al*., 2008). The low levels of conjugated dienes (CD), peroxides and MDA, if apparently could lead to think of a smaller oxidation, actually seem to depend on the more rapid evolution of the oxidative mechanism taking place in this kind of cooking method. Figure 1 shows that higher amount of peroxides accumulates in baking (31.45±1.17 mEq O_2/kg lipid) and barbecuing rainbow trout fillets when compared with the frying ones. Furthermore, when compared with grilled, boiled, oven and microwave baked ones, the CD and peroxides values decreased for all fried (215-220 °C) samples of silver catfish (*Rhamdia quelen*), probably because of their decomposition into secondary oxidation products which might have been lost in the frying oil or transformed in protein adducts (Weber *et al*., 2008).

The interaction between lipid oxidation products and proteins is hypothesized even by Talab (2014) for explaining the decreasing effect of cooking method on carp lipid oxidation. Evaluating the effects of different cooking methods on PV and TBARS of raw, fried, microwave and halogen cooked carp fish cutlets, the author found that cooking decreased both primary and secondary oxidation products. Particularly, PV recorded values were 3.69, 2.98, 2.80 and 2.60 meq O_2/kg for raw, fried, microwave and halogen cooked samples while TBARS of raw, fried, microwave and halogen cooked carp cutlets were 1.20, 1.18, 1.09, and 1.01 mg MDA/kg, respectively.

Similarly, Wu and Mao (2008) supposed that the high temperatures accompanying drying processes during microwaving could speed up the breakdown of peroxides into their carbonyl components, and thus the peroxide value, and more generally the primary oxidation products, may remain low. For this reason, conjugated dienes and peroxides did not differ from both raw silver catfish (Weber *et al.*, 2008) and raw grass carp fillets (*Ctenopharyngodon idellus*) (Wu and Mao, 2008), while a significant increase in secondary oxidation products was observed both in catfish and in carp baked in microwave and conventional ovens. These data were supported by previous research (Tokur, 2007) in which baking and barbecuing significantly increase TBARS levels (5.78±0.94 and 8.40±0.51 mg MDA/kg muscle) in rainbow trout fillets in comparison with the smoking process (1.82±0.16 mg MDA/kg muscle).

The oxidative impact of cooking and its fast develop can be noted by the abundance of aldehydes and chetons (volatiles), mainly arising from unsaturated fatty acid degradation. Several volatile molecules belonging to the main odorant categories (furanones, pyrazines, aldehydes, chetons and other intermediate products from Maillard reactions, such as 5-methylfurfural), can be detected in cooked fish or be loose in the cooking medium, as in case of boiled fish (Morita *et al*., 2003).

Interestingly, in spite of having a poor organoleptic profile, steam-cooked bighead carp (*Hypophthalmichthys nobilis*) was not rejected by consumers. Strangely, hedonic score resulted higher for steamed carp than for the oven-baked one, so that 64% of the panellists liked very much or moderately the appearance and flavour of the steamed carp (Freeman, 1999).

It is nevertheless true that some volatile compounds from lipid oxidation, like (Z)-4-heptenal and hexanal (Prost *et al*., 1998), above odour threshold, could be responsible for off-flavours and consequently can deteriorate the organoleptic profile of cooked fillets.

By the way, it is important to remember that many other molecules, such as proteins, are responsible for aromatic profile. The weak odour of steamed horse mackerel for example, when compared with grilled and fried samples could be explained by the concentration at lower levels of alanine, aspartic acid, glutamic acid, glycine, and proline which are responsible for flavour and taste in seafood and seafood products (Ruiz-Capillas and Moral, 2004). For this reason, it is not possible to strictly link the organoleptic profile exclusively to lipid oxidation.

Anyway the lipid stability of baked fish can be affected by the presence of antioxidative agents in dietary treatment. Jittinandana *et al*. (2006) compared the results between rainbow trout fed with low and high dietary vitamin E supplementation showing a significant difference in TBA after baking (0.67 vs 1.20 mg MDA/kg).

Conclusion

The complexity of lipid oxidation is reflected on the large variety of molecules that can be generated and on the various factors which affect it. Hence, every step in fish supply chain seems to be important for preserving lipid integrity and native quality. Feeding antioxidant is a recent practice to arise antioxidant power in muscle. However, some issues emerge from this review. Firstly, it is quite clear that the main antioxidants such as α-tocopherol and astaxanthin act during the initiation phase of lipid oxidation by acting as scavengers of ROS and ROMs species. Thanks to this ability, the antioxidants can play a central role to limit the detrimental effects of stress (pre-slaughter or at killing) and storage. In this sense, lycopene modulates oxidative status via activating host defence system during stressful conditions while α-tocopherol acts preferentially in long term frozen storage. The growing efficiency carvacrol>rosemary>thymol has to be taken into consideration while using new antioxidants such as essential oils (thymol, carvacrol, rosemary extract). Finally, it has to consider that, in some cases increasing concentration of the antioxidants in the diet is not always associated with increasing lipid stability. Further investigations on feeding field are recommended. It could be of interest to evaluate the role of new alternative

protein and/or lipid sources (such as insect meals) on lipid composition and stability of the fillets. Even the utilization of agriculture by-products rich in polyphenols (for example those derived from olive oil production chain) may be considered both for their role as potential antioxidants and an environmental point of view.

Stress just before or at slaughter can greatly threaten flesh quality both immediately and after storage. Stress induces the activation of numerous metabolic pathways, that often involve the production of very reactive molecular species, such as hydroperoxides. Their presence can be the main cause of increasing level of lipid oxidation products both immediately after death or during storage. A common operation such as bleeding can significantly reduce both reactive molecules and hemoglobin, which is recognized as a great pro-oxidant. However, stress response seems to be strictly species-specific, so different species of fish can react differently to the same killing method and show different pattern in lipid degradation. Future outlooks on the connection between stress at slaughter and oxidative stability, as well as the evaluation of the effects of new killing procedures on both animal welfare and fish quality should be conducted.

Temperature and length are two critical points of storage phase which has to be considered even by consumers. Frozen storage at very low temperatures (-30, -40 °C) confirms to be the best storage practice, while the refrigerated one shows some limit. Particularly, considering lipid oxidation, it has to be underlined that 7 days of refrigerated storage is accepted as the maximum storage length for many species of fish.

Finally, if it is true that cooking can make safer a product regardless its storing age, it is also true that age can compromise aromatic profile of cooking fillets. However, on one hand it could be interesting to evaluate the effect of new cooking methods, such as under-vacuum cook or air-frying, on lipid oxidation. On the other, it should be deepened the connections between oxidation, volatiles composition and consumers perception and acceptance. Perhaps the creation of acceptance predictive models based on lipid stability could even be useful.

In conclusion, many steps long the production-supply chain of fish can lead lipid deterioration and many interactions between them may contribute to alter flesh quality. Thus, feeding antioxidant, reducing stress, good storage practices, if associate with an appropriate cooking method (low temperature, short time), seem to preserve such a fragile and extremely important lipid fraction from farm to fork.

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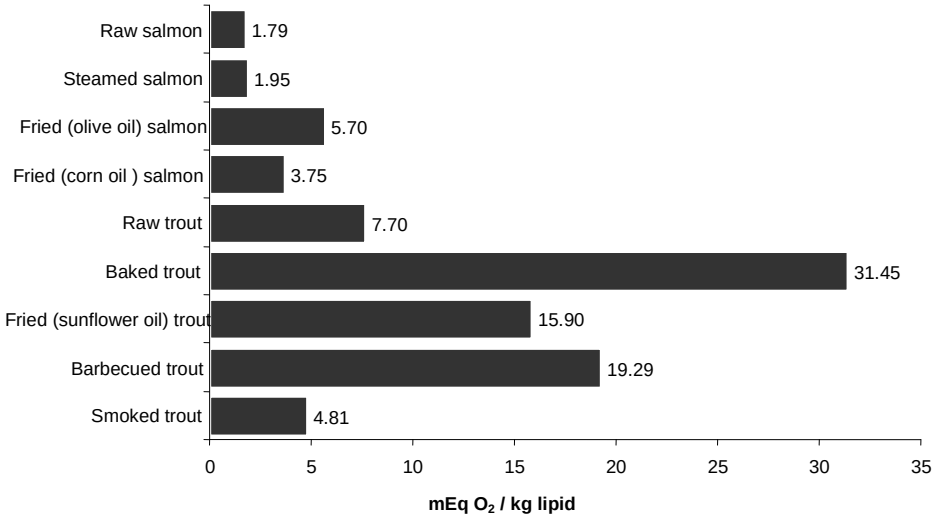
Table 1. Effect of antioxidant supplementation diet on secondary lipid oxidation products (TBARS, mg MDA/kg fillet) of different species. Data retrieved from Stéphan *et al*. (1995), Jensen *et al.* (1998), and Huang *et al.* (2003).

Antioxidant	Quantity	Feeding length	Species	Storage length	Temperature (°C)	TBARS (mg MDA/kg fillet)	References
α-tocopherol	20 mg/kg	34 weeks	Turbot	6 months	-20	0.259	Stéphan *et al*. (1995)
α-tocopherol	70 mg/kg	34 weeks	Turbot	6 months	-20	0.063	Stéphan *et al*. (1995)
α-tocopherol	320 mg/kg	34 weeks	Turbot	6 months	-20	0.029	Stéphan *et al*. (1995)
α-tocopherol/ astaxanthin	100/40 mg/kg	6 months	R. trout	12 months	-28	0.39	Jensen *et al*. (1998)
α-tocopherol/ astaxanthin	100/40 mg/kg	6 months	R. trout	18 months	-28	0.47	Jensen *et al*. (1998)
α-tocopherol/ astaxanthin	600/40 mg/kg	6 months	R. trout	12 months	-28	0.39	Jensen *et al*. (1998)
α-tocopherol/ astaxanthin	600/40 mg/kg	6 months	R. trout	18 months	-28	0.51	Jensen *et al*. (1998)
α-tocopherol	65 mg/kg	8 months	Hybrid tilapia	7 days	4	2.88	Huang *et al*. (2003)
α-tocopherol	200-300 mg/kg	8 months	Hybrid tilapia	7 days	4	1.08	Huang *et al*. (2003)
α-tocopherol	65 mg/kg	8 months	Hybrid tilapia	8 weeks	-40	5.76	Huang *et al*. (2003)
α-tocopherol	200-300 mg/kg	8 months	Hybrid tilapia	8 weeks	-40	4.32	Huang *et al*. (2003)

Figure1. Peroxides accumulation on rainbow trout (Al-Saghir *et al*., 2004) and Atlantic salmon (Tokur, 2007) cooked by different methods.



PAPER II

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Stress during slaughter increases lipid metabolites and decreases oxidative stability of farmed rainbow trout (*Oncorhynchus mykiss*) during frozen storage

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Abstract

The consequences of slaughter on the formation of lipid metabolites and oxidative stability of fish muscle during long term frozen storage (- 10 °C) were evaluated using farmed rainbow trout killed by asphyxia in air or percussion. The level of major adenine nucleotides and their related compounds was determined in order to check the stress level during slaughter. Plasma lipid metabolites were studied through the determination of eicosanoids and docosanoids such as prostaglandins, leukotrienes, tromboxanes, isoprostanes, resolvins, hydroxides, hydroperoxides, coming from eicosapentaenoic (EPA), arachidonic (ARA), and docosahexaenoic (DHA) acids. In addition, lipid oxidative stability of fillets was monitored. Results revealed that stress during slaughter can greatly influence oxidative stress and oxidative stability of rainbow trout fillets. In fact, asphyxia, which was the most stressful, induced a higher production of some lipid mediators such as hydroperoxides and EPA-derived prostaglandins, such as 12-HpHEPE/15-HpHEPE and PGD3/PGE3. As a consequence, fillets derived from asphyxiated fish were less stable in terms of oxidative stability and showed lower shelf-life.

Key words: eicosanoids, rainbow trout, stress biomarker, lipid oxidation, TBARS

1. Introduction

The consequences of stress and/or muscle activity related to slaughter have been widely investigated during the last decades (Poli, Parisi, Scappini, & Zampacavallo, 2005; van de Vis et al., 2003). In many species, slaughter stress has been shown to greatly influence the post-mortem biochemical processes, mostly by causing anaerobic glycolysis in muscle and increasing ATP degradation rate (Tejada, 2009). Moreover, stress has been proved to affect flesh quality during storage, by measuring freshness index K-value (Sigholt, Erikson, Rustad, Johansen, Nordtvedt, & Seland, 1997), quality index (van de Vis et al., 2003), pH, color and water holding capacity (Simitzis, Tsopelakos, Charismiadou, Batzina, Deligeorgis, & Miliou, 2014). Finally, Wendelaar Bonga (1997) reported that stress responses in fish concern complex mechanisms involving stimulation of oxygen uptake and transfer, mobilization of energy substrates, reallocation of energy away from growth and reproduction, and mainly suppressive effects on immune functions. These stress responses may in many ways resemble inflammatory processes.

Eicosanoids and other lipid metabolites derived from enzymatic and non enzymatic oxidation of polyunsaturated fatty acids (PUFAs) have been employed as markers of lipid stress conditions. It has been well documented that some of these compounds can play an important role in immune and inflammatory responses in fish (Rowley, Knight, Lloyd-Evans, Holland, & Vickers, 1995; Rowley et al., 2012). Particularly, 4-series leukotrienes (LT), lipoxine (LX), 12-hydroxy-eicosatetraenoic acid (12-HETE), and 12-hydroxy-eicosapentaenoic acid (12-HEPE) generated through the action of lipoxygenases (LOX) were found to be produced *in vitro* by head kidney leukocytes extracted from rainbow trout under biological stimuli (Knight, Lloyd-Evans, Rowley, & Barrow, 1993; Rowley et al., 2012). Moreover, cyclooxygenase (COX) products, such as 2-series prostaglandins (PG) and tromboxanes (TX), were found in leucocytes (Knight et al., 1993) and thrombocytes (Lloyd-Evans et al., 1994) of rainbow trout underlining the role of these lipids in immunomodulation and other pro-inflammatory responses.

Recently, some authors have suggested a connection between stress conditions and the production of eicosanoids in different tissues of fish. Oxley, Jolly, Eide, Jordal, Svardal, and Olsen (2010) studied the effect of including different levels of plant sources in diet and acute stress *pre-mortem* on eicosanoids production and COX activity. In the same way, Olsen, Svardal, Eide, and Wargelius (2012) monitored cyclooxygenase activity in different tissues (midgut and hindgut segments, gills, liver, head kidney and white muscle) of Atlantic salmon subjected to acute stress. Results highlighted that stress had a profound effect on the intestinal eicosanoid content,

inducing a temporal increase in the expression of cox2a in tissues. Moreover, Chung, Lee, and Lee (2013) assessed changes of the production of oxidized lipid products in marine fish (*Oryzias latipes*) after acute exposure to H_2O_2. The consequences of the increase of the enzymatic activity and the production of these lipid metabolites on final fish quality are still uncertain.

Despite many authors having studied COX and LOX activities toward the production of arachidonic (ARA, 20:4ω6) and eicosapentaenoic (EPA, 22:5ω3) acids-derived eicosanoids (Furne, Holen, Araujo, Lie, & Moren, 2013; Rowley et al., 1995), in the last decade lipid products derived from oxidation of docosahexaenoic acid (DHA, 22:6ω3) have been also identified. Hong, Tjonahen, Morgan, Lu, Serhan, and Rowley (2005) have reported the presence of strong anti-inflammatory compounds derived from DHA, the di- and tri-hydroxy-containing bioactive products like neuroprotectin D1(PD1), resolvin D5 (RvD5), resolvin D1 (RvD1) and resolvin D2 (RvD2) in rainbow trout brain cells cultures. The 14*S*-hydroxy-docosanoids acid and 17*S*-hydroxy-docosanoids acid from DHA, signatures of DHA conversion by lipoxygenases, were also identified.

This work is aimed to compare the effects of two different slaughter methods (asphyxia and percussion) on the production of lipid oxygenated mediators in plasma of farmed rainbow trout and therefore, on the shelf life of the resulting trout fillets during postmortem storage. For such scope, lipid metabolites were identified and quantified using a SPE-LC MS/MS methodology and correlated with the stress during slaughtering. As a final goal, the effect of the killing method on the oxidative stability was determined in a frozen storage experiment (-10 °C) by monitoring the rate of oxidation in trout fillets at different storage times. The study was completed with measurements of lipid composition in plasma and muscle of rainbow trout and the occurring degradation of nucleotides for the assessment of stress conditions during slaughter.

2. Materials and methods

2.1 Preparation of fish samples and storage conditions

Rainbow trout (*Oncorhynchus mykiss*) were obtain from a fish farm located in San Michele all'Adige (Trento, Trentino Alto Adige, Italy) where they have been fed with a commercial feed (42% crude protein, 22% crude fat; Veronesi S.p.A., Verona, Italy) until 1127 ± 258 g. Fourteen fish were killed by percussion as control group, and other 14 were exposed to asphyxia in air until death. These two killing methods were chosen

among the commonly utilized methods reviewed by EFSA (2009) because they resulted in the highest (percussion) and poorest (asphyxia) animal welfare. Immediately after death, all the fish were submitted to extraction of blood from the caudal vein and the samples were collected in heparinized syringes containing 1 mM TRIS buffer, and 30 Units of sodium heparine, as anticoagulant, for each mL of final solution. Then fish were gutted and filleted, and the fillets were frozen at -80 °C. Plasma and fillets were sent to the CSIC (Consejo Superior de Investigaciones Científica) of Vigo (Spain) where samples were analyzed both for oxidative stress and oxidative stability. Firstly, plasma and muscle were characterized for fatty acid profiles (FAs). Then, ATP content of muscle and eicosanoids of plasma were evaluated as stress markers. Finally, shelf life of trout fillets was assessed by sensory analysis and the measures of secondary oxidation products of muscle, expressed as malondialdheyde (TBA-test), were monitored for 165 days maintaining samples frozen (-10°C) in order to observe the oxidative stability of fillets.

The experiment was approved according to "The regulations in Animal Experimentation" in the Department of Agri-Food Production and Environmental Sciences, University of Florence and conducted by certified personnel.

2.2 Chemical analyses

2.2.1 Lipid content and extraction

Lipids were extracted from fish muscle and plasma according to the method of Bligh and Dyer (1959) and quantified gravimetrically. The extracted lipids were used for the analysis of fatty acid profiles.

2.2.2 Fatty acid profiles of muscle and plasma

Fatty acids were methylated according to the method of Lepage and Roy (1986) and nonadecanoic acid (C19:0) was used as an internal standard. The fatty acid methyl esters (FAMEs) were analyzed by GC according to Christie (1982).

2.2.3 Nucleotides

ATP and derived nucleotides were determined as proposed by Özogul, Taylor, Quantick, and Özogul (2000). Briefly, 1.5 g of muscle were homogenised with 10 mL of perchloric

acid 6% (PCA, Sigma-Aldrich, St. Luis, MO, USA). After a centrifugation at 3200×g at 4 °C for 10 minutes (Allegra® X-12R, Beckman Coulter Inc., Brea, CA, USA) and holding at -20°C for 20 min the samples were filtered using Filter-Lab® 100 mm filter paper (Filtros Anoia, S.A., Barcelona, Spain). The extract was adjusted to pH 6.8-7 using 0.6 and 0.1 M potassium hydroxide (Sigma-Aldrich), filtered and finally it was brought to 25 mL volume with 50 mM phosphate buffer (pH 7). Before HPLC injection, 100 µL of sample were filtered using a 13 mm GHP 0.2 µm filter (Waters, Milford, MA, USA).

HPLC analyses were made using an Alliance® HPLC Model 2695 (Waters, Milford, MA, USA) apparatus, equipped with a photodiode array detector model 2996 (Waters) and a multi λ fluorescence detector (Waters). The column was a ZORBAX Eclipse XDB-C8, 4.60 x 75 mm, particle diameter 3.5 µm (Agilent, Santa Clara, CA, USA), used at a temperature of 35 °C.
Nucleotide standards [adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (Ino), hypoxanthine (Hx)], and tetrabutylammonium bromide were purchased from Sigma-Aldrich Chemical Company (Poole, Dorset, UK). Acetonitrile was purchased from Merck KGaA (Darmstadt, Germany) and HPLC grade water was obtained from Scharlab S.L. (Sentmenat, Spain). Separation was performed in continuous gradient elution using two mobile phases. Phase A was 50 mM phosphate buffer / 10 mM tetrabutylammonium bromide dissolved in HPLC grade water and adjusted to pH 7 with 0.1 M potassium hydroxide. The solution was prepared daily and filtered through a 0.2 µm 47 mm GHP membrane (Waters). Phase B was acetonitrile. The injection volume was 10 µL and detection was monitored at 254 nm. The total separation time was 12 min with a rate flux of 1 mL/min. The results were expressed as mM nucleotides / g muscle, and they were used to calculate K-value = [(Hx + Ino)/(Hx + Ino + IMP + AMP + ADP + ATP)] * 100 (Karube, Matsuoka, Suzuki, Watanabe, & Toyama, 1984).

2.2.4 Eicosanoids and docosanoids from plasma

PUFA derivatives together with free ARA, EPA, and DHA were quantified according to the methodology previously developed by Dasilva, Pazos, Gallardo, Rodríguez, Cela, and Medina (2014) using SPE extraction prior to LC-MS/MS analysis. Briefly, plasma samples (300 µL) were diluted with 30% cold methanol (v/v), to a final volume of 1.2 mL. The internal standard 11-HETE-d8 was added to each sample. The spiked samples were incubated on ice during 10 min and then centrifuged at 3200 x g for 10 min, at 4 ºC, to remove any precipitated proteins which might cause interferences. The clear supernatant and washes of the resultant pellet with 30% methanol were collected in

amber glass vials and subjected to SPE on Oasis-HLB cartridges (60 mg, 3mL; Waters, Milford, MA, USA). After that, extracts were evaporated to dryness under a fine stream of nitrogen; the residue was dissolved in 100 µL ethanol and analyzed by LC/MS/MS in a Waters C18-Symmetry column, 150×2.1 mm, 3.5 µm (Milford, MA, USA) using a binary eluent system of water (A) and methanol (B), both with 0.02% (v/v) of formic acid, as mobile phases. The flow rate was set at 0.2 mL/min; the column effluent was directly introduced in the ESI without splitting, and injection volume was set to 10 µL. Operating conditions of the ESI source were negative ion mode with a gas flow rate of 40 units, spray voltage of 5.5 kV, capillary temperature of 300 ºC and S-lens radio-frequency level of 60%. The quantification of target compounds was made using the most intense, or selective, transition for each analyte and identification was helped comparing the MS/MS spectra, recorded in the range from 90 to 400 m/z units.

2.2.5 Sensory Analysis

Sensory detection of rancid odors was evaluated by an expert panel formed by four trained specialists in descriptive analysis of marine off-flavors. The determination was performed in a room designed for such purpose, after the samples had been held during 10 min at room temperature. Approximately 10 g were placed in separate sterile polystyrene Petri dishes and put on a tray of ice. The panelist detected the rancidity/painty odors using a structured scale, from 8 (absolutely fresh) to 1 (putrid).

2.2.6 TBARS

The 2-thiobarbituric acid reactive substances (TBARS) were measured according to Vyncke (1970). The results were expressed as mg of malondialdehyde (MDA) equivalents/kg sample using a calibration curve determined with eight standard solutions of TEP (1,1,3,3,-Tetraethoxypropane) at concentration ranging from 0.2 to 3.1 µM.

2.3 Statistical analysis

Data were analysed using the General Linear Model procedures of the statistical analysis software SAS 9.1 (2004) for Windows. A one-way ANOVA tested the stunning method as fixed effect.

3. Results and discussion

3.1. ATP degradation

In order to test the occurrence of stress during slaughter, degradation of ATP and the formation of its corresponding catabolites were investigated in muscle. The concentration (μmol nucleotides / g muscle) of ADP, AMP, IMP, Ino, and Hx in fillets of farmed trout killed with asphyxia or percussion is shown in Table 1.

Samples taken soon after slaughter contained only low concentrations, if any, of ADP, and traces of AMP while ATP level was below the detection limit (ND) for both sample groups. These findings are in agreement with the fact that stress associated with slaughter of the fish leads to a rapid loss of ATP and initiation of the degradation sequence (Tejada, 2009).

The rapid conversion of ATP to its further catabolites was confirmed by the high amounts of IMP, and Ino. IMP was the most abundant nucleotide in both killing groups followed by Ino. Despite no significant differences being found, IMP content was lower in trout killed by asphyxia (5.4 μmol/g muscle) when compared with those killed by percussion (6.1 μmol/g muscle), while the opposite trend was reported for the final degradation products (Ino and Hx). These data revealed the possible influence of the killing procedures on stress, suggesting a major effect on asphyxiated fish.

Wills, Zampacavallo, Poli, Proctor, and Henehan (2006), evaluating the effect of different slaughter methods on nucleotide contents, found an IMP concentration of 10 and 4 μmol/g muscle for air asphyxiated and percussion stunned rainbow trout, respectively. The IMP level obtained in this work is higher than 5.4 μmol/g muscle. However, that difference might be explained with the global nucleotides profile. In the present study Ino and Hx were also reported in abundance compared with the data of Wills et al. (2006) who had low levels for almost all nucleotides, with a very small amount of Ino and Hx. Although the degradation of ATP up to IMP is very fast, the degradation of IMP is relatively slow (Tejada, 2009) so that differences between Ino and Hx concentration might be attributed to a higher muscular activity due to stress during slaughter.

In order to confirm that percussive stunning method was less stressful than asphyxia, K-value (Karube et al., 1984) was calculated as index of tissue stress. Results in Table 1 show a significant higher K-value ($p < 0.05$) for the asphyxia group (29 %) than for the percussion one (18 %), demonstrating that asphyxia induced more stress in fish.

3.2 Muscle and plasma fatty acids

All fish used in this study showed a muscle lipid content around to 5 %; the total fatty acid (TFA) composition is reported in Table 2. As expected, no statistical differences

were found in the lipid content between fish subjected to two killing methods, and only few statistical differences were found in the fatty acid profile of muscle. Regarding the TFA composition of plasma, no significant differences were found in its main constituents between the asphyxia and percussion groups (Table 2).

PUFA represented the majority of total fatty acids in muscle, composed primarily by ω6 and by a smaller amount of ω3. Although a certain caution is required in the comparison of the results with previous studies, since the FA composition depends considerably on diets (Baron, Svendsen, Lund, JokuSEMn, Nielsen, & Jacobsen, 2013), the presence of vegetable sources in the feed for the farmed rainbow trout of the present trial is reflected mainly by the percentage of linoleic acid (18:2ω6) that reached up 27 % of TFA. Linoleic acid is the precursor for the synthesis of arachidonic acid (ARA, 20:4ω6) which was the third major component of the ω6 fraction, providing 1% of TFAs in muscle.

Despite the abundance of the ω6 fraction, the PUFA composition (reported in Table 2) affected the ω6/ω3 ratio that was 1.3, about 3 times lower than the maximum value (4.0) recommended by UK Department of Health (Committee on Medical Aspect of Food Policy, 1994).

As regards to long chain ω3 PUFAs, precursors of eicosanoids and other lipid mediators, the major contributors were docosahexaenoic (DHA, 22:6ω3), eicosapentaenoic (EPA, 20:5ω3), and α-linolenic (ALA, 18:3ω3) acids. Their content agreed with the values proposed for farmed rainbow trout fed commercial feeds (Baron et al., 2013; Blanchet, Lucas, Julien, Morin, Gingras, & Dewailly, 2005; Haliloğlu, Bayır, Necdet Sirkecioğlu, Mevlüt Aras, & Atamanalp, 2004) containing both fish meal and fish oil. No differences were found between asphyxia and percussion groups.

Regarding the lipid composition of plasma, the total SFAs were found to be around 25 %, being mainly composed by palmitic, stearic, and myristic acids. Among MUFAs, the most abundant were oleic and palmitoleic acids. Total PUFAaccounted for 58.5 % of TFA of which 41 % were ω3 PUFAs and 17.7 % belonged to ω6 series. The most representative long chain fatty acid was DHA, followed by linoleic acid, and EPA.

3.3 Lipid mediators

Table 3 summarizes the plasma levels of the free fatty acids (ARA, EPA, and DHA) which are the main precursors of eicosanoids and docosanoids. The relative levels of these plasma circulating free fatty acids (FFA) showed that DHA was the preponderant

free fatty acid in plasma, followed by EPA and ARA in both groups of fish. The concentration order (DHA>EPA>ARA) was in agreement with the TFA composition found in plasma. Despite no significant differences being found between methods of slaughtering in the concentration of these fatty acids, the results showed a tendency to lower free ARA, EPA, and DHA concentrations in asphyxiated trout than in percussion-slaughtered one. The percentage of decrease was the same for the three fatty acids (around 13 %).

The lipid mediator profile of plasma in percussion-slaughtered and asphyxiated rainbow trout presented different molecules derived from ARA, EPA, and DHA (Table 3). The oxidized products detected were: isoprostane $F_{2\alpha}$ (8-isoPGF$_{2\alpha}$), prostaglandin E_2 (PGE_2), ±11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid (11-HETE), and leukotriene B_4 (LTB_4) (from ARA); prostaglandin D_3/prostaglandin E_3 (PGD_3/PGE_3), 12(S)-hydroperoxy-5Z-,8Z,10E,14Z,17Z-eicosapentaenoic acid and 15(S)-hydroperoxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid (12-HpEPE/15-HpEPE), ±5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid (5-HEPE), ±12- hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid (12-HEPE), and ±15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid (15-HEPE) (from EPA); and ±4-hydroxy-5E,7Z,10- Z,13Z,16Z,19Z-docosahexaenoic acid (4-HDoHE), ±11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-docosahexaenoic acid (11-HDoHE), ±17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid (17-HDoHE) (from DHA). Finally, isoprostane $F_{3\alpha}$ (8-isoPGF$_{3\alpha}$), thromboxan B_3 (TXB_3) (from ARA), 17(S)-hydroperoxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid (17-HpDoHE), resolvin D1 (RvD1), and neuroprotectin D1 (PD1) (from DHA) were searched but not detected.

Considering the percussion-slaughtered as the group with less stress induced during slaughter, the results indicated that 17-HDoHE, resulting from LOX activity on DHA, appeared to be the main lipid metabolite in plasma followed by the EPA resulting product, 12-HEPE. Other products also derived from the action of LOX towards DHA, as 4-HDoHE and 11-HDoHE, were present in significant concentrations. 5-HEPE derived from EPA was also detected reaching higher concentrations than 15-HEPE. Finally, low amounts of LTB_4 were also found. Hydroperoxides from EPA and DHA were below the detection limit and RvD1 and PD1 were not detected.

Concerning the COX derived products, levels of PGE_2, 8-isoPGF$_{2\alpha}$, 11-HETE, PGD_3/PGE_3, 8-isoPGF$_3$, and TXB_3 were investigated. PGE_2 was the main eicosanoid followed by 11-HETE; however, the latter was two times lower than PGE_2. The content of 8-isoPGF$_{2\alpha}$ was found only on the order of 10^{-6} ng/ mL, while no traces of PGD_3/PGE_3, 8-isoPGF$_{3\alpha}$, nor TXB_3 were detected in the percussion group. Under these conditions, the overall lipid mediator profile of percussion-slaughtered trout was the following 17-HDoHE >

12-HEPE > 4-HDoHE > 5-HEPE > 11-HDoHE > PGE_2 > 15-HEPE > 11-HETE > LTB_4 > 8-iso$PGF_{2\alpha}$.

Regarding the profile of asphyxiated rainbow trout, Table 3 reveals that the main difference between both methods of slaughtering was the high amount of hydroperoxides, 12-HpEPE/15-HpEPE, derived from 12 and 15 LOX activity on EPA. They were found at a concentration level three orders of magnitude higher than the other lipid metabolites. These compounds were not detected in the percussion trout. Other differences between the two methods were the lower levels of 5-HEPE, 4-HDoHE and 17-HDoHE detected in the asphyxiated trout compared with the percussion-slaughtered ones. The LOX oxidation product 12-HEPE was the second main compound found in plasma of asphyxiated trout at similar concentrations to those previously mentioned in the percussion group and 11-HDoHE, 15-HEPE, and LTB_4 were found as well. Neither RvD1 nor PD1 were detected in the asphyxiated group.

According to the profile of the percussion group, PGE_2 was the main COX product followed by 11-HETE in the asphyxiated group; 8-iso$PGF_{2\alpha}$ was also detected whereas no traces of 8-iso$PGF_{3\alpha}$ nor TXB_3 appeared. Interestingly, PGD_3/PGE_3 were only detected in this group and they were not found in the percussion trout. It must be also noticed that the levels of PGE_2 found in the asphyxiated group were lower than those found in the percussion trout. Under this condition, the overall profile of the asphyxiated trout was 12-HEPE >> 17-HDoHE > 11-HDoHE > 4-HDoHE > 5-HEPE > PGE_2 > 15-HEPE > 11-HETE > LTB_4 > PGD_3/PGE_3 > 8-iso$PGF_{2\alpha}$.

Therefore, the comparison between asphyxia and percussion groups revealed different trends for oxidized products. 12-HpEPE/15-HpEPE and PGD_3/PGE_3 were detected only in the asphyxiated trout whilst they were not found in the percussion group. Lower levels of 17-HDoHE, 5-HEPE, 4-HDoHE, 15-HEPE, PGE_2, and 11-HETE were reported for asphyxia group than percussion, even though statistical differences ($p < 0.05$) were found only for 5-HEPE, 17-HDoHE, and PGE_2.

Previous studies on fish subjected to stressing conditions occurring during confinement have investigated COX derivates products, such as prostaglandins, as biomarkers, although no clear trends for PG synthesis in response to stress could be uniquely discerned (Olsen et al., 2012; Oxley et al., 2010). According to Balvers et al. (2012) prostaglandins levels in plasma under stress stimuli were down-regulated. The results for PG found in the present study confirmed that stress condition at slaughter decreased PGE_2 levels. In contrast, Olsen et al. (2012) reported that only the isoform 2a of COX clearly responds to stress with an upregulation in the main tissues of Atlantic salmon (*Salmo salar*), whilst the other two (COX1 and COX2b) remain unaltered. That

finding supports the results obtained for PGD_3/PGE_3, derived from EPA, which appeared only in the asphyxia group, revealing a possible increase of COX activity on EPA in response to stress. This opposite trend between PGE_2 (from ARA) and PGD_3/PGE_3 (from EPA) may reflect that stressing killing methods might shift the COX activity towards EPA-derived species which are considered less inflammatory (Tocher, 2003), in order to minimize the impact of oxidative stress.

The present study also revealed that 12-HpEPE/15-HpEPE could be considered as good markers of stress because they were highly produced under stress conditions while they were not detected in the percussion group. Hydroperoxide intermediates have been suggested to be involved in the biosynthesis of some eicosanoids in fish (Rowley, Lloyd-Evans, Barrow, & Serhan 1994), and the identification of HpEPE found in this work demonstrated such hypothesis. Hydroperoxy derivatives are the primary products of LOX that are easily reduced to hydroxydes by glutathione peroxidase (GPX) (Guichardant et al., 2011). This enzyme has been well characterized in fish during the past few years in order to associate its expression with stress during life. In particular, increasing stress time was associated with a down-regulation of GPX (Malandrakis, Exadactylos, Dadali, Golomazou, Klaoudatos, & Panagiotaki, 2014). An increase of the activity of LOX, together with an inhibition of GPX activity caused by stress during slaughter, might explain the notable presence of HpEPE in the asphyxiated fish and the absence in the percussion-slaughtered ones. Moreover, the same trend in enzyme modulation seems to be confirmed by the significant decrease in other lipid mediators such as 5-HEPE and 17-HDoHE, from EPA and DHA, respectively, in the asphyxiated rainbow trout.

More recently, DHA was found to be a fairly good substrate of LOX in humans to produce various hydroxylated end-products after reduction of the hydroperoxide intermediates by GPX. They are 4-HDoHE, 7-HDoHE, and 11-HDoHE (Lagarde, Bernoud-Hubac, Calzada, Véricel, & Guichardant, 2013) and all of them were found in the present trial in plasma of rainbow trout. In addition to this, those derivatives of DHA have been described as precursors of a bioactive family, called resolvins and protectins, a class of compounds with active anti-inflammatory and inflammation resolving properties in mammals (Masoodi, Mir, Petasis, Serhan, & Nicolaou, 2008). In the present study, RvD1 and PD1 were sought in plasma but they were not detected. Although in the present study neither resolvins nor protectins were detected, the presence of their precursors, 4-HDoHE, 7-HDoHE, and 11-HDoHE provided evidence of LOX activity on DHA in fish plasma. Moreover, the decreasing trend of their levels caused by the most stressful slaughter method may suggest the degradation of 4-HDoHE, 7-HDoHE, and 11-HDoHE in order to reduce stress conditions.

Therefore, results for both LOX and COX products revealed that the method of slaughtering seems to affect the oxidative response in plasma of rainbow trout in a complex way. The present findings revealed that 12-HpEPE/15-HpEPE acids and PGE_3/PGD_3 could be considered as good stress biomarkers due to their presence in the asphyxia group but not in the percussion one. Moreover, that trend together with the reduction of PGE_2 level supported the hypothesis that slaughter stress condition shifted lipid mediator synthesis towards less inflammatory species derived from EPA. At the same time, the general decrease of secondary products derived from both EPA and DHA might be due to an inhibition of GPX activity or to a later degradation in more oxidized forms (Rowley et al., 1995). It is also important to take into consideration that increasing hydroperoxide concentrations could promote oxidative processes inducing loss of fish quality.

3.4 Oxidative stability of fillets

The formation of oxidation by-products measured by TBARS level in fillets from asphyxiated and percussion-slaughtered trout is summarized in Fig. 1. Fillets belonging to both groups kept low values during the first 75 days of storage at -10 ºC. After that, fillets of the asphyxiated trout showed an increment of their TBARs values, raising from 0.27 up to 1.67 mg MDA equivalents/ kg muscle, with the maximum level of 1.98 mg MDA equivalents/ kg muscle at 135 days. In contrast, fillets of the percussion-slaughtered trout show a smaller increase after 105 days of storage, increasing from 0.29 to 0.52 mg MDA equivalents/ kg muscle. As for the asphyxiated rainbow trout, the highest level of oxidation was reached after 135 days (0.69 mg MDA equivalents/ kg muscle).

Interestingly, difference between the two killing methods emerged after 75 days of storage, when TBARS for the asphyxiated fish started to increase. As shown in Fig. 1, asphyxiated rainbow trout started to be oxidized one month before the other group, showing a noticeably tendency to a faster increase in rancidity than the percussion-slaughtered group. In addition to this, significant differences ($p < 0.05$) between killing methods were found at 135 and 165 days. In both cases, the asphyxiated trout was found to be more oxidized than the other. These results were in agreement with sensory analysis. Asphyxiated fillets showed a slight rancid off-flavor by the 105^{th} day of frozen storage and significant rancidity by the 135^{th} day. Instead, a slight rancid odor was not detected for the whole storage period in the fillets from the percussion group
.

That trend seems to reveal that stressful killing method influenced oxidative stress during frozen storage, both reducing the length of induction phase and increasing the rate of lipid oxidation. Finally, since the interaction between peroxides and lipid oxidation is well known, it could be supposed that the higher level of hydroperoxide levels found in the asphyxiated rainbow trout might have negatively affected the flesh oxidative stability, as demonstrated by the highest level of lipid oxidation reached.

4 Conclusion

The present trial revealed that the killing method affected both the formation of lipid oxygenated metabolites and the oxidative stability of farmed rainbow trout. As a consequence of the greater enzymatic activity under stress conditions, the resulting higher concentration of hydroperoxides seemed to affect the oxidative stability of the asphyxiated rainbow trout flesh during the frozen storage, leading as result to a decrease of the shelf life of frozen trout fillets in terms of rancidity. Noticeably, 12-HpEPE/15-HpEPE and PGE_3/PGD_3, EPA-derived metabolites, could be considered as good markers of stress because they were highly produced under stress conditions (asphyxia) while they were not detected in the percussion group. Therefore, the present investigation indicated that slaughter method can largely affect the concentration of lipid oxygenated products and then the development of oxidation during post-mortem storage. This in turn could reduce their commercial shelf-life, due to a higher susceptibility to develop rancidity.

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Table 1. ATP and related catabolites mean concentration (µmol nucleotides/ g muscle) in fillets of trout slaughtered by asphyxia or percussion.

	Asphyxia	Percussion	Significance	rsd[1]
ATP	N.D.[2]	N.D.[2]	-	-
ADP	0.259	0.219	0.397	0.06
AMP	0.091	0.098	0.805	0.03
IMP	5.411	6.109	0.694	2.38
Ino	2.021[b]	1.153[a]	0.036	0.45
Hx	0.327	0.200	0.174	0.12
K-value	29.21[b]	18.72[a]	0.049	6.04

Lowercase superscript letters indicate statistically significant differences ($p < 0.05$).

[1]rsd: residual standard deviation.

[2]N.D.: not detected.

Table 2. Total lipids of muscle and fatty acid composition (in % of total fatty acids) of muscle and plasma of trout slaughtered by asphyxia or percussion.

	Muscle				Plasma			
	Asphyxia	Percussion	Significance	rsd[1]	Asphyxia	Percussion	Significance	rsd[1]
Total lipids, %	4.81	4.88	0.847	0.416				
14:0	2.15	2.08	-	0.097	1.00	1.05	0.577	0.111
16:0	14.01	13.81	0.740	0.70	18.21	18.06	0.880	1.343
16:1ω7	2.78	2.72	0.687	0.185	1.24	1.20	0.782	0.193
18:0	3.86	3.84	0.931	0.227	5.85	5.30	0.705	1.971
18:1ω9	18.34	18.21	0.854	0.824	10.18	10.25	0.891	0.692
18:1ω7	2.43[a]	2.52[b]	0.040	0.037	1.64	1.64	0.995	0.446
18:2ω6	27.78	27.24	0.723	1.755	11.59	11.56	0.977	1.241
18:3ω3	2.82	2.72	0.477	0.145	0.90	0.74	0.259	0.183
20:2ω6	1.20	1.32	0.091	0.064	1.79	1.82	0.951	0.659
20:3ω6	0.78	0.80	0.750	0.082	1.60	1.76	0.515	0.344
20:4ω6 (ARA)	0.97	1.12	0.169	0.108	2.07[a]	2.46[b]	0.014	0.159
20:4ω3	0.72[b]	0.55[a]	0.045	0.069	0.43	0.28	0.448	0.257
20:5ω3 (EPA)	2.96	3.08	0.520	0.202	7.62	7.78	0.508	0.320
22:5ω3	1.54	1.45	0.137	0.057	2.09	2.01	0.783	0.390
22:6ω (DHA)	14.23	15.07	0.621	1.907	29.60	29.90	0.917	3.959
ΣSFA	20.98	20.66	0.682	0.896	26.19	25.53	0.416	1.068
ΣMUFA	25.51	25.37	0.889	1.153	15.72	15.85	0.887	1.209
ΣPUFA	53.50	53.96	0.652	1.165	58.01	58.63	0.679	1.995
ω3	22.62	23.29	0.721	2.116	40.86	41.01	0.958	3.649
ω6	30.88	30.67	0.887	1.624	17.15	17.62	0.729	1.835
DHA/EPA	4.91	4.75	0.600	0.336	3.89	3.83	0.868	0.484
ω6/ω3	1.31	1.38	0.619	0.162	0.42	0.44	0.834	0.081
DHA/ALA	0.55	0.53	0.779	0.081	2.58	2.64	0.889	0.606

Lowercase superscript letters indicate statistically significant differences ($p < 0.05$).

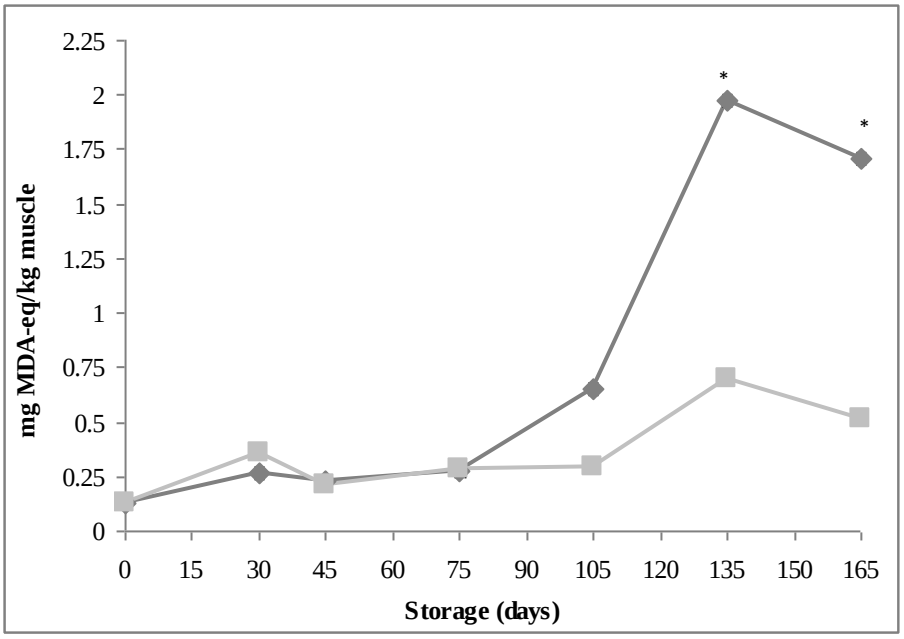
[1]rsd: residual standard deviation.

Table 3. Concentration levels of FFAs (DHA, EPA, and ARA), and lipid mediators in plasma of trout slaughtered by asphyxia or percussion. Results are expressed as µg/ mL of plasma for FFAs and 12-HpEPE/15-HpEPE, as ng/ mL plasma for other lipid mediators.

	Asphyxia	Percussion	Significance	rsd[1]
DHA	1.73	1.96	0.605	0.594
EPA	0.70	0.82	0.421	0.192
ARA	0.25	0.29	0.713	0.136
8-isoPGF$_{2\alpha}$	1.36E- 6	1.16E-6	0.235	2.14E-7
PGE$_2$	2.52[a]	2.84[b]	0.002	0.091
11-HETE	0.76	0.86	0.303	0.123
LTB$_4$	0.24	0.23	0.956	0.247
PGD$_3$/PGE$_3$	0.13	ND	0.136	0.112
12-HpEPE/15-HpEPE	0.10	ND	0.134	0.086
5-HEPE	3.92[a]	6.27[b]	0.049	1.350
12-HEPE	12.60	12.23	0.344	0.500
15-HEPE	1.25	1.60	0.092	0.243
4-HDoHE	4.98	6.58	0.242	1.740
11-HDoHE	5.48	5.98	0.115	0.378
17-HDoHE	8.19[a]	17.58[b]	0.040	5.094
8-isoPGF$_{3\alpha}$	ND	ND	-	-
TBX$_3$	ND	ND	-	-
17-HpDoHE	ND	ND	-	-
RvD1	ND	ND	-	-
PD1	ND	ND	-	-

Lowercase superscript letters indicate statistically significant differences ($p < 0.05$).
[1]rsd: residual standard deviation.

Fig. 1 Secondary lipid oxidation products during 165 days of frozen storage (- 10 °C), expressed as mg MDA-equivalents/ kg muscle, in fillet of trout slaughtered by asphyxia (black line) or percussion (gray line).



*: significant difference ($p<0.05$) between the two groups at the specific time.

PAPER III

Carbon monoxide as stunning/killing method on farmed Atlantic salmon (*Salmo salar*): effects on lipid and cholesterol oxidation

RUNNING TITLE Effect of carbon monoxide on lipid oxidative stability

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ABSTRACT

BACKGROUND: Carbon monoxide (CO) has been recently utilized as a new stunning/killing procedure for Atlantic salmon (*Salmo salar*). Its effect on lipid and cholesterol oxidation of farmed A. salmon fillets were evaluated at two times of refrigerated (2.5°C) storage, T0 (64h after death) and T14 (14 days from T0). The use of CO was compared with the commonly utilized percussive method (P).

RESULTS: Fatty acid profile, primary (conjugated dienes) and secondary oxidation products (TBARS), cholesterol oxidation products (COPs) and carotenoids were unaffected by killing method. Despite the low oxidative status of lipid (0.66 and 0.60 mg malondialdehyde kg^{-1} muscle in P and CO, respectively), cholesterol was found highly oxidized (0.17 and 0.13 mg COPs kg^{-1}). Storage significantly affected oxidative stability of fish muscle by increasing oxidation products. Interestingly, TBARS content doubled while the increase for COPs was not homogeneous: α- and β-epoxycholesterol increased by 25%, whereas triol and 7-ketocholesterol increased by 48 and 62%, respectively.

CONCLUSION: The quality of salmon fillets just after slaughtering and after 14 days of refrigerated storage at 2.5°C did not change, irrespective to the killing method adopted, suggesting that CO method may be applied without any detrimental effect on the quality of fish fillets.

Keywords

Carbon monoxide, killing, Atlantic salmon, TBARS, COPs.

INTRODUCTION

The interest in the consequences of stress at slaughter on animal welfare and post-mortem biochemical processes have been largely investigated during the last years.[1-3] In many fish species, slaughter stress was shown to greatly influence both welfare[4] and fillets quality during storage, altering the freshness index K-value,[5] pH, color and water holding capacity.[6,7] However, the influence of slaughter conditions on oxidative stability of fillets during storage is still unclear.

Recently, there has been some focus on the use of carbon monoxide (CO) as a new stunning/killing procedure for Atlantic salmon (*Salmo salar,* Linnaeus 1758)[8] and as color stabilizer during storage in tilapia fillets (*Oreochromis* spp.).[9] CO binds irreversibly to the oxygen binding sites of hemoglobin, myoglobin and neuroglobin creating hypoxia.[8] The fish do not respond directly to CO gas,[10] and it has the potential to be a sedative with a low stress response. The binding of CO also creates a cherry-red carboxy hemo-/myoglobin complex that increases redness of both the fillets and gills during storage.[11,12] CO binding may also affect oxidative stability of the meats as the heme iron may be prevented from catalyzing lipid oxidation.[9,13] Nevertheless, more studies are required to investigate the effects of CO treatment ***in vivo*** on other quality parameters of fillets, like lipid oxidative stability.

Lipid peroxidation in foods constitutes a complex chain of free radical reactions giving rise to a wide range of molecules with numerous sensorial and biological effects. Oxidation of polyunsaturated fatty acids (PUFA) for instance leads to the production of aldehydes like heptenal which are responsible of an overall off-flavor.[14] Lipid peroxidation will also produce many cholesterol oxidation products (COPs) having cyto-toxic, mutagenic and carcinogenic effects[15,16] in addition to being involved in several chronic diseases like atherosclerosis, diabetes and kidney failure.[17]

The aim of the present study was to assess whether stunning/killing of A. salmon with CO affects fillet nutritional quality or lipid peroxidation during storage compared to fish stunned/killed by percussion. Analyses were carried out after 64 hours and 14 days post slaughter in fillets stored at 2.5°C.

MATERIALS AND METHODS

Preparation of fish samples and storage conditions

The experiments was carried out at the Institute of Marine Research (Matre, Norway). A. salmon (weight: 1.104 ± 0.125 kg) were kept in two tanks containing aerated full strength seawater at 10°C. Fish in tank 1 were utilized as control and killed by percussion (P). Fish in tank 2 were flushed with 100% food grade carbon monoxide (CO) (Yara Praxair, Oslo, Norway), using a ceramic diffuser (wedge lock base unit, Point Four Systems Inc., Richmond, Canada) for 20 minutes at 2-3 bar until swimming ceased. They were then killed by percussion. Fifteen fish from each tank were examined for the present study. Immediately after death, the fish were transferred into polystyrene boxes and covered with ice. After *rigor* resolution at 64h (T0) fish belonging to both

groups were filleted and the right fillets analyzed for proximate composition, oxidation profile and carotenoid content. The same parameters were analyzed on the left fillets of CO and P groups after 14 days (T14) of refrigerated storage at 2.5°C. In both cases, the cranial region (10 cm) were utilized.
The experiment was approved according to "The regulations in Animal Experimentation" in Norway and conducted by certified personnel.

Proximate composition

Moisture, crude protein (Nx6.25), and ash contents were determined using 950.46, 976.05, and 920.153 AOAC[18] methods, respectively. For lipid analysis, a sample of fish muscle (approximately 10 g) was ground and extracted using chloroform and methanol according to Folch et al.[19] Total lipids were measured gravimetrically. The lipid extract was then utilized for the analysis of fatty acid profiles, cholesterol, COPs, vitamin A (Vit. A) and carotenoids.

Fatty acids

Fatty acids (FA) in lipid extract were trans-esterified to methyl esters (FAME) using a base-catalyzed trans-esterification.[20] The FA composition was determined by gas-chromatography (GC), using a Varian GC 430 gas chromatograph equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 capillary column (30 m × 0.32 mm i.d., 0.25 µm film and polyethylene glycol bonded phase; Supelco, Bellefonte, PA, USA), purchased from Agilent (Palo Alto, CA, USA). The oven temperature was held at 100°C for 2 min, increased to 160°C over 4 min at the rate of 12°C min^{-1}, and then increased to 220°C over 14 min at the rate of 3°C min^{-1} and kept at 220°C for 25 min. The injector and the detector temperatures were set at 220°C and 300°C, respectively. One µL of sample in hexane was injected into the column with the carrier gas (helium) kept at a constant flow of 1.5 mL min^{-1}. The split ratio was 1:20. Chromatograms were recorded with the Galaxie Chromatography Data System 1.9.302.952 (Agilent, Palo Alto, CA, USA) computing integrator software. Fatty acids were identified with reference to standards (Supelco 37 Comp. FAME Mix, Supelco, Bellefonte, PA, USA). The individual fatty acids were quantified using tricosanoic acid (C23:0) (Supelco, Bellefonte, PA, USA) as internal standard. Fatty acids were expressed as a percentage of total FAME. From the fatty acid profile, the following indexes were calculating:
- Atherogenic index (AI) according to the formula[21]
[C12:0 + (4 × C14:0) + C16:0] / (ΣPUFA n3 + ΣPUFA n6 + ΣMUFA);
-Thrombogenic index (TI), according to the formula
[C14:0 + C16:0 + C18:0] / [0.5 × ΣMUFA) + (0.5 × ΣPUFA n6) + (3 × ΣPUFA n3) + (ΣPUFA n3/ΣPUFA n6)];

- Hypocholesterolaemic/hypercholesterolaemic FA ratio (HH): according to the formula[22] (C18:1 n9 + C18:2 n6 + C20:4 n6 + C18:3 n3 + C20:5 n3 + C22:5 n3 + C22:6 n3) / (C14:0 + C16:0) ;
- Polyene index (PI) as (C20:5 n3 + C22:6 n3) / C16:0;
- n3/n6 ratio which is ΣPUFA n3/ΣPUFA n6.

Lipid oxidation products

Conjugated dienes (CD) content in the lipid extract was measured by a colorimetric method[23] using hexane as solvent. Conjugated dienes were quantified at 233 nm (50 Scan spectrophotometer, Varian equipped with a Cary Win UV Software; Palo Alto, CA, USA) and using a molar extinction coefficient of 25200 (mol L^{-1})$^{-1}$ cm^{-1}. The results are expressed as mol CD kg^{-1} sample.

The 2-thiobarbituric acid reactive substances (TBARS) were measured using the colorimetric method[24] at 532 nm. Briefly, TBARS were extracted in TCA (5%), then added with TBA 0.04 mol L^{-1}. The products were quantified, after 20 min of incubation at 93°C, with reference to calibrations curves of TEP (1,1,3,3,-tetra-ethoxypropane) in 5% (w/v) TCA (0.8 to 8 µmol L^{-1}).

Cholesterol and Cholesterol Oxidized Products (COPs)

The content of cholesterol and COPs in fish fillets was determined in the total lipids.[25] One hundred and fifty µL of dihydrocholesterol in chloroform (2 mg mL^{-1}, by Steraloids, Newport, RI, USA) and 25 µL of a solution of 19-hydroxycholesterol in n-hexane/isopropanol (1 mg mL^{-1}, in 4:1, by Steraloids, Newport, RI, USA) were added to 300 mg of intramuscular lipids as internal standards for cholesterol and COPs, respectively. Firstly, 300 mg of total lipids were dissolved in n-hexane:isopropanol (4:1, v/v) and directly cold saponified. One-tenth of the unsaponifiable matter was utilized for the determination of total cholesterol, whereas the remaining part (9/10) was purified by NH_2-SPE cartridge for COPs purification. Cholesterol and COPs were then silylated,[25] dried under a nitrogen stream and dissolved in n-hexane. Both cholesterol and COPs were identified by GC-FID (GC 2000 plus, Shimadzu, Columbia, MD, USA) equipped with a VF 1-ms apolar capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Varian, Palo Alto, CA, USA). For cholesterol and COPs determination, 2 µL of sample in hexane were injected into the column with the carrier gas (hydrogen) flux at 1 mL min^{-1} and the split ratio was 1:10. The run was carried out in constant pressure mode. The oven temperature was held at 250°C for 1 min, increased to 260°C over 20 min at the rate of 0.5°C min^{-1}, and then increased to 325°C over 13 min at the rate of 5°C min^{-1} and kept at 325°C for 15 min. The injector and the detector temperatures were set at 325°C.[25] The chromatograms were recorded with the LabSolution software (Shimadzu, Columbia, MD, USA). Cholesterol and COPs were calculated by comparing the area of samples and internal standards and expressed as g kg^{-1} of muscle.

Carotenoids and vitamin A content in fillets

The content of carotenoids and vitamin A in fish fillets was determined in the lipid extracts. Lipid extracts were added 0.7 µL apocarotenal as internal standard and then cold saponified.[26] Unsaponifiable matter was resuspended in 200 µL of hexane/isopropanol (4:1) solution. Finally, 20 µL of each sample were quantified using a Prostar HPLC (Varian) equipment with UV-DAD and a C_{18} reverse phase column (ChroSEMp HPLC Columns SS 250mm×4.6mm with ChroSEMp guard column Omnispher 5 C_{18}).[27,28] The mobile phases were (A) methanol:acetonitrile:water (10:70:20), and (B) methanol:ethylacetate (70:30). The flow was 90:10 of mobile phase A and B respectively at 1 mL min^{-1} for 15 min followed by 50:50 (1 mL min^{-1}) for 5 minutes followed by 0:100 at 1.5 mL min^{-1} for 10 min. Carotenoids were detected at 450 nm while vitamin A (Vit. A) was detected at 325 nm and quantified by an external calibration curve, obtained from retinol at concentrations ranging from 0.045 µg mL^{-1} to 7 mg mL^{-1}. Both carotenoids and Vit. A were expressed as µg kg^{-1} muscle.

Statistical analysis

Data related to proximate composition of fillets was submitted to ANOVA by the PROC GLM of the Statistical Analysis System (SAS),[29] where Killing method (K: Percussion, CO), Storage time (S: T0, T14) and the Killing method × Storage time (K × S) interaction were included in the model as fixed effects. The remaining data were analyzed as completely randomized design with repeated measures, using the MIXED procedure of SAS.[29] The model included the fixed effects of the Killing method (K: Percussion, CO), of the Storage time (S: T0, T14) and the Killing method × Storage time (K × S) interaction, while the individual fish was included in the model as random effect nested within the killing method. The covariance structure was compound symmetry, which was selected on the basis of Akaike's information criterion of the mixed model of SAS. Statistical significance of the killing effect was tested against variance of fish nested within killing method according to repeated measures design theory.[30]
Multiple comparisons among means were performed using the Tukey's test and were considered significant for p values <0.05. The coefficients of the residual (after the above model) correlations between the analyzed parameters were also calculated.

RESULTS

Proximate composition and fatty acid profile

Table 1 summarizes the proximate composition of fillets. Killing method seemed to marginally affect only protein ($p<0.01$) and ash content ($p<0.05$), whereas storage time did not have significant effect for any of the parameters considered.
The average lipid content was around 80 g kg^{-1} muscle, irrespective of treatment.

The FA composition of total lipid is given in Table 2. There were no effects of fish slaughtering method. Total saturated FA (SFA) was around 14.6% of total FA in each group, being mainly composed of palmitic acid (C16:0), which accounted for 9%. Monounsaturated FA (MUFA), were dominated by oleic (C18:1 n9), and eicosenoic (C20:1 n9) acids, which combined accounted for 90% of MUFA. The PUFA, amounting

45% of the FA, was primarily composed of n3 FA. Particularly, α-linolenic (C18:3 n3), eicosapentaenoic (C20:5 n3, EPA), and docosahexaenoic (C22:6 n3, DHA) acids accounted for 20% of total FA and for 80% of total PUFA n3. The n6 fraction was dominated by linoleic acid (C18:2 n6) (85% of n6 FA), while arachidonic acid (ARA, C20:4 n6) was found only in small amounts (0.40%, data not shown). Total PUFA and n3 FA contents significantly decreased during storage, in fillets from both P and CO groups.
Finally, no significant differences between slaughtering methods or storage times were found in the case of nutritional indices as n3/n6 ratio, AI, TI, and HH (Table 3).

Lipid oxidation products

The primary and secondary oxidation products CD, TBARS and COPs are reported in Table 4. Slaughter method did not affect theses parameters 64h post slaughter (T=0). However, storage time increased both CD and TBARS ($p<0.05$) in the fish fillets after 14 days of storage. The interaction of storage with killing method was not significant, revealing that CO and P fish had the same oxidation pattern.

Cholesterol content in fillets from P or CO salmons was around 0.4 g kg^{-1} muscle (Table 4). Neither killing method nor storage time affected cholesterol content in the fillets. Four different types of COPs were analyzed in lipid extracts: α-epoxycholesterol, β-epoxycholesterol, 7-ketocholesterol, cholestantriol (triol). There were no significant differences in COPs content between the slaughter methods. However, fillets from CO group tended to have lower amounts of COPs, especially α- and β-epoxycholesterol (Table 4).

The total COPs content increased ($p<0.05$) after 14 days of storage time (Table 4). The increase pattern was not homogeneous: α- and β-epoxycholesterol increased by 25%, whereas triol and 7-ketocholesterol increased by 48 and 62%, respectively.

Carotenoid content

Vit. A, total carotenoids, and astaxanthin contained in fish fillets are reported in Table 4. No significant differences between the two slaughter methods were found for Vit. A, total carotenoid, and astaxanthin even though CO-slaughtered fish showed a higher contents than the percussion ones. However, their values remain unchanged during refrigerated storage. Finally, the total carotenoid value was in agreement with the pattern of its main constituents, showing to not be affected by killing method and storage time.

Correlation

A correlation pattern (Pearson test, $\alpha = 0.05$) was evaluated for lipid composition and oxidative parameters. Results are summarized in Table 5. For a better data comprehension, only significant correlations are here examined. MUFA and n6 FA

fraction were strongly correlated with TL (r= 0.64; p<0.001; r= 0.45; p<0.01, respectively), whereas a weak positive correlation between cholesterol content and TL (r= 0.28; p<0.05) was observed. Interestingly, a negative correlation was found between TL and PUFA (r= -0.73; p<0.001), n3 (r= -0.71; p<0.001), EPA (r= -0.27; p<0.05), DHA (r= -0.70; p<0.001), and PI (r= -0.54; p<0.001).
Moreover, n3 and n6 FAs were negatively correlated (r= -0.81; p<0.001), whereas positive correlations were observed between MUFA and n6 FA (r= 0.87; p<0.001), EPA and n3 FA (r= 0.49; p<0.001), and DHA and n3 FA (r= 0.90; p<0.001).

As regard the primary oxidation products, CD content was positively correlated with TL (r= 0.43; p<0.01), cholesterol (r= 0.26; p<0.05), and n6 FA (r= 0.25; p<0.05) content and negatively with DHA content (r= - 0.25; p<0.05), and with PI (r= -0.27; p<0.05). Finally, total cholesterol content was positively correlated with MUFA (r= 0.28; p<0.05) and n6 FA (r= 0.45; p<0.01) content and negatively with SFA (r= -0.26; p<0.05), n3 (r= -0.29; p<0.05), DHA (r= -0.38; p<0.01) content and with PI (r= -0.31; p<0.01).

DISCUSSION

The interest in the consequences of stress at slaughter on animal welfare and post-mortem biochemical processes have been largely investigated.[1-3] In many fish species, slaughter stress was shown to greatly influence both welfare[4] and fillets quality during storage,[5-7] even if some aspects of quality, as oxidative stability, are still unclear. Recently, CO has been proved to be a stunning/killing method causing a low stress response,[10] however its impact on fillets quality and stability during storage has not been investigated yet. The results of the present study show that the quality of salmon fillets just after slaughtering and after 14 days from rigor resolution of refrigerated storage at 2.5°C is not significantly affected by the killing method. This suggest that CO may be utilized without any detrimental effect on the quality of fish fillets.

Specifically, results showed only a tendency of CO derived flesh to contain lower values of FA and cholesterol oxidation products and higher carotenoid values than the percussion ones, even if no significant differences emerged. That lack of significance might be attributed to the presence of natural antioxidant defenses, as carotenoids, that may reduce the effect of the killing method on the oxidation susceptibility of A. salmon flesh. Further investigations may be useful in order to confirm the effect of CO-slaughter method on flesh quality perhaps in other species, which do not contain antioxidants. In contrast, the present study confirmed that storage length is the main factor affecting lipid oxidation, as previously found in other species as European sea-bass (*Dicentrarchus labrax*, Linnaeus 1758),[7] Coho salmon (*Oncorhynchus kisutch,* Walbaum 1972),[31] and sardine (*Sardinella pilchardus,* Walbaum 1972).[32]

Concerning nutritional values, A. salmon is a fatty species and present results agree with this classification. Despite the high percentage of fat contained in fish muscle, fat characteristics are very interesting for human nutrition as a consequence of the n3 FA fraction predominance, particularly EPA and DHA, on both n6 and SFA ones.[33] Moreover, also the values of other health indexes as AI, TI, and HH (0.21, 0.13,

and 5.8, respectively) confirmed the optimal nutritional fat characteristics from salmon fillets. Finally, analysis of correlation revealed that fat content and composition are strictly linked. Fat content match MUFA and n6, confirming that muscle contained mainly triacylglycerols that stored preferentially palmitic, oleic, linoleic, and γ-linolenic acids, as reported in literature.[34]

Cholesterol content is another nutritional aspect that should be considered. In the present trial, its content in fillet samples was lower (around 0.4 g kg^{-1}) than previously reported for wild (0.52 g kg^{-1}) and farmed (0.62 g kg^{-1}) A. salmon.[35] Despite cholesterol is reported to play a central role in many metabolic processes,[36] adults are recommended to not exceed 300 mg of cholesterol per day,[37] which means that an abundant portion (almost 300 g) of A. salmon would not exceed that quantity. Interestingly, in contrast with previous findings,[32] data obtained from correlation analyses showed that cholesterol content and TL are not independent, even if the correlation coefficient is quite low (r= 0.28; Table 5). Moreover, cholesterol and DHA are negatively correlated. This fact, together with the opposite relation obtained for cholesterol and MUFA and for cholesterol and n6, may be explained with the widely reported prevalence of fat deposits on structural lipids, where DHA is preferentially stored.[34]

However, CO stunning/killing method seemed not to preserve FA and cholesterol from oxidation, even if no detrimental effects were found in this study. Particularly, the FA composition of TL extract was quite stable through the storage. According to previous studies on salmonids, such as Rainbow trout (*Oncorhynchus mykiss,* Walbaum 1972)[38] and A. salmon,[39] FA showed a low oxidation rate. Particularly, CD and TBARS values confirmed that fillets from A. salmon were not prone to oxidation,[39] despite their high content of TL. In effect, data obtained in the present study showed that TBARS values after 14 days of storage were much lower than 8 mg malondialdehyde kg^{-1},[40] which is the limit of acceptability proposed for most fish species. Nevertheless, a marginal but significant decrease in total PUFA content was observed as a significant effect of the storage, due to the oxidation of the n3 FA, especially DHA. Indeed, the PUFA decrease was mainly due to DHA lost, which accounted for the 85%. This confirmed that n3 FA are the main substrates of oxidative processes. This result is also supported by the presence of a significant correlation between primary oxidation products and DHA (Table 5).

Moreover, cholesterol is susceptible to be oxidized in COPs that have shown adverse effects on human health.[17] Indeed, COPs seem to be involved in the initiation and progression of several chronic diseases, such as atherosclerosis, neurodegenerative disorders, diabetes and kidney failure. Generally, COPs are inserted in the threshold of unclassified compounds, which corresponds to 0.15 μg per person per day;[41] however a specific threshold of toxicological concern has not been set yet. In the present study, four of the most common COPs, as α– and β–epoxycholesterol, triol, and 7-ketocholesterol were detected already above the threshold at the killing (Table 4) in both percussion and CO-slaughtered fish. This fact can be explained in agreement with a recent study,[42] which reports that the degree of unsaturation of food fats may greatly influence and induce oxysterol production.

All COPs detected at the beginning and at the end of the storage period were secondary oxidation products of cholesterol; in fact α– and β–epoxy epimers originated from the reaction between cholesterol and 7 α– and β– hydroxycholesterol which, in turn, are produced from the dismutation of the 7-hydroperoxides. In the presence of water and in acid medium, the epoxy ring of α-epoxycholesterol and β-epoxycholesterol can undergo to open, producing cholestantriol. Finally 7-ketocholesterol, in the same manner as α- and β–epoxycholesterol, derives from the dismutation 7-hydroperoxides.[43]

The presence of epoxy derivatives, as α- and β-epoxycholesterol, might be partly due to the interaction of sterols with hydrogen peroxide, which is released by microbial enzymes naturally present in muscle tissues.[44] Hence, their small increase found in P and CO derived fillets might indicate a small microbial growth during refrigerated storage. Furthermore, these epoxy compounds can be easily converted into triols in the presence of water,[44] explaining the high values ranged by triol at the end of the experiment together with 7-ketocholesterol that generally apply for 50% of total COPs.[45] These results, coupled with the lack of primary oxidation products of cholesterol such as 7-hydroperoxides, suggested that the cholesterol oxidation process was at a late stage after 14 days of storage.

In salmon, fish pigmentation is recognized as an important quality attribute and it is associated with the accumulation of dietary carotenoids, mainly astaxanthin, in intramuscular fat.[46] In the present study, carotenoids tended to decrease (-7%; Table 4) during storage both in P and CO-slaughtered fish even if no significant differences between T0 and T14 were found. At the same time, the results also revealed that carotenoids, within both P and CO groups, affected lipid and cholesterol oxidation in the same way (absence of significant r values). Two possible explanations may be hypothesized: the astaxanthin did not act as antioxidant, as previously reported,[38] or the carotenoid fraction could minimize the effect of killing method on oxidative damage. This latter is sustained by the findings about the stability of redness color of A. salmon fillets of fish killed by percussion or CO.[10] Authors, in fact, indicated that the unchanged color was due to the presence of red pigments more than the ability of CO at retaining color.[9,13]

In conclusion, the present study showed that the use of CO as stunning/killing method did not affect oxidative stability of A. salmon during short-term refrigerated storage. Thus, considering that CO improves animal welfare by reducing stress at killing,[8,12] it would be interesting to study the effect of this treatment on other fish species.

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Table 1. Proximate composition (g kg^{-1} of muscle) of Atlantic salmon (*Salmo salar*) killed by percussion (P) or carbon monoxide (CO). Both groups were stored at 2.5°C till T14.

	Killing (K)		Storage (S)		Significance			rsd[1]
	P	CO	T0	T14	K	S	K×S	
Total lipids	82.0	78.3	77.9	82.5	n.s	n.s.	n.s.	11.5
Crude protein	208.3[a]	212.3[b]	209.6	211.0	**	n.s.	n.s.	5.5
Ash	13.2[b]	12.6[a]	12.8	12.9	*	n.s.	n.s.	1.0
Water	677.6	682.5	680.2	679.8	n.s.	n.s.	n.s.	10.5

[1]rsd: residual standard deviation.
Within criterion, a, b: $p<0.05$
n.s.: not significant;
* $p<0.05$; ** $p<0.01$.

Table 2. Fatty acids profile of muscle total lipids (g kg^{-1}) of Atlantic salmon (*Salmo salar*) stunned/killed by percussion (P) or carbon monoxide (CO). Both groups were stored at 2.5°C until T14.

	Killing (K)		Storage (S)		Significance			rsd[1]
	P	CO	T0	T14	K	S	K×S	
C16:0	9.01	9.01	8.97	9.04	n.s.	n.s.	n.s.	0.23
C18:1 n9	32.47	32.33	32.29	32.51	n.s.	n.s.	n.s.	0.76
C18:2 n6	11.40	11.43	11.39	11.45	n.s.	n.s.	n.s.	0.30
C18:3 n3	4.78	4.77	4.78	4.77	n.s.	n.s.	n.s.	0.17
C20:1 n9	3.85	3.86	3.83	3.87	n.s.	n.s.	n.s.	0.18
C20:5 n3 (EPA)	5.01	4.97	5.04	4.93	n.s.	n.s.	n.s.	0.37
C22:6 n3 (DHA)	10.67	10.94	10.97	10.63	n.s.	n.s.	n.s.	0.96
ΣSFA	14.70	14.66	14.62	14.74	n.s.	n.s.	n.s.	0.31
ΣMUFA	45.74	45.58	45.51	45.80	n.s.	n.s.	n.s.	0.70
ΣPUFA	39.56	39.76	39.86[b]	39.46[a]	n.s.	**	n.s.	0.63
Σn3	24.74	24.93	25.06[b]	24.09[a]	n.s.	*	n.s.	0.80
Σn6	14.06	14.08	14.05	14.09	n.s.	n.s.	n.s.	0.23
n3/n6	1.76	1.77	1.78	1.74	n.s.	n.s.	n.s.	0.08

The fatty acids C12:0, C13:0, C14:0, C14:1 n5, C15:0, C15:1, C16:1 n9; C16:1 n7, C16:2 n4, C16:3 n4, C16:4 n1, C17:0, C17:1, C18:0, C18:1 n7, C18:3 n6, C18:3 n4, C18:4 n3, C18:4 n1, C20:0, C20:1 n11, C20:1 n7, C20:2 n6, C20:3 n6, C20:3 n3, C20:4 n6, C20:4 n3, C21:0, C21:5 n3, C22:0, C22:1 n11, C22:1 n9, C22:1 n7, C22:2 n6, C22:4 n6, C22:5 n6, C22:5 n3, C24:0, and C24:1 n9, in percentage <3%, were also detected but not reported in the table for brevity. They were utilized to calculate the fatty acid groups.

[1]rsd: residual standard deviation.
Within criterion, a, b: p<0.05
n.s.: not significant;
* p<0.05; ** p<0.01.

Table 3. Nutritional indices of Atlantic salmon (*Salmo salar*) killed by percussion (P) or carbon monoxide (CO). Both groups were stored at 2.5°C until T14.

[1]rsd: residual standard deviation

	Killing		Storage		Significance			rsd[1]
	P	CO	T0	T14	K	S	K×S	
PI[2]	1.71	1.76	1.75	1.72	n.s.	n.s.	n.s.	0.17
AI[3]	0.22	0.22	0.22	0.21	n.s.	n.s.	n.s.	0.01
TI[4]	0.13	0.13	0.13	0.13	n.s.	n.s.	n.s.	0.01
HH[5]	5.82	5.84	5.85	5.81	n.s.	n.s.	n.s.	0.22

[2]PI, Polyene Index;
[3]AI, Atherogenic index;
[4]TI, Thrombogenic index;
[5]HH, hypocholesterolaemic/hypercholesterolaemic FA ratio.
n.s.: not significant.

Table 4. Conjugated dienes (CD), cholesterol, TBARS, COPs, Vitamin A and carotenoids content in Atlantic salmon (*Salmo salar*) killed by percussion (P) or carbon monoxide (CO). Both groups were stored at 2.5°C until T14.

	Killing		Storage		Significance			rsd[1]
	P	CO	T0	T14	K	S	K×S	
CD (mol kg^{-1} muscle)	0.27	0.25	0.24[a]	0.28[b]	n.s.	**	n.s.	0.04
TBARS (mg kg^{-1} muscle)	0.66	0.60	0.45[a]	0.81[b]	n.s.	**	n.s.	0.34
Cholesterol (g kg^{-1} muscle)	0.42	0.38	0.38	0.42	n.s.	n.s.	n.s.	0.14
Tot. COPs (g kg^{-1} muscle)	0.017	0.014	0.011[a]	0.020[b]	n.s.	*	n.s.	0.09
α-epoxycholesterol	0.004	0.003	0.003	0.004	n.s.	n.s.	n.s.	0.02
β-epoxycholesterol	0.004	0.003	0.003	0.004	n.s.	n.s.	n.s.	0.02
Triol	0.005	0.004	0.003[a]	0.006[b]	n.s.	*	n.s.	0.03
7-ketocholesterol	0.004	0.004	0.002[a]	0.006[b]	n.s.	*	n.s.	0.04
Vit. A (µg kg^{-1} muscle)	115.5	121.6	122.3	114.8	n.s.	n.s.	n.s.	83.2
Total carotenoids (µg kg^{-1} muscle)	7116.7	9508.9	8616.0	8009.6	n.s.	n.s.	n.s.	44.3
Astaxanthin (µg kg^{-1} muscle)	4801.4	5610.4	5270.5	5141.2	n.s.	n.s.	n.s.	2597.5

[1] rsd, residual standard deviation.
Within criterion, a, b: $p<0.05$
n.s.: not significant; * $p<0.05$; ** $p<0.01$.

Table 5. Residual correlation coefficients (*r*) between weight of Atlantic salmon and chemical parameters analyzed in fillet samples

	TL	Chol	α-Epoxy	Triol	7-Keto	Tot. COPs	Vit A	Tot. car	PI	SFA	MUFA	PUFA	*n*-3	*n*-6	*n*-3/*n*-6	EPA	DHA
TBARS	−0.09	−0.25*	−0.3	0.03	0.04	0.07	−0.28*	−0.15	0.14	−0.18	0.05	0.03	−0.00	0.06	−0.02	−0.20	0.07
CD	0.43**	0.26*	−0.06	−0.17	−0.34	−0.29	0.27	0.13	−0.27*	−0.02	0.15	−0.16	−0.22	0.25*	−0.24	−0.15	−0.25*
TL	1.00	0.28*	0.05	−0.08	−0.31	−0.15	0.22	0.07	−0.54***	0.01	0.64***	−0.73***	−0.71***	0.45**	−0.65***	−0.27*	−0.70***
Cholesterol		1.00	−0.07	0.10	−0.01	0.03	0.21	−0.12	−0.31**	−0.26*	0.28*	−0.18	−0.29*	0.45**	−0.37**	0.02	−0.38**
β-Epoxycholesterol			−0.43*	0.85***	0.78***	0.93***	−0.09	−0.27	0.32	−0.27	−0.17	0.33	0.22	0.08	0.11	−0.11	0.29
α-Epoxycholesterol			1.00	−0.66**	−0.61**	−0.35	0.07	−0.15	−0.03	0.13	0.09	−0.17	−0.03	−0.24	0.07	−0.21	0.14
Triol				1.00	0.85***	0.88***	−0.12	0.02	0.26	−0.07	−0.27	0.34	0.25	−0.01	0.16	0.14	0.19
7-Ketocholesterol					1.00	0.89***	−0.09	0.00	0.29	0.12	−0.42*	0.43*	0.36	−0.19	0.31	0.30	0.25
Total COPs						1.00	−0.10	−0.14	0.35	−0.01	−0.35	0.41*	0.35	−0.16	0.29	0.09	0.36
Vitamin A							1.00	0.22	−0.77***	0.46	−0.37**	0.19	0.27	−0.42**	0.33**	0.26	0.12
Astaxanthin								0.92***	−0.24	0.47**	−0.17	−0.03	0.03	−0.25	0.11	0.27	−0.12
Total carotenoids								1.00	−0.29*	0.43**	−0.11	−0.08	−0.03	−0.17	0.04	0.23	−0.19
PI									1.00	−0.35**	−0.15	0.36**	0.31**	−0.05	0.23	−0.05	0.41**
SFA										1.00	−0.48**	0.02	0.23	−0.67***	0.40**	0.55***	0.02
MUFA											1.00	−0.89***	−0.95***	0.87***	−0.97***	−0.62***	−0.78***
PUFA												1.00	0.97***	−0.64***	0.89***	0.41**	0.89***
n-3													1.00	−0.81***	0.98***	0.49***	0.90***
n-6														1.00	−0.92***	−0.60***	−0.66***
n-3/*n*-6															1.00	0.55***	0.85***

*$P < 0.05$;
**$P < 0.01$;
***$P < 0.001$.

PAPER IV

Mechanical Separation Process applied to different species of interest for European aquaculture: effect on lipid oxidation.

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Abstract

Mechanical separation systems are a good option to create new fish products and open new market, however studies on the effect on quality of mechanical treatment on species of interest for European aquaculture, such as European sea bass, gilthead sea bream, and rainbow trout are currently scarce. Thus, the effect on colour, nutritional quality, and lipid stability was considered immediately after separation process and after 90 days of frozen storage. Results revealed that mechanical separation technique significantly affected colour and lipid stability of the three studied species. Increases in lightness (L*) and secondary oxidation products were observed, together with a decreased of antioxidant capacity. Nutritional value instead was unaffected by treatment. Thus, mechanical separation process could represent a new way to better exploit species of interest for European aquaculture, but oxidative processes during the treatment have to be limited and controlled.

Keywords: MSM, TBARS, fishburger, antioxidant capacity.

1. Introduction

Products development in aquaculture sector has been very limited during these last years (EC, 2013). In this sense, mechanical separation systems are a good option for create new fish products and open new market. In accordance with Regulation (EC) No 883/2004, mechanically separated meat (MSM) is a product obtained by removing remaining meat from bones using mechanical means, where the normal structure of the muscle fibre is mostly lost or modified in such a way that it is not comparable with regular meat. During the last decades, MSM has grown in importance, especially in poultry and pigs sectors, raising a production of 700 000 t in 2007 (EC, 2010). Concerning seafood industry, no specific restrictions about MSM utilized are presented in EU Regulation and mechanical separation treatment may represent a new technology in fish supply chain.

Recently, MSM obtained from Nile tilapia (*Oreochromis niloticus*) (Freitas et al., 2012; Kirschnik et al., 2013; Marengoni et al., 2009; Fogaça et al., 2015), and Brazilian catfish (*Brachyplatystoma vaillantii*) (Oliveira et al., 2015) by-products have been chemically and sensory characterized. However, many areas have to be explored. For example, the utilization of mechanical separation (MS) on the whole fish has to be investigated, as well as its effects on European seawater and freshwater species. Indeed, MS has been utilized for the recovery of fish by-products whereas it may be interested to use it to exploit no marketable European farmed fish, such as the undersized or damaged ones. That will entail the utilization of whole fish, rich in fat and protein, and not only the frame derived from filleting process.

Lipid oxidation is a very important event leading the loss of nutritional values and food quality, especially for fish, due to the high presence of polyunsaturated fatty acids (PUFAs). However, the extent of such as mechanism in new products, like MSM of fish is not yet well investigated. Thus, it seems reasonable to check the effect on lipid stability of MS technology applied to sea and freshwater European farmed species in order to understand its possible role for the creation of new products.

2. Materials and methods

2.1 Preparation of fish samples and storage conditions

Different species of sea and freshwater were used for the present trial. Eighteen European sea bass (*Dicentrarchus labrax*) and 18 gilthead sea bream (*Sparus aurata*) were purchased from a fish farm located in Orbetello (Grosseto, Italy). Finally 18 rainbow trout (*Oncorhynchus mykiss*) were purchased from a farm located in the north west of Tuscany (Lucca, Italy). Fish were killed by percussion and immediately after death, fish were transferred into polystyrene boxes, covered by ice, and moved to the industry where six fish for each species were minced by the MSM machine Baader 60-1 (Lubecca, Germany). Then, the remained fish and the MSM were brought

to DISPAA (Florence, Italy) where all the whole fish were filleted. Whereas six fillets (right) for each species were stored as fillet (WF samples), six fillets (left) for each species were grounded by using a New Style Chopper (Westmark Gmbh, Elspe, Germany) in order to obtain 6 fish-burger (FB samples), while six MSM-fish burger were obtained from MSM (MSM samples). Three samples for each treatment and each species were analysed at time 0 (T0), while the other samples were analysed after storage at – 20 °C for 90 days (T90). Three replicates of WF, FB, and MSM for the three species were analysed for: colour, total lipids, fatty acid composition, primary (conjugated dienes) and secondary (tiobarbituric acid substances, TBARS) oxidation products, and antioxidant capacity.

2.2 Colour

A Dr Lange Spectro-colour® colorimeter (Keison International Ltd, UK) equipped with a Spectral qc 3.6 software was utilized for colorimetric measurement. Colour was measured in triplicate on the epaxial-cranial sites of fillet (WF) and in three points of the burgers (FB and MSM). Colour measurements were carried out according to the CIELab system (CIE, 1976). Lightness (L*), redness index (a*), yellowness index (b*), Hue, and Chroma were recorded.

2.3 Fatty acids

The total lipid content of the samples was determined according to Folch et al. (1957) method and fatty acids (FA) in lipid extract were trans-esterified to methyl esters (FAME) using a base-catalyzed trans-esterification followed by a boron trifluoride catalyzed esterification (Morrison & Smith, 1964). The FA composition was determined by gas chromatography (GC) using a Varian GC 430 gas chromatograph equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 capillary column (30 m × 0.32 mm i.d., 0.25 µm film and polyethylene glycol bonded phase; Supelco, Bellefonte, PA, USA), purchased from Agilent (Palo Alto, California, USA). The oven temperature was held at 100 °C for 2 min, increased to 160 °C over 4 min, then increased to 220 °C over 14 min and finally kept at 220 °C for 25 min. The injector and the detector temperatures were set at 220 °C and 300 °C, respectively. One µL of sample in hexane was injected into the column with helium as carrier gas kept at a constant flow of 1.5 mL/min. The split ratio was 1:20. Chromatograms were recorded with the Galaxie Chromatography Data System 1.9.302.952 (Agilent) computing integrator software. Fatty acids were identified by comparing the FAME retention time with the standard Supelco 37 component FAME mix (Supelco). Fatty acids were quantified through calibration curves using tricosanoic acid (C23:0) (Supelco) as internal standard. This analysis was not carried out in FB samples, because the similarity of composition of these samples and WF samples.

2.4 Lipid oxidation products

Conjugated dienes (CD) content in the lipid extract were measured by the colorimetric method (Srinivasan et al., 1996) using hexane (Sigma Aldrich, St. Luis, MO, USA) as solvent. Conjugated dienes were quantified at 232 nm (50 Scan spectrophotometer, Varian equipped with a Cary Win UV Software; Palo Alto, CA, USA) and using a molar extinction coefficient of 29000 mL /mmol cm. The results are expressed as mmol hidroperoxides/kg lipid.

The 2-thiobarbituric acid reactive substances (TBARS) were measured using the colorimetric method decrypted by Vynke (1970) at 532 nm. Briefly, TBARS were extracted in TCA (5%), then added with TBA 0.02mol/L. After 40 min of incubation at 97 °C oxidation products were quantified with reference to calibrations curves of TEP (1,1,3,3,-tetra-ethoxypropane) in 5% (w/v) TCA (0.2 to 3.1 µmol/L).

2.5 Antioxidant capacity

Samples of fresh burgers (3 g) were extracted with 10 ml of ethanol. The antioxidant capacity was performed on ethanol extracted samples according the minor modifications reported in Mancini et al. (2015) to the methods of Re et al. (1999) for ABTS reducing activity assay (ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)), of Blois (1958) and Jung et al. (2010) for DPPH scavenging activity (DPPH, 2,2-diphenyl-1-picrylhydrazyl) and Descalzo et al. (2007) for FRAP assay method (ferric reducing ability).

2.6 Statistical analysis

The statistical analysis was performed using SPSS version 17.0 software (SPSS Inc. Illinois, USA). Normality of data distributions was tested by the Kolmogorov-Smirnov test. Fatty acids were subjected to one-way analysis of variance (ANOVA) with 'treatment' as a fixed effect, using the Bonferroni post-hoc test to check the significance of the differences among levels (WB, FB and MSM samples). The primary and secondary oxidation products and antioxidant capacity were subjected to two-way ANOVA with 'treatment' and 'storage' and their interaction as fixed effect, using Bonferroni post hoc test to check again the significance of the differences among levels (WB, FB and MSM samples), and storage (T0 and T90).

3. Results and Discussion

Table 1 presents the results of ANOVA for colour values. Treatment significantly affected colour for European sea bass, gilthead sea bream, and rainbow trout. The differences in colour parameters are similar for the seawater species, indeed the L*, a* and b* of WF are significantly lower than the minced fillet (FB) and MSM burger. On

the other hand, trout fillet showed a significantly lower lightness (L*) and higher redness (a*) compared to MSM and minced fillet. No significant differences were observed for b* values in trout. The fish fillet colour is linked with heme-based pigment, physical structure of muscle, and the amount of unbound water influences light scattering. Since sea bass and sea breams are white fish, it is reasonable to suppose that changes of pigments under high-pressure treatment are of minor importance, so that these colour changes may be attributed to modifications of protein matrix as reported by Chéret, Chapleau, Delbarre-Ladrat, Verrez-Bagnis, & Lamballerie (2005). The redness in trout is due to astaxanthin, and the significantly higher a* could be related to the significant interaction TxS. Indeed, storage time increased lightness and redness in trout, as previously reported (Choubert, & Baccaunaud, 2006). Evolution of colour during storage can be associated with enzymatic and non-enzymatic reactions resulting in degradation of myofibrillar proteins and disorganization of myofibrils (Cherét et al., 2005). These modifications were observed also in the white flesh fish. According to that, the storage of all treated fish led to a significant decrease in L* value after 90 days. However, L* value did not reach the state in which colour starts darkening which was set to be about 58 (L* value) (Ochiai, Chow, Watabe & Hashimoto, 1988). These results emphasised the importance of using white flesh fish (sea bass and sea bream, for example) to develop fish products, as supported by Bito (1965) who assessed that the colour of the white fish burgers was more stable than that of tuna stored at the same temperature, which lost its colour after 2 months.

The fatty acid (TFA) composition of European sea bass, gilthead sea bream, and trout fillets and MSM immediately after treatment is reported in Table 2. No statistically differences were found in the fatty acid profile between fillets and MSM for none of the considered species. Although a certain caution is required when the results are compared with previous studies, it seems that the raw material for MS process deeply influences fatty acid composition. Indeed, when MSM is obtained by filleting residues (Oliveira et al., 2015), lipid fraction is mainly constituted by SFA (around 54 %), and MUFA (34 %) whilst the most important PUFA applied for 10 % of total fatty acid. On the contrary, when whole fish, degutted and without head, is utilised as in the present research, MSM fatty acid profile reflected that of the fillet. In conclusion, using no marketable fish instead of fish by-products may result in a high quality MSM chemical composition despite the species utilised.

The fatty acid composition of European sea bass, gilthed sea bream, and rainbow trout samples instead fell within previous data about farmed fish (Badiani et al., 2013; Grigorakis, 2007; Tibaldi et al., 2015; Secci, Parisi, Dasilva, & Medina, 2016).

The total amount of saturated fatty acids (SFA) in muscle was found to be around 20 % in seawater species, whilst trout stopped at around 15 %. Regardless the

quantitative difference, this fraction was found to be mainly composed of palmitic (16:0), stearic (18:0), and myristic (14:0) acids in all the species. In general, the dominance of these three fatty acids has been reported previously in farmed fish fed with different ratio of marine and plant feed ingredients (Baron et al., 2013; Timm-Heinrich, Eymard, Baron, Nielsen, & Jacobsen, 2013). Among monounsaturated fatty acids (MUFA), the most abundant lipids in were oleic (C18:1ω9), and palmitoleic (C16:1ω7) with some species-specific differences. Particularly, gilthead sea bream resulted in the lowest oleic content, applying for 15 % of total fatty acids, whereas rainbow trout contained almost the 10 % more than the other. The opposite trend was found for palmitoleic acid which resulted to be the highest in gilthead sea bream and the lowest in rainbow trout. In both cases, oleic and palmitoleic values for European sea bass were more similar to sea bream than to trout, confirmed the affinity of these two marine species (Grigorakis, 2007). Oleic acid is often reported to be the most abundant MUFA in the lipids and it is one of those more affected by replacement of fish oil by plant oil in feeds (Baron et al., 2013).

As regards to muscle PUFA, their amount ranged from 45 % (sea bass) to 52.5 % of trout but the main differences lean on its composition. Indeed, PUFA fraction of sea water species are mainly composed by ω3 (around 72 %) and the ω6 represented about 23 % of total polyunsaturated fatty acids. Freshwater instead had 52 % of ω6 and 41 % of ω3. Even in that case, results confirmed the differences between marine and freshwater fish highlighted by Tocher (2003). However, the dominance of C18:2ω 6 on ω6 fraction of PUFA has been reported both in marine (Badiani et al., 2013; Tibaldi et al., 2015) and in freshwater farmed species (Secci, Parisi, Dasilva, & Medina, 2016) though its percentage seemed to be strictly connected with the sources of feed ingredients (Baron et al., 2013). Specifically, present results revealed that C18:2 ω6 applied for 82 % and 86 % in sea bass and trout, respectively, in agree with the 87 % and 90 % previously obtained for the same species (Badiani et al., 2013; Secci, Parisi, Dasilva, & Medina, 2016).

The major contributors to ω3 fraction were docosahexanoic acid (DHA) for all the three considered species, followed by eicosapentaenoic acid (EPA) in Eurpean sea bass (around 9 % of PUFA ω3) and gilthead sea bream (19 % of PUFA ω3), and gamma-linolenic acid (C18:3ω3) in rainbow trout samples.

Concerning lipid stability during treatment and storage, such as high values of PUFAs ω3 fraction could be the main cause of lipid degradation of MSM during the mechanical treatment. Indeed, results revealed that MS treatment significantly affected lipid oxidation of seawater fish whereas no effect emerged on trout (Table 3). Specifically, primary oxidation products, obtained by measuring conjugated dienes content, were affected nor by treatment or storage in sea bass and trout samples, whilst CD content of sea bream was found to be significantly affected by treatment

and storage. Nonetheless, the extent of lipid oxidation was underlined by TBARS values. Globally, treatment significantly affected lipid oxidation of the three species. Mechanical separation process seemed to promote lipid oxidation in seawater species immediately after treatment, being TBARS more than two times higher in MSM than in WF. Burgers obtained from minced meat resulted in an intermediate level of oxidative status (Table 3). Moreover, gilthead sea bream appeared the most susceptible species to be oxidised by treatment, by raising 7.26 mg MDA/ kg sample, that is a value near the threshold of 8 mg MDA/ kg sample for the rancid perception, as proposed by Shormüller (1968). Lipid fraction of trout instead seemed not to be affected by mechanical separation process.

However, present results are not in complete agreement with previous findings. Results by Fogaça et al. (2015) on the effect of MSM on tilapia (*Oreochromis niloticus*) showed a low oxidative value (1.03 mg MDA/ kg tissue) despite the high lipid content (around 7 %). Lowest value was obtained for no-washed MSM from tilapia by Kirschnik et al. (2013) who found a TBARS content around 0.5 mg MDA/ kg tissue which however, raised up to 0.7 mg MDA/kg tissue after 90 days at -18 °C. However, it has to be note that these studies were conducted on fish filleting waste which had a lowest PUFA percentage (Oliveira et al., 2015) than data in the present research.

Concerning storage, it significantly affected TBARS values of all the studied species, in agreement with previous studies (Indergård, Tolstorebrov, Larsen, & Eikevik, 2014; Secci, Parisi, Dasilva, & Medina, 2016). At T0, trout was found in a lower oxidative status in comparison with the other two species, by being three times lower than the values obtained for sea bream and almost half the sea bass one. Such as difference was in agreement with a previous study that showed the scarce susceptibility of rainbow trout to be oxidised, both for its low content of ω3 fraction and for carotenoid content (Secci, Parisi, Dasilva, & Medina, 2016). PUFA ω3 may be responsible instead for the three times higher TBARS contained in sea bream than in sea bass. Indeed, the 5 % of difference in ω3 amount of two species may increase sea bream lipid susceptibility to oxidation.

After 90 days of frozen storage, secondary lipid oxidation products doubled in sea bass and sea bream samples, whereas increased 4 times in rainbow trout. The rate obtained for sea bass was in agreement with that obtained by Simitzis et al. (2014) who found that TBARS in percussion killed fish doubled during 90 days at -20 °C. On the other hand, oxidation in trout highly increased confirming that astaxanthin seems to protect against the very early stages of lipid oxidation but not during the long term frozen storage (Jensen, Birk, JokuSEMn, Skibsted, & Bertelsen, 1998).

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods. The ABTS, DPPH, and FRAP have been widely used to test the ability of compounds to act as free radical

scavengers and thus to evaluate the antioxidant activity (Mancini et al., 2015). All the treated fish, irrespective of the species, showed some reducing ability which probably could be attributed to the intrinsic antioxidant system of the muscle (Table 4). Particularly, the presence of astaxhanthin in trout muscle may be responsible for the highest global antioxidant capacity, especially ABTS value, because it can improve scavenging and antioxidant activity. However, treatment significantly reduced antioxidant capacity in all the studies species. As reported by Pazos, González, Gallardo, Torres, & Medina (2005), under post mortem conditions, the endogenous antioxidants are consumed sequentially and the loss coincides with fish muscle lipid oxidation development. Present results are in agreement with this pattern, because storage was found to significantly affect both antioxidant capacity and lipid oxidation. Specifically, storage significantly reduced the antioxidant assay whereas significantly increased TBARS content. Moreover, as suggested by Gómez-Estaca et al. (2011) higher oxidative stability should be expected from samples with higher reducing ability during refrigeration or under other oxidizing conditions.

3. Conclusions

In conclusion, mechanically separation process significantly affected quality of the derived-products in terms of colour, antioxidant capacity and oxidative stability. Globally, MSM of seawater species resulted more damaged by mechanical treatment than that of trout, maybe because of the high content of ω3. However, using no marketable fish instead of fish waste may result in a high nutritional quality MSM despite the species utilised. Thus, mechanical separation process could represent a new way to better exploit species of interest for European aquaculture, but oxidative processes during the treatment have to be limited. Washing MSM, as proposed by other authors (Kirschnik et al., 2013) could be an option to wash out pro-oxidant molecules, such as heme, however further researches on the utilisation of antioxidant during the process or added to the MSM are suggested.

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Table 1. Colour parameter values of European sea bass, gilthead sea bream, and rainbow trout fillets (WF), minced (FB) and MSM burger at T0 and after 90 days of frozen storage (T90).

		Treatment (T)				Storage (S)			TxS
Species	Parameter	WF	FB	MSM	SEM[1]	T0	T90	SEM	
European sea bass	L*	42.65^{b}	47.41^{a}	49.04^{a}	1.07	47.55^{a}	45.18^{b}	0.46	N.S.
	a*	-1.40^{b}	0.59^{a}	0.15^{ab}	0.48	-0.29	-0,15	0.13	N.S.
	b*	1.35^{b}	5.19^{a}	5.57^{a}	0.43	2.26^{b}	5.81^{a}	0.23	N.S.
Gilthead sea bream	L*	39.05^{b}	46.50^{a}	44.37^{a}	1.19	46.66^{a}	39.95^{b}	0.77	N.S.
	a*	-2.50^{b}	-0.64^{a}	-0.24^{a}	0.75	-0.12^{b}	-2.13^{a}	0.19	N.S.
	b*	0.77^{b}	5.36^{a}	6.57^{a}	1.57	2.16^{b}	6.31^{a}	0.73	N.S.
Rainbow trout	L*	28.81^{b}	39.68^{a}	38.79^{a}	1.98	42.70^{a}	28.82^{b}	1.21	N.S.
	a*	3.05^{a}	0.44^{b}	0.56^{b}	0.73	2.11^{a}	0.59^{b}	0.53	0.02
	b*	10.24	12.11	11.06	0.10	11.38	10.89	0.64	0.01

[1] SEM: Standard Error of the Mean

Within criterion, a, b, c: $p<0.05$;

NS, Not Significant ($p>0.05$).

Data were obtained from three replicates.

Table 2. Fatty acids profile (g/100g of total fatty acids) of European sea bass, gilthead sea bream, and rainbow trout fillets (WF) and MSM burger immediately after treatment (T0).

	European sea bass				Gilthead sea bream				Rainbow trout			
	WF	MSM	Sign.	SEM[1]	WF	MSM	Sign.	SEM	WF	MSM	Sign.	SEM
Total lipid %	9.59	10.49	NS	1.83	9.87	7.76	NS	0.643	8.06	6.65	NS	0.840
C14:0	3.56	3.43	NS	0.039	4.24	4.10	NS	0.145	1.45	1.44	NS	0.011
C16:0	13.42	13.72	NS	0.296	12.58	12.59	NS	0.444	10.75	10.40	NS	0.263
C16:1ω7	4.80	4.43	NS	0.039	6.39	6.36	NS	0.158	2.47	2.37	NS	0.106
C18:0	2.31	2.46	NS	0.025	2.83	2.61	NS	0.057	3.24	2.98	NS	0.094
C18:1ω9	17.98	18.94	NS	0.720	14.94	14.83	NS	0.408	24.39	23.77	NS	0.313
C18:2ω6	9.05	8.67	NS	0.131	8.17	8.02	NS	0.323	25.82	26.24	NS	0.332
C18:3ω3	1.86	1.91	NS	0.020	1.29	1.31	NS	0.087	4.12	4.23	NS	0.088
C20:1ω9	3.49	3.73	NS	0.086	1.42	1.53	NS	0.056	1.38	1.36	NS	0.123
C20:5ω3	10.12	9.52	NS	0.165	10.41	10.32	NS	0.313	3.08	3.13	NS	0.137
C22:1ω11	3.03	3.38	NS	0.101	1.25	1.41	NS	0.093	0.71	0.70	NS	0.074
C22:5ω3	3.05	2.85	NS	0.126	7.66	7.37	NS	0.223	1.81	1.64	NS	0.054
C22:6ω3	15.91	15.75	NS	0.807	15.83	16.90	NS	0.517	11.00	11.95	NS	0.362
ΣSFA	20.17	20.47	NS	0.299	20.72	20.32	NS	0.629	16.00	15.35	NS	0.342
ΣMUFA	33.43	34.68	NS	0.883	27.99	28.07	NS	0.410	31.70	30.94	NS	0.556
ΣPUFAω6	11.40	10.89	NS	0.175	10.59	10.44	NS	0.315	29.68	30.17	NS	0.236
ΣPUFAω3	33.53	32.67	NS	1.019	38.06	38.71	NS	0.150	21.83	22.76	NS	0.347
ΣPUFA	46.39	44.85	NS	1.178	51.29	51.60	NS	0.247	52.30	53.71	NS	0.330

C12:0, C13:0, C14:1ω5, C15:0, C15:1, C16:1ω9; C16:2ω4, C16:3ω4, C16:4ω1, C17:0, C17:1, C18:1ω7, C18:3ω6, C18:3ω4, C18:4ω1, C20:0, C20:1ω11, C20:1ω7, C20:2ω6, C20:3ω6, C20:3ω3, C20:4ω6, C20:4ω3, C21:0, C21:5ω3, C22:0, C22:1ω9, C22:1ω7, C22:2ω6, C22:4ω6, C22:5ω6, C24:0, and C24:1ω9 were also detected but not reported because in percentage <3%. They were utilized to calculate Σ.

[1] SEM: Standard Error of the Mean

NS, Not Significant ($p>0.05$).

Data were obtained from three replicates.

Table 3. Primary (CD, mmol Hp/kg sample) and secondary (TBARS, mg MDA/kg sample) oxidation products in European sea bass, gilthead sea bream, and rainbow trout fillets (WF), and minced (FB) and MSM burger at T0 and after 90 days of frozen storage (T90).

		Treatment (T)				Storage (S)			TxS
Species	Parameter	WF	FB	MSM	SEM[1]	T0	T90	SEM	
European sea bass	CD	0.42	0.39	0.46	0.04	0.43	0.41	0.05	N.S.
	TBARS	1.10^{b}	1.37^{b}	2.34^{a}	0.28	1.11^{b}	2.09^{a}	0.23	N.S.
Gilthead sea bream	CD	0.44^{a}	0.37^{ab}	0.34^{b}	0.03	0.35^{b}	0.42^{a}	0.025	N.S.
	TBARS	2.72^{c}	5.40^{b}	7.26^{a}	0.575	3.72^{b}	6.53^{a}	0.469	N.S.
Rainbow trout	CD	0.22	0.25	0.21	0.017	0.21	0.24	0.014	N.S.
	TBARS	3.15	3.09	2.11	0.732	0.72^{b}	4.85^{a}	0.589	N.S.

[1] SEM: Standard Error of the Mean

Within criterion, a, b, c: $p<0.05$;

NS, Not Significant ($p>0.05$).

Data were obtained from three replicates.

Table 4. Antioxidant capacity, expressed as ABTS (mmol/kg sample), DPPH (mmol/kg sample), and FRAP (mmol/kg sample), in European sea bass, gilthead sea bream, and rainbow trout fillets (WF), minced (FB) and MSM burger at T0 and after 90 (T90) days of frozen storage.

		Treatment (T)				Storage (S)			TxS
Species	Parameter	WF	FB	MSM	SEM[1]	T0	T90	SEM	
European sea bass	ABTS	0.28^{a}	0.21^{b}	0.27^{a}	0.02	0.28^{a}	0.23^{b}	0.017	0.00
	DPPH	0.12^{a}	0.13^{a}	0.07^{b}	0.00	0.11^{a}	0.10^{b}	0.003	0.00
	FRAP	0.22	0.21	0.21	0.02	0.23^{a}	0.20^{b}	0.013	0.00
Gilthead sea bream	ABTS	0.15^{b}	0.13^{b}	0.21^{a}	0.01	0.11^{b}	0.22^{a}	0.009	0.00
	DPPH	0.07^{a}	0.06^{b}	0.02^{c}	0.00	0.03^{b}	0.08^{a}	0.002	0.00
	FRAP	0.13	0.15	0.11	0.01	0.15^{a}	0.11^{b}	0.010	0.18
Rainbow trout	ABTS	0.48^{a}	0.39^{b}	0.29^{c}	0.02	0.34^{b}	0.43^{a}	0.016	0.00
	DPPH	0.09^{a}	0.08^{a}	0.05^{b}	0.01	0.08^{a}	0.06^{b}	0.004	0.00
	FRAP	0.21^{a}	0.20^{a}	0.13^{b}	0.02	0.22^{a}	0.12^{b}	0.013	0.09

[1] SEM: Standard Error of the Mean

Within criterion, a, b, c: $p<0.05$;

NS, Not Significant ($p>0.05$).

Data were obtained from three replicates.

6. CONCLUSIONS

The overall aim of this study was to assess the effects of different extrinsic factors (killing method, storage, mechanical separation technique) on lipid oxidative stability of fillets from different farmed species.

In conclusion, the present PhD thesis showed that:

- ✓ killing method (asphyxia) affect both the formation of lipid oxygenated metabolites and the oxidative stability of farmed rainbow trout. Asphyxia is more stressful than percussion slaughtered method, as revealed by K-value. During such as stress, a greater enzymatic activity may be discerned thank to the higher concentration of hydroperoxides and other lipid oxygenated metabolites, especially EPA-derived. Noticeably, 12-HpEPE/15-HpEPE and PGE3/PGD3, EPA-derived metabolites, could be considered as good markers of stress because they were highly produced under stress conditions (asphyxia) while they were not detected in the percussion group. In addiction stress at slaugher seemed to shift the enzymatic activity (LOX and COX) torwards EPA instead of ARA, producing less inflammatory species. Therefore, the high presence of pro-oxidant molecules, as Hp, affects the oxidative stability of the asphyxiated rainbow trout flesh during the frozen storage, leading as result to a decrease of the shelf life of frozen trout fillets in terms of rancidity.

- ✓ New stunning/killing procedure, such as carbon monoxide utilisation, can be apply without any detrimental effect on oxidative stability of farmed Atlantic salmon. Cholesterol shows to be highly prone to be oxidatise.

- ✓ The mechanical separation treatment of fillets from European sea bass, gilthead sea bream, and rainbow trout can be utilised in order to exploit no marketable fish, such as the undersized or damaged ones. Derived meat (MSM) has a high quality, especially in term of nutritional value. Its fatty acids composition indeed resembles that of whole fish. However, mechanical process can induce oxidative damages. Use of washing or add antioxidant could limit them, but further investigations are needed.

- ✓ Storage confirms to be a critical step during the whole supply fish chain. Particularly, highest the storage temperature highest lipid oxidation values. Frozen storage should be suggested in order to preserve the quality of fish fillets and avoid lipid losses.

ANNEX I

Formation of lipid mediators in farmed rainbow trout under stress conditions

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Fish response to stress might resemble inflammatory process in which a series of derived oxidation metabolites from PUFAs seemed to play an important role. Recently, many authors focused on the connection between stress and eicosanoids production in different tissues of fish. C20 long chain fatty acids such as arachidonic (ARA, C20:4n6), eicosapentaenoic (EPA, C20:5n3), and dihomo-gamma-linolenic (DGLA, C20:3n6) are precursors of many lipid mediators: 4-series leukotrienes (LT), lipoxine (LX), 12-hydroxy-eicosatetraenoic acid (12-HETE), 12-hydroxy-eicosapentaenoic acid (12-HEPE), 2-series prostaglandins (PG), and tromboxanes (TX). As well, DHA is the precursor of 14S-hydroxy-docosanoids (14-HDoHE), 17S-hydroxy-docosanoids (17-HdOHE), neuroprotectin D1, resolvin D5, resolvin D1, and resolvin D2.

The present work focused on the formation of lipid metabolites in plasma of farmed rainbow trout (*Oncorhynchuss mykiss*) subjected to stress conditions by means of a slaughtering under asphyxia. Control trout were a set of non stressed fish using percussion slaughter method. The lipid metabolites were studied through the determination of several eicosanoids and docosanoids, such as prostaglandins, leukotrienes, tromboxanes, isoprostanes, resolvins, hydroxides, hydroperoxides, coming from EPA, ARA, and DHA, using SPE extraction prior to LC-MS/MS analysis.

The present trial revealed that stress during slaughter widely affected the formation of lipid oxygenated metabolites. Lipid mediators were found highly concentrated in stressed fish rather than in control. Metabolic biomarkers of stress were identified as well. The results suggest that suffering during slaughter affected the enzymatic oxidative response of fish shifted eicosanoid synthesis towards less inflammatory species derived from EPA. Additionally, the larger endogenous concentration of lipid oxygenated products formed can affect the stability of the final fish flesh leading a major susceptibility to oxidation.