EFFECT OF OXIDIZED DIETARY LIPID ON GROWTH, MUSCLE AND LIVER QUALITY OF JUVENILE ATLANTIC COD (*Gadus morhua*), AND THE PROTECTIVE ROLE OF VITAMIN E

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BY

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ABSTRACT

The effects of oxidized dietary lipid and the role of vitamin E on growth performance, health status, and muscle and liver quality of juvenile Atlantic cod (*Gadus morhua*) were evaluated over a 9-week feeding period. Four isonitrogenous experimental diets containing fresh or oxidized fish oil with or without added vitamin E (α-tocopherol or mixed tocopherols) were fed to juvenile cod in duplicate tanks. The highly oxidized lipid used had a peroxide value of 94 meq/kg oil.

No significant change ($P>0.05$) on growth performance or feed utilization was observed when oxidized dietary lipid was used. The erythrocyte osmotic fragility (EOF), referred to as susceptibility to hemolysis, of fish fed oxidized oil without added vitamin E was high in comparison with those fed unoxidized oil. Supplementation of α-tocopherol appeared to decrease the hemolysis, but mixed tocopherols had no significant effect ($P>0.05$) on EOF.

The proximate composition of fish whole body was also affected by oxidized oil and vitamin E. Fatty acid composition of liver total lipid reflected that of dietary lipid. However, muscle lipid contained high levels of polyunsaturated fatty acids (PUFA) compared to that of liver lipid, as expected. Both muscle and liver fatty acid composition followed the same trend among the treatments as those of dietary fatty acids. Fish fed fresh oil had a higher proportion of polyunsaturated fatty acids (PUFA) in muscle and liver lipid than those fed oxidized oil. Fatty acid composition of neutral lipids and phospholipids in fish muscle tissue was also analyzed. A larger quantity of

eicosapentaenoic acid (EPA) was present in neutral lipid fraction, while phospholipids contained a higher amount of docosahexaenoic acid (DHA).

Oxidized oil significantly ($P<0.05$) decreased the deposition of α-tocopherol in liver but not in muscle. Gamma- and δ-tocopherols from dietary tocopherols mixture were retained at very low levels in liver but higher retention was observed in muscle. The oxidative state of both liver and muscle, as measured by the 2-thiobarbituric acid reactive substances (TBARS) and headspace propanal, negatively correlated with tissue vitamin E levels.

It is suggested that oxidized oil affected juvenile Atlantic cod by causing vitamin E deficiency in certain tissues and that these effects could be alleviated by supplementation of a sufficient amount of vitamin E through diet. It is also indicated that mixed tocopherols were good antioxidants for Atlantic cod, although less effective than α-tocopherol alone in many tissues with the exception of muscle, where γ- and δ-tocopherols were deposited at considerably high levels. Due to the fact that muscles are the major edible part of fish, these results may be useful to fish farmers for producing fish with better oxidative stability and hence extended shelf life during storage.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AMPL	Acetone mobile polar lipid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
EEA	Essential amino acid
EFA	Essential fatty acid
EOF	Erythrocyte osmotic fragility
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FAO	Food and agriculture organization
FCE	Feed conversion efficiency
FFA	Free fatty acid
FID	Flame ionization detection
FPH	Fish protein hydrolysate
Fr-FO	Fresh fish oil
GC	Gas chromatography
GPX	Glutathione peroxidase
HSI	Hepatosomatic index
HPLC	High performance liquid chromatography

LPC	Lysophosphatidylcholine
MDA	Malondialdehyde
MONO	Monounsaturated fatty acid
MS	Mass spectrometry
NL	Neutral lipid
NPU	Net protein utilization
Ox-FO	Oxidized fish oil
PA	Phosphatidic acid
p-AnV	*para*-Anisidine value
PBS	Phosphate buffer solution
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PER	Protein efficiency ratio
PG	Propyl gallate
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
PV	Peroxide value
RBC	Red blood cell
RCS	Reactive chlorine species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAT	Saturated fatty acid
SE	Sterol ester

SGR	Specific growth rate
SOD	Superoxide dismutase
SPH	Sphingomyelin
ST	Sterol
TAG	Triacylglycerol
TBA	Thiobarbituric acid
TBARS	Thiobarbituic acid reactive substances
TBHQ	*tert*-Butylhydroquinone
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TMP	Tetramethoxypropane
TMR	Transmethylation reagent
UV-DAD	Ultra violet-diode array detector
VSI	Viscerosomatic index
WHO	World health organization

CHAPTER 1

LITERATURE REVIEW

1.1 Biology of Atlantic cod

Atlantic cod, *Gadus morhua*, has been the most important groundfish and dominant commercial species in the North Atlantic for centuries (Bowering and Atkinson, 2003). It belongs to Gadidae (the codfish) family, which contains 59 species, including haddock (*Melanogrammus aeglefinus*) and pollock (*Pollachius virens*). Atlantic cod inhabit cold waters (0°C-15 °C) overlying the continental shelves of the Northwest and the Northeast Atlantic Ocean, from western Greenland south to Cape Hatteras, North Carolina, and are most abundant from the coast of northern Labrador to the Nantucket Shoals region off Massachusetts (Lear, 1984). They are distributed over a wide range of depth zones, from inshore shallow water (about 5 m) to the edge of continental shelf (in water as deep as 600 m) (Lear, 1984). Cod is a medium to large marine fish with an average weight of 2 to 3 kg and an average length of 60 to 70 cm (Committee on the Status of Endangered Wildlife in Canada, 2003). It has three dorsal fins, two anal fins, and a broom shaped tail, and can be easily distinguished from most other fish species. The color of its back, which is covered with small and smooth scales, depending upon the habitat, ranges from grey or green to reddish brown with speckled spots. It has a large mouth with a projecting jaw, and the gill openings are wide (Lear, 1984). Cod are omnivorous, feeding at dawn or dusk on a variety of invertebrates, fishes, crabs, clams, and incidental plants.

At the first stage of life, cod exist as eggs and then larvae in the upper 50 meters of the ocean. Food availability and water temperature are the primary factors that affect habitat

suitability for cod during this early stage. After the larval stage, cod juveniles settle near the bottom and remain there for a period of 1 to 4 years to reduce the risk of predation. As cod grow older, they appear to avoid cold temperature, and the distribution becomes increasingly diverse. The age of maturity, referred to as the age at which 50% of females are reproductive, differs among the stocks. Cod in the Newfoundland and Labrador population mature at 6 years, while the Georges Bank cod stocks mature at 2.5 years (Committee on the Status of Endangered Wildlife in Canada, 2003).

Codfish migrate extensively each year. The pattern of this migration depends on numerous factors including season, geographic location, food supply, water temperature, and life history stage (e.g., spawning). The distance of migration ranges from tens of kilometers for some inshore populations to hundreds of kilometers for others. As a prolific fish species, cod releases a large number of eggs. For example, a female cod about 80 cm long produces about two million eggs, while a cod of about 130 cm produces over 11 million eggs (Lear, 1984). Spawning occurs over a wide area of the continental shelf and a wide range of bottom depths. The time of spawning also varies for different stocks. For example, spawning begins in May on the south coast of Newfoundland, while cod off Labrador and northern Newfoundland spawn from March to May (Committee on the Status of Endangered Wildlife in Canada, 2003). The mortality rate of fertilized cod eggs is immense. Of the millions of eggs each female spawns, only about one survives planktonic juvenile predators to become a mature cod. As a result of the extremely low reproduction and survival rates coupled with the long-term and large scale overfishing, Atlantic cod is now widely acknowledged to be an endangered species.

1.2 History of cod fishery and cod farming

Atlantic cod played a key role in the early colonization of North America. It has been credited as being a main reason for the settlement of Atlantic Canada, especially Newfoundland and Labrador (Bowering and Atkinson, 2003). It was cod that brought the first Europeans to Newfoundland in the late fifteenth century. The Portuguese began fishing the Newfoundland waters in the early 1500s followed by French and Spanish Basques. The cod fishery continued to expand both in Newfoundland and all along the Atlantic coast where cod were plentiful. By the late 1800s, the annual catch of cod in Newfoundland had reached 400,000 tons. Cod became an important economic commodity in the international market. A market for dried cod was developed in Europe by Norwegians, and versatile products of cod such as smoked or frozen roes, "cheeks" and "tongues" emerged in the cod trade and these were valued worldwide. Catches of cod from the northwest Atlantic reached a peak of 2,000,000 tons during the 1960s, but started to drop severely from the 1970s. Overfishing for decades led to a significant decline in wild cod stocks. According to government record, the annual yield of Atlantic cod decreased to around 250,000 tons throughout the 1980s. Fishing annually removed more than 70% of Newfoundland's northern cod commercial biomass in the late 1980s. In 1992, the Canadian Minister of Fisheries and Oceans declared a ban on fishing northern cod in order to allow the stocks to recover. In 2002, cod landings were only 35,718 tons, which reflected a decrease of 81% compared to 1992. The dramatic decline of wild cod stocks resulted in a shortage of cod product provision and thus the enhancement of its market value, necessitating the farming of cod as a commercial activity.

Atlantic cod is a cold-water fish with a high biological potential for aquaculture. Due to its high fecundity, ability to spawn naturally in captivity, high growth rate at low temperature and acceptable feed conversion efficiency, Atlantic cod is regarded as an excellent candidate for commercial farming (Howell, 1984). Atlantic cod culture has a history of over 100 years. The first attempt to cultivate cod started in Norway, at the Flødevigen Biological station, in 1884. At approximately the same time, a cod hatchery was built and operated in Woods Hole, USA (Moksness *et al.*, 2004). From 1920 to 1950, a large number of newly fertilized cod eggs were incubated in the laboratory, and released into the coastal waters of Norway and USA (Moksness *et al.*, 2004). However, complete artificial culture of cod from eggs to mature fish under laboratory conditions was only accomplished in 1977 (Hognestad, 1984). Intensive farming began in the middle of the 1980s. In Newfoundland, small cod taken in the commercial fishery were grown up in pens (Brown and Puvanendran, 2002). However, farming was unpredictable and labor intensive. Large scale cultivation of cod larvae has been possible for some years, until the profitable commercial cod product was hindered by the low market value and high production costs that far exceeded the possible revenues (Morais *et al.*, 2001). Recently, the overexploitation and depletion of natural stocks as well as the enhanced market value of cod have brought a renewed interest in this species (Adault, 2001; Morais *et al.*, 2001). A recent study on cod production in the United Kingdom revealed that the prospects for commercial cod aquaculture appeared more promising than a couple of years earlier (Dalton and Waning, 2004). Atlantic cod farming has attracted great attention in the North Atlantic region, including Northern Europe and Canada, and a commercial cod aquaculture industry is developing.

1.3 Uses of cod products

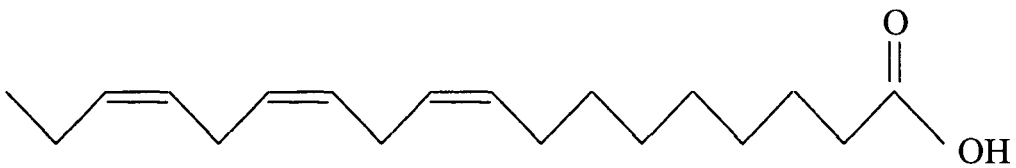
1.3.1 Cod fillet

Atlantic cod is one of the most widely utilized fish in the western world. This fish has a mild flavor and delicate texture and is used as a well recognized food fish. The flesh of cod is composed of firm, non-oily, white tissue that deteriorates relatively slowly compared to that of other marine fish. Cod fillets, either in fresh, dried, frozen, salted or smoked form, can be baked, poached, steamed, grilled, sauteed, deep-fried for fish and chips, seafood salads, casseroles and chowders, which are popular dishes for fish lovers. As a lean fish, Atlantic cod is low in calories and serves as a rich source for proteins and essential nutrients such as n-3 fatty acids as well as iodine and some B vitamins (Hoppner and Lampi, 1989; Anderson *et al.*, 2002).

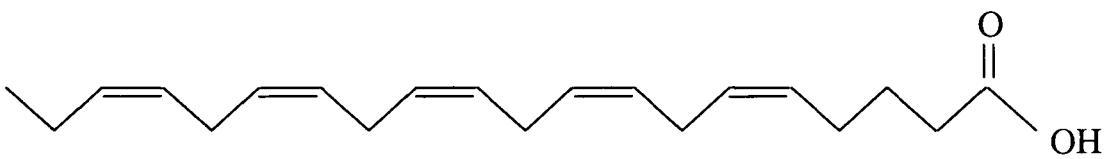
1.3.2 Cod liver oil

In addition to food applications, cod is also known for production of cod liver oil. Atlantic cod has a large and fatty liver containing a high proportion of lipid (70% of the liver weight) (Shahidi and Dunajski, 1994). Cod liver oil is rich in polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been shown to be beneficial to human health, and serves as an excellent source for supplementation of these fatty acids as well as fat soluble vitamins, principally A and D. Unlike the n-6 (or ω6) fatty acids found in most vegetable oils, both EPA and DHA belong to the n-3 (or ω3) fatty acids family (Figure 1.1), which has attracted increasing interest in recent years from nutritionists and health professionals. With vegetable oils being the main source of our edible oils, the ratio of n-6/n-3 fatty acids in

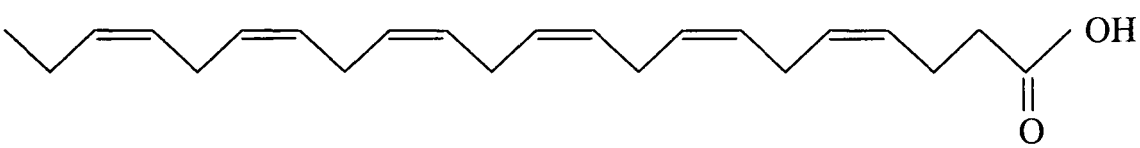
Figure 1.1 Chemical structures of n-3 fatty acids: ALA, α-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid



α-linolenic acid (ALA)



eicosapentaenoic acid (EPA)



docosahexaenoic acid (DHA)

the human diet (20/1 to 50/1) far exceeds the ratio recommended (5/1 to 10/1) by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). Insufficiency of n-3 fatty acids in the diet has been linked to a number of diseases and health disorders.

Recognition of the health benefits associated with consumption of n-3 fatty acids (mainly from seafoods) is one of the most promising developments in human nutrition and disease prevention research in the past three decades. According to our current knowledge, long-chain n-3 PUFA play an important role in the prevention and treatment of coronary artery disease (Schmidt *et al.*, 2000), hypertension (Howe, 1997), diabetes (Krishna Mohan and Das, 2001), arthritis and other inflammatory (Babcock *et al.*, 2000), and autoimmune disorders (Kelly, 2001), as well as cancer (Rose and Connolly, 1999; Akihisa *et al.*, 2004). Long chain n-3 PUFA are essential for normal growth and development, especially for brain and retina (Anderson *et al.*, 1990). Supplementation of n-3 PUFA during pregnancy, especially during the last trimester is highly recommended. The beneficial effects of PUFA have also been ascribed to their ability to lower serum triacylglyerols (Howell *et al.*, 1998), to increase membrane fluidity and to reduce thrombosis by conversion to eicosanoids (Kinsella, 1986). Both EPA and DHA, induced increases in the serum concentrations of the corresponding fatty acids as well as their relative contents in platelets (Vognild *et al.*, 1998). The n-3 PUFA are also attractive from a nutritional point of view because there is growing evidence that they provide specific physiological functions against thrombsis, cholesterol build-up and allergies (Kimoto *et al.*, 1994). The health benefits of n-3 PUFA from marine oils have recently been reviewed (Shahidi and Miraliakbari, 2004; Shahidi and Zhong, 2005a).

Cod liver oil contains 32% n-3 PUFA (Copeman and Parrish, 2004), that are hard to obtain in sufficient amounts from our regular diet, and is frequently used in nutrient supplementation as well as medical treatment. It is also a good source for vitamin A (1000IU/g) and vitamin D (10 IU/g). The fatty acid composition of cod liver oil includes 23% saturated fat, 47% monounsaturated, 7% EPA and 11% DHA (Shahidi and Miraliakbari, 2004). The characteristic "fishy" odor or taste of raw cod liver oil was a barrier to its use by some consumers. Nevertheless, convenient, easy digestable and good tasting cod liver oil products are now commercially available following purification, encapsulation and addition of flavorants to mask the original taste of the raw oil. More recently, concentration of n-3 PUFA from marine fish oil has been studied and applied in industrial production. It has been suggested that PUFA concentrates devoid of saturated fatty acids are much better than marine oils themselves since they allow keeping the daily intake of total lipids as low as possible (Haagsma *et al.*, 1982). Concentration of PUFA or their esters can be accomplished by certain techniques, including fractional vacuum distillation (Ackman *et al.*, 1973), low-temperature crystallization (Brown and Kolb, 1955), chromatographic separation such as HPLC (Roggero and Coen, 1981; Halgunset *et al.*, 1982a; Aveldano *et al.*, 1983) and silver resin chromatography (Adolf and Emken, 1985), supercritical fluid extraction (Nilsson *et al.*, 1988), urea complexation (Grompone, 1992; Wanasundara and Shahidi, 1999), and enzyme-assisted hydrolysis (Wanasundara and Shahidi, 1998), among others, as summarized by Shahidi and Zhong (2005a).

1.3.3 Cod by-products

Although fillet and liver are the main products in the cod industry, the utilization of by-products from cod processing has been widely studied (Venugopal and Shahidi, 1995). Fish by-products contain proteins with excellent amino acid composition and digestibility (Anderson *et al.*, 2002; Sovik and Rustad, 2004). It was found that cod heads, viscera, frames and skins had over 90% pepsin digestibility, suggesting that these by-products can be used as animal feed ingredients as well as possibly for human consumption after hydrolytic degradation (Bechtel and Johnson, 2004). Hydrolysis of fish by-products can be performed by enzymatic and microbial processes, providing fish protein hydrolysate (FPH) as final products, which have uses in the food industry as thickeners and stabilizers, among others (Kim *et al.*, 1997; Slizyte *et al.*, 2004; Sovik and Rustad, 2005; Slizyte *et al.*, 2005a; Slizyte *et al.*, 2005b). Additionally, collagen and gelatin have been prepared from cod skin and bones, and used as a processing aid or ingredients in food (Sadowska *et al.*, 2003). By-products from cod are also a source of enzymes for biotechnological applications (Gildberg, 2004). For example, intestinal tryptic enzymes in cod have been found to be useful in pharmaceutical ointment preparations for treatment of skin irritations (Bjarnason, 1996). Another valuable component of cod by-products is lipid. Cod roes are currently exploited for lipid production, particularly phospholipids and n-3 long chain PUFA (Dumay *et al.*, 2004). However, commercial viability of these processes and products remains to be evaluated.

As already noted, Atlantic cod has been a well recognized and utilized fish species, and is now attracting growing interest from fish farmers. The versatile applications of cod

products have provided a huge opportunity for cod farming, a rapidly expanding sector of aquaculture.

1.4 Introduction of cod farming

1.4.1 Farming conditions

Successful farming of cod depends on several factors, including proper physical farming conditions, feeding methods, dietary variables, and management of disease and mortality. Generally, in order to obtain optimal fish performance (survival, growth and reproduction), fish must be cultured within the range of their environmental requirements. Farmed fish interact with their environment in two main ways: through environmental requirements such as light, temperature and water quality, and via their surroundings and biota such as their excreted products, wastes, diseases, parasites and their infrastructure requirements of culture sites (Black, 1998). As farmed fish have no means of physical escape, unlike wild fish, the environment must be appropriate for their physiological adaptation, and must be able to buffer culture-induced changes in water or sediment quality which have detrimental effects on fish (Black, 1998).

Water quality is the most important aspect of cod farming conditions. Seawater after certain treatments such as filtration and ultraviolet treatment is supplied for culture of marine fish. Water quality, in terms of temperature, dissolved oxygen, salinity, ammonia, carbon dioxide, hydrogen sulfide and algae blooms, is monitored regularly and adjusted to the optimal levels for the specific fish and the specific developmental stage of the fish (Black, 1998; Howell and Baynes, 2004). Oxygen content is an essential factor in fish farming because of its vital role in driving metabolic processes of fish. Suppressed food

intake has been observed when oxygen is in short supply (Black, 1998). Sub-optimal dissolved oxygen levels also give rise to increased stress and disease susceptibility, reduced growth, survival and feed conversion efficiency (Howell and Baynes, 2004). Water currents coupled with aeration are mostly employed for supply of oxygen to fish tanks in on-land aquaculture. Water temperature has a marked influence on fish growth through the effect on appetite, digestion and metabolic rate. With respect to Atlantic cod, the optimum temperature is 9.7-13.4°C for larvae, and 11-15°C for small size fish (50-1000 g), and 9-12°C for large size fish (1.5-2.5 kg) (Howell and Baynes, 2004). Water temperature acts in synergy with other environmental parameters such as oxygen concentration, and photoperiod (Black, 1998). Other water quality factors such as salinity, ammonia, carbon dioxide, hydrogen sulfide and algae blooms also have impacts to some extent on fish performance, which have been reviewed by Howell *et al.* (1998, 2004), Olsen *et al.* (2004), Black (1998), Pickering (1998), and Sayer (1998), among others. Growth and reproduction of farmed cod can be affected by light conditions. The quality, intensity and photoperiod of light are the three factors that need to be considered to achieve optimum performance. Comparisons of effects of different sources of light (natural or artificial light); various light intensity and photoperiod on farmed fish have been studied (Sayer, 1998; Howell and Baynes, 2004; Hemre *et al.*, 2004; Svasand *et al.*, 2004). Knowledge of microbial interactions (Vadstein *et al.*, 2004), parasites (Sommerville, 1998), immune defence systems (Manning, 1998), stress responses (Pickering, 1998) as well as disease management (Vadstein *et al.*, 2004) is also indispensable for growth and mortality rate control.

1.4.2 Feeding methods

Feeding methods play an important role in the success of fish culture from both biological and economical points of view. Farmed fish, other than wild species, obtain the required nutrients for their growth and maintenance from feed provided by farmers. The growth performance and product quality are much dependant on the quality and quantity of feed. It has been proven that improvements in feed and feeding regime could reduce the time required for grow-out, increased protein retention and decreased cod liver size (Rosenlund *et al.*, 2004). Meanwhile, feed and feeding account for a high proportion of the cost of aquaculture production. Therefore, fish farmers should balance both aspects to attain maximum profit from fish farming. The time, frequency and quantity of feed supply should be optimized to ensure that they meet the demand, but without being excessive (Jobling, 2004).

1.5 Cod nutrition

Fish nutrition is one of the most important issues in aquaculture, especially in intensive fish culture. A strong correlation of nutrient consumption with growth performance, immune response and disease control has been noticed (Lall, 1988; Lall and Oliver, 1993). Diet variables significantly affect the yield, time for grow-out, sensory quality and nutritional value of farmed fish. Researches aiming at assessing the qualitative and quantitative nutritional requirement of cultured fish have been carried out to establish the optimum feed composition for given fish species. The development of aquaculture feed is usually based on nutrient requirements, digestion, absorption and retention of major nutrients, and energy utilization from various feed ingredients (Lall *et al.*, 2003). In most

nutrition research, juvenile fish are generally selected for feeding trials as they are more vulnerable to unfavorable nutritional conditions than larger fish, and the pronounced response such as depressed growth and deficiency symptoms are easier to observe (Jobling, 1998).

1.5.1 Carbohydrates

A typical diet for carnivorous fish such as cod should be low in carbohydrate due to their limited capacity for carbohydrate digestion, and high in protein due to their potential for a high growth rate (Stoss and Ottera, 2004). Carbohydrate may serve as a cheap energy source; nevertheless, the main reason for inclusion of carbohydrates in diet formulation is their binding properties. During feed preparation, gelatinization of starch occurs under certain treatments such as heating, moisturization, pressurization, and mechanical shear, and imparts thickening and gel-forming properties to the mixture of diet ingredients (Jobling, 2004). Carbohydrates take part in fish metabolism as well as working as a processing aid. It was found that feeds containing small amounts of highly available starches resulted in protein sparing and growth stimulation compared with those without starch (Rosenlund *et al.*, 2004). Cereals, maize, potato and their by-products are commonly used for starch supply. In addition to starch, other carbohydrates including alginate, cellulose ethers, and lignin and hemicellulose derivatives may be used as thickening and gelling agents in fish feeds (Jobling, 2004). However, starch digestion is limited in cod owing to the low activities of both digestive and metabolic enzymes, and depends on the concentration, source, and physical state (gelatinization grade) of the starch (Rosenlund *et al.*, 2004). Therefore, low levels (Halgunset *et al.*, 1982b) of

digestible carbohydrate are recommended in feed formulation for cold-water marine species (Jobling, 2004).

1.5.2 Proteins

Fish require an adequate supply of dietary protein, as it is a source of both nutrients and energy. Evidence suggests that cod farming demands a high protein content in the diet to promote growth rate and feed utilization, without causing excessive accumulation of lipid in the liver (Morais *et al.*, 2001). The quality of protein depends upon the composition of its fundamental components, amino acids, which are also important precursors or nitrogen sources for a variety of biomolecules (Olsen *et al.*, 2004). Amino acids, as the constituents of proteins, can be classified into essential amino acids (EAA) and non-essential amino acids. Essential amino acids are defined as those can not be synthesized or can not be synthesized in sufficient amounts to allow maintenance of good growth rates (Jobling, 2004). The EAA for fish include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, cystine and tyrosine (Olsen *et al.*, 2004). The EAA requirements of certain fish species appear to correlate with the EAA profile of their whole body or muscle proteins, indicating that information of body protein composition can be used for determining EAA requirement of farmed fish (Kaushik, 1998). The EAA pattern of Atlantic cod muscle protein is given in Table 1.1.

The level of protein inclusion in feeds differs among fish species. Formulated diets for intensive farming of carnivorous fish typically contain 40-50% protein (Jobling, 1998). The recommended level of protein in cod growout diets is 48-60% (Lall and Nanton,

Table 1.1 The essential amino acid composition of the fillet of Atlantic cod (*Gadus morhua*).

Essential Amino Acids	Content (g/kg protein)
Arginine	55
Histidine	22
Isoleucine	39
Leucine	72
Lysine	88
Methionine	28
Phenylalanine	39
Threonine	39
Tryptophan	11
Valine	39

Adapted from (Jobling, 2004).

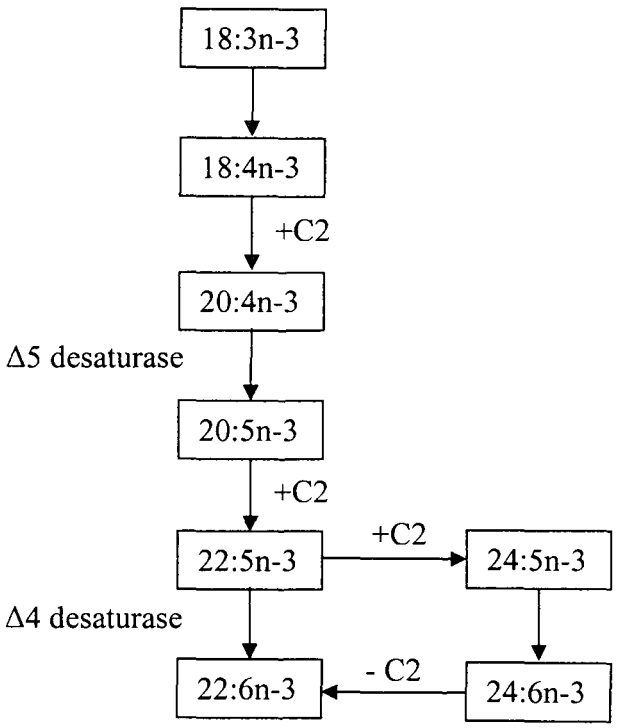
2002). However, it was found that protein retention in Atlantic cod decreased when protein supply was over 46%, and the best protein retention was observed in fish given diets containing 32-46% protein (Rosenlund *et al.*, 2004). Generally, fish meals, silages, and other fish-based products are considered as high quality sources of protein for farmed fish. Nevertheless, the large demand for wild fish for aqua feeds has brought concerns about the non-sustainable overexploitation of marine species. Many researchers have addressed the issue of replacement of fish meal with plant proteins in fish diets (Turchini *et al.*, 2003).

1.5.3 Lipids

Lipid is probably the most thoroughly studied macronutrient in aquaculture. Dietary lipids play an important role in fish nutrition for providing both essential fatty acids and energy as well as assisting the absorption of fat-soluble vitamins (Lee *et al.*, 2003). Dietary lipids have a protein-sparing effect that allows a more efficient use of protein in diets and lower nitrogen loss (Morais *et al.*, 2001; Regost *et al.*, 2003). It has been found that reasonably increased dietary lipid levels improved growth, feed conversion efficiency and protein utilization, thus reducing nitrogen excretion in farmed fish (Steffens, 1993; Izquierdo *et al.*, 2003). Lipids are generally stored in depot organs or adipose tissue as triacylglycerols (Howell *et al.*, 1998), a neutral and abundant storage lipid which provides metabolic energy through oxidative catabolism (Durazo-Beltran *et al.*, 2003). Phospholipids (PL), grouped as polar lipids, are a major component of cellular membranes, and are of both structural and functional importance (Jobling, 1998). Both neutral lipids and phospholipids contain essential fatty acids (EFA) but in different

proportions. Aquatic animals are characterized by having PL containing a high fraction of EFA. EFA in all animals are the polyunsaturated fatty acids (PUFA) that cannot be synthesized *in vivo* and must be supplied by diet to satisfy the requirement for normal cellular function (Bell, 1998). EFA are divided into two families depending on the positions of the double bonds in PUFA molecules: n-6 and n-3 families. Fish have an absolute requirement for long chain n-3 PUFA such as eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), and almost certainly for n-6 PUFA such as arachidonic acid (20:4 n-6, AA) (Howell *et al.*, 1998). Generally, n-3 fatty acids dominate n-6 fatty acids by a factor of 5-20 in marine food webs (Olsen *et al.*, 2004). In most of the aquatic organisms the function of both n-3 and n-6 PUFA in the tissue appears to be particularly structural/functional and their use as an energy source is limited (Durazo-Beltran *et al.*, 2003). EPA, DHA and probably AA are important structural/functuional and physiological components of cell membranes and are thought to contribute to permeability, enzyme activity and other functions in polar lipids of biomembranes (Lee *et al.*, 2003). Some aquatic species, including freshwater fish and invertebrates, can elongate and desaturate dietary linoleic acid (18:2n-6) or linolenic acid (18:3n-3) (Figure 1.2) to satisfy or partially satisfy their nutritional requirements for PUFA like AA, EPA and DHA, and thus biosynthetic ability varies from species to species (Durazo-Beltran *et al.*, 2003). Marine fish are incapable of these conversions and a dietary source of these EFA is necessary for their normal growth. The EFA requirements of marine fish species range from 0.5 to 2.0% of the dry feed, and are influenced by the temperature and salinity of water (Lee *et al.*, 2003). The best sources of long chain n-3 PUFA are marine fish oils. Marine fish oils have been used as a traditional

Figure 1.2 Pathways of elongation and desaturation of 18:3 n-3 as identified in rainbow trout (Bell, 1998)


18:3n-3
18:4n-3
+C2
20:4n-3
Δ5 desaturase
20:5n-3
+C2
22:5n-3
+C2
24:5n-3
Δ4 desaturase
22:6n-3
- C2
24:6n-3

source of lipid in feeds for intensive fish culture. However, the demand for fish oil by the aquaculture industry is predicted to exceed available resources within the next decade (Rosenlund *et al.*, 2001). Therefore, the search for alternative lipid sources for aqua feeds is a prerequisite for further development of aquaculture. Investigations have been undertaken in partial replacement of fish oils with plant oils in formulated feeds. The lipid content in fish feeds should be optimized, as insufficient dietary lipid may cause deficiency of n-3 PUFA in fish, whereas excessive dietary lipid may affect fish product quality. Increasing the dietary lipid level can result in increased lipid concentration in fish whole body and increased viscerasomatic index (VSI) and viscera lipid concentration, which is considered a waste of digestible calories from feed, and against the economical concern of fish processors as well (Chaiyapechara *et al.*, 2003). Optimization of the lipid/protein ratio in feed of Atlantic cod has been discussed (Lie *et al.*, 1988; Morais *et al.*, 2001). In addition to optimized lipid content in fish diet, proper storage of feed and addition of antioxidants are important. These protect against lipid oxidation in feed and against *in vivo* oxidative stress of fish, which could affect the health status of fish and the flavors and nutritional value of fish products. Vitamins E and C are the commonly used natural antioxidants in fish diet, in place of synthetic antioxidants that are not favored by consumers. Details about lipid oxidation will be discussed in the following section.

1.5.4 Micronutrients

Micronutrients are those dietary components that are required by animals in relatively small amounts for the maintenance of optimal health, growth and reproduction (Jobling, 2004). Micronutrients consist of essential minerals, vitamins and vitamin-like substances

such as carotenoids. Essential minerals including trace and ultra-trace minerals have a variety of functions in fish. Calcium, magnesium and phosphorus are important structural components of skeleton; sodium, potassium and chlorine contribute to acid-base balance and the maintenance of cell homeostasis; zinc and copper serve as co-factors in enzymatic biochemical reactions (Jobling, 2004). A general discussion on minerals in fish nutrition is available (Lall, 1989). Vitamins are complex organic compounds that are required in trace amounts but fulfil several vital functions such as acting as co-enzymes (Jobling, 2004). Vitamin supplementation through diets is strongly recommended to avoid deficiency symptoms and consequently achieve good growth and survival rate of farmed fish. Vitamin-like substances such as carotenoids, inositol and choline play a part in fish metabolism and function as constituents of cell membrane phospholipids as well as previtamins. Detailed information of micronutrients and their biological and physiological roles in fish is given in Table 1.2.

Generally, in order to develop efficient and economical feed formulas for aquaculture, basic information is required on nutrition requirements of the species cultivated, the chemical composition, organoleptic properties and digestibility of feed ingredients, as well as utilization and economical cost in feeds. Concepts in the formulation and preparation of a complete fish diet have been reviewed (Lall, 1991). With respect to Atlantic cod farming, a diet containing high amounts of protein (48-60%), low carbohydrate (10-15%), and low lipid (<15%) with a sufficient amount of n-3 long chain PUFA (1-1.5% EPA and DHA) and well fortified with vitamins and trace elements is recommended for initial feed formulations of cod grow-out diets (Lall and Nanton, 2002).

Table 1.2 Micronutrients and their functions [1]

Biological or physiological role	Micronutrients
Minerals	
Ionic regulation (electrolytes)	Sodium, potassium, chlorine
Acid-base balance	Calcium, sodium, chlorine
Structural functions: bone/skeletal tissue, cell membranes	Calcium, phosphorus, magnesium, sulfur
Nerve impulse transmission and muscle contraction	Calcium, sodium, potassium
Respiratory pigment (haemoglobin)	Iron
Component of hormones	Iodine, sulfur
Enzyme structure and function: component of enzyme, co-factor or component of co-factor, activator or regulator	Zinc, selenium, cobalt, manganese, chromium, vanadium
Vitamins	
Normal growth, vision, reproduction	Retinol (vitamin A)
Calcium and phosphate metabolism/regulation	Cholecalciferol (vitamin D)
Antioxidants	Tocopherols (vitamin E), Ascorbic acid (vitamin C)
Energy metabolism	Thiamin (vitamin B1), Niacin (nicotinic acid), Pantothenic acid (vitamin B5)
Nerve function	Thiamin (vitamin B1), Niacin (nicotinic acid), Pantothenic acid (vitamin B5), Cyanocobalamine (vitamin B12)
Blood clotting	Menadione (vitamin K)
RBC formation and function	Cyanocobalamin (vitamin B12)
Muscle and RBC function	Tocopherols (vitamin E)
Fatty acid synthesis, glucose metabolism	Biotin (vitamin H)
Embryonic development, gut function	Folacin (folate)
Collagen synthesis, immune responses	Ascorbic acid (vitamin C)
Vitamin-like substances	
Antioxidant, provitamin A	Carotenoids
Cell membrane phospholipids	Inositol (myo-inositol), Choline
Chemical signal transmission	Inositol (myo-inositol)
Neurotransmission functions	Choline
Lipid/fatty acid metabolism	Choline, Carnitine

[1]Adapted from (Jobling, 2004).

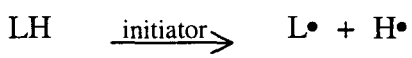
1.6 Lipid oxidation

1.6.1 Introduction of lipid oxidation

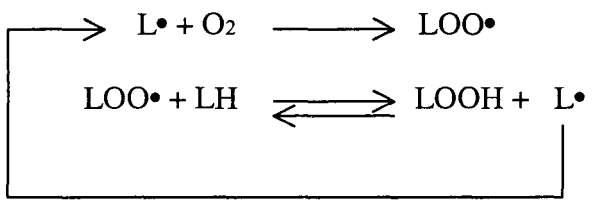
Lipid oxidation is a major cause of food quality deterioration and has been a challenge for manufacturers and food scientists alike. Concerns about lipid oxidation have also been addressed in the aquaculture industry due to the large requirement of long-chain n-3 PUFA in fish diet and the high susceptibility of these PUFA to oxidation. Lipid oxidation may occur both in dietary oil and in fish body fat; the former may have a potential effect on the growth and health status of the fish receiving the oxidized feed, while the latter may ultimately influence the flavor and nutritional qualities of fish products.

Lipids are susceptible to oxidative processes in the presence of catalytic systems such as light, heat, enzymes, metals, metalloproteins and microorganisms, giving rise to the development of off-flavors and loss of essential fatty acids, fat-soluble vitamins and other bioactives. Lipids may undergo autoxidation, photooxidation, thermal oxidation and enzymatic oxidation under different conditions, most of which involve some type of free radical and/or oxygen species (Shahidi, 2000). Autoxidation is the most common process leading to oxidative deterioration and is defined as the spontaneous reaction of atmospheric oxygen with lipids (Gordon, 2001). Unsaturated fatty acids are generally the reactants affected by such reactions, whether they are present as free fatty acids, triacylglycerols (as well as diacyglycerols or monoacylglycerols) or phospholipids (Gordon, 2001). It has been accepted that autoxidation of unsaturated fatty acids occurs via a free radical chain reaction that proceeds through three steps of initiation, propagation, and termination (Kamal-Eldin *et al.*, 2003). A simplified scheme explaining the mechanism of autoxidation is given below.

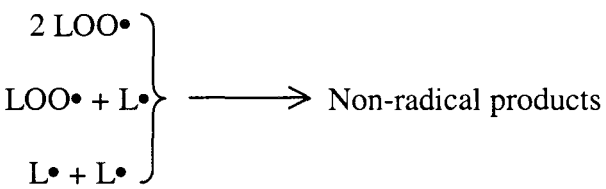
Initiation:



Propagation:



Termination:



Oxidation normally proceeds very slowly at the initial stage, and reaches a sudden increase in oxidation rate after the induction period. Lipid hydroperoxides have been identified as primary products of autoxidation; decomposition of hydroperoxides yields aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds, known as secondary oxidation products. These compounds constitute the basis for measurement of oxidative deterioration of lipids.

1.6.2 Methods of measuring lipid oxidation

Numerous analytical methods are routinely used for measuring lipid oxidation in foods. It is important to select a proper and adequate method for a particular application. Methods to monitor lipid oxidation in foods can be classified into five groups based on what they

measure: the absorption of oxygen, the loss of initial substrates, the formation of free radicals, and the formation of primary and secondary oxidation products (Dobarganes and Velasco, 2002). The available methods for measuring lipid oxidation have been summarized by Shahidi and Zhong (2005b).

Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) are the most frequently used parameters for evaluation of lipid oxidation. The PV represents the total content of hydroperoxides. Lipid oxidation involves the continuous formation of hydroperoxides as primary oxidation products which may break down to a variety of non-volatile and volatile secondary products (Melton, 1983; Dobarganes and Velasco, 2002). The formation rate of hydroperoxides outweighs their rate of decomposition during the initial stages of oxidation, and this becomes reversed at later stages. Therefore, peroxide value (PV) is an indicator of the initial stages of oxidative change (Riuz *et al.*, 2001a). A number of methods have been developed for determination of PV, among which the iodometric titration, ferric ion complex measurement spectrophotometry, and infrared spectroscopy are most commonly used (Yildiz *et al.*, 2003).

The TBA test measures the concentration of malondialdehyde (MDA), a secondary oxidation product formed as a result of the degradation of PUFA and related compounds. The MDA can react with thiobarbituric acid (TBA) to form a pink MA-TBA complex that is measured spectrophotometrically at its absorption maximum at 530-535 nm. The extent of oxidation is reported as the TBA value and is expressed as milligrams of MDA equivalents per kilogram sample or as micromoles of MDA equivalents per gram of sample. This is because alkenal and alkadienals also react with the TBA reagent and produce a pink colored chromogen. Thus the term thiobarbituric acid reactive substances

(TBARS) is now used instead of MDA. The TBA test is one of the most extensively adopted assays in aquaculture for measuring lipid oxidation in fish tissues. Fish samples are used directly without lipid extraction, thus avoiding further oxidation during processing.

Headspace propanal is also a recommended indicator for lipid oxidation in foods that are high in n-3 fatty acids, such as marine fish products. Propanal is one of the major secondary products formed during the oxidation of n-3 fatty acids and is a main volatile responsible for off flavor during processing and storage of food products. Gas chromatographic analysis of headspace propanal provides a simple and rapid means of determining lipid oxidation in foods. It allows the injection of clean aliquots of volatile compounds from the headspace produced from foods or biological systems and does not require sample preparation (Medina *et al.*, 1999). Other methods have also been employed to evaluate lipid oxidation in foods, such as conjugated dienes, *p*-anisidine value (*p*-AnV), carbonyl value and sensory tests, among others. Selection of a proper method is important and should be based on the system under investigation and the state of oxidation itself.

1.7 Antioxidants

1.7.1 Antioxidant mechanism

Antioxidants are substances that when present in foods at low concentrations compared to that of an oxidizable substrate markedly delay or prevent the oxidation of the substrate (Shahidi, 2000). Antioxidants that fit in this definition include free radical scavengers, inactivators of peroxides and other reactive oxygen species (ROS), chelators of metals,

and quenchers of secondary lipid oxidation products that produce rancid odors (Decker, 1998). These substances may occur naturally in foods, such as tocopherols, ascorbic acid and some phenolic compounds, or be synthesized and used as food additives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ). Antioxidants have also been used in the health-related area because of their ability to protect the body against damage caused by ROS as well as reactive nitrogen species (RNS) and those of chlorine (RCS) (Shahidi, 1997). Antioxidants can be broadly classified by their mechanism of action as primary antioxidants, which break the chain reaction of oxidation by hydrogen donation and generation of more stable radicals; and secondary antioxidants, which slow the oxidation rate by several mechanisms, including chelation of metals, regeneration of primary antioxidants, decomposition of hydroperoxides and scavenging of oxygen, among others (Shahidi and Zhong, 2005c). Primary antioxidants such as tocopherols and some phenolic compounds inhibit the chain reaction of oxidation by acting as hydrogen donors or free radical acceptors, as shown below.

$$ROO\bullet + AH \longrightarrow ROOH + A\bullet$$

The inhibitor reaction is considered in competition with the propagation reaction of lipid oxidation and yields stable products that will not initiate new free radicals or bring about a rapid oxidation by a chain reaction (Nawar, 1996).

Addition of antioxidants has been carried out in many food systems in order to minimize or negate oxidative deterioration of lipid. Recently, indirect incorporation of antioxidants through animal feed has attracted more attention due to the growing resistance of consumers against the direct inclusion of food additives in food products. Natural

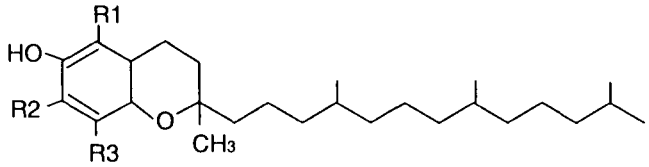
antioxidants such as tocopherols, ascorbate and retinol are added into fish feed to prevent the oxidative damage of dietary oils, and possibly, to afford potential protection to fish tissues by increasing the concentrations of the antioxidants *in vivo* (Baker and Davis, 1997a).

1.7.2 Tocopherols

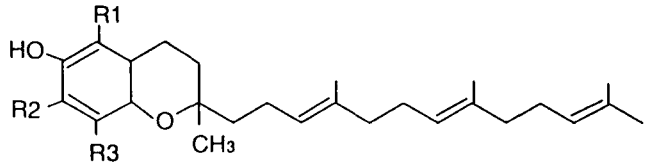
Tocopherols and tocotrienols, also known as vitamin E, are natural antioxidants that are extensively used to increase the oxidative stability of foods. They are distributed widely in plant tissues. The main commercial source of natural tocopherols is the soybean oil. Tocotrienols, less common than tocopherols, are present in palm oil and rice bran oil, as well as cereals and legumes (Reische *et al.*, 1998). Tocopherols and tocotrienols are classified into α-, β-, γ- and δ-, depending on their chemical structures (Figure 1.3).

Among tocopherols, α-tocopherol is the most commonly used vitamin E homologue because it has the highest vitamin E activity and occurs abundantly in natural sources. In aquaculture, α-tocopherol is added to fish feed as an antioxidant to prevent oxidation of dietary lipid, and as a supply of vitamin E to satisfy the nutritional requirement. Vitamin E is an indispensable nutrient in fish nutrition in order to maintain flesh quality, immunity, normal resistance of red blood corpuscles to hemolysis, permeability of capillaries and heart muscle (Sau *et al.*, 2004). The importance of vitamin E in aquaculture has been reviewed by Watanabe (1990). Other than its vitamin E activity, α-tocopherol is a potent biological antioxidant that can protect biological membranes and lipid components containing unsaturated fatty acids against attack by oxygen free radicals (Huang and Huang, 2004). Improved fish survival, growth, and health status by

Figure 1.3 Chemical structures of tocopherols and tocotrienols



R1	R2	R3	
CH_3	CH_3	CH_3	α-tocopherol
CH_3	H	CH_3	β-tocopherol
H	CH_3	CH_3	γ-tocopherol
H	H	CH_3	δ-tocopherol



R1	R2	R3	
CH_3	CH_3	CH_3	α-tocotrienol
CH_3	H	CH_3	β-tocotrienol
H	CH_3	CH_3	γ-tocotrienol
H	H	CH_3	δ-tocotrienol

supplementation of α-tocopherol have been observed (Tocher *et al.*, 2003). Alpha-tocopherol also serves as an antioxidant in the post-mortem tissues. Dietary α-tocopherol supplement partly inhibits the oxidative processes responsible for muscle lipid darkening during cold storage (Stephan *et al.*, 1995).

Although α-tocopherol has been the focus of vitamin E studies, γ- and δ-tocopherol are claimed to have stronger antioxidant activity *in vitro*. Generally, the antioxidant activity of tocopherols is in the order of δ- > γ- > β- > α-tocopherol (Shahidi, 2000). Oils such as canola or soybean oil containing the most α-linolenic acid (18:3n-3) seems to be richer in γ-tocopherol than in α-tocopherol but δ-tocopherol usually plays a lesser role. This suggests that γ-tocopherol is superior to α-tocopherol as a natural antioxidant in the milieu of vegetable oil triacylglycerols (Ackman *et al.*, 1997). Results from an *in vitro* test revealed that γ-tocopherol was 1.4 times as effective as α-tocopherol in inhibiting oxidation of polyunsaturated fatty acids (Wu *et al.*, 1979). Nevertheless, α-tocopherol has been considered a more potent antioxidant than γ-tocopherol *in vivo*. The difference among tocopherol homologues in their transportation, deposition, distribution and retention in animal tissues may probably account for the varied biological antioxidant efficiency *in vivo*. Investigations have been undertaken to evaluate the impact of different tocopherols in diet on the quality of farmed fish (Sigurgisladottir *et al.*, 1994; Ackman *et al.*, 1997; Hamre and Lie, 1997; Hamre *et al.*, 1998). Mixed tocopherols are cheaper sources than purified or synthetic α-tocopherol, which helps reduce the economical cost of fish farming. Ample quantities of natural tocopherol mixtures can be obtained from by-products of vegetable oils production such as the distillate of soybean oil deodorization.

1.8 Oxidized dietary lipid in aquaculture

Dietary lipids serve as a good energy source resulting in a sparing action on dietary protein and also provide EFA resulting in good growth and feed utilization. However, these lipids are susceptible to oxidation owing to their high PUFA content. The positive nutritional value of n-3 PUFA in fish lipids may become a negative factor for fish if diets are not properly prepared or stored to avoid lipid oxidation (Watanabe and Takeuchi, 1989).

Many studies on cultured fish have reported reduced growth of fish consuming oxidized oils (Hashimoto *et al.*, 1966; Watanabe and Takeuchi, 1989; Stephan *et al.*, 1991; Baker and Davis, 1996a; Tocher *et al.*, 2003). One of the possible reasons could be the reduced palatability of the rancid oils that causes food rejection of fish. Furthermore, digestibility of nutrients was found to be influenced by lipid oxidation. It was hypothesized that oxidized oils interfere with proper functioning of the surface cells of intestinal villae, thus reducing the absorption of nutrients (Ackman *et al.*, 1997). Oxidized oils are also known to be toxic for various fish species. Lipid oxidation is thought to be highly deleterious, causing biomembrane damage and being implicated in several pathological conditions in fish (Tocher *et al.*, 2003). Oxidized dietary lipid induced diseases like exudative diathesis, muscular dystrophy and depigmentation, as demonstrated by Watanabe and Takeuchi (1989). Secondary oxidation products such as aldehydes, ketones, alcohols, alkanes and alkenes are the main toxic metabolites responsible for the adverse effects of rancid oils on fish. Some of these compounds, especially aldehydes, are capable of binding the amino groups of protein, nucleic bases, the N bases of phospholipids and the SH groups of sulfhydryl compounds (Hamre *et al.*, 2001). Fatty acid alkoxy radicals

decomposed from hydroperoxides also react strongly with various biomolecules such as protein and DNA (Hamre *et al.*, 2001). However, the adverse impression of lipid oxidation on cultured fish is quite a controversial issue as some fish have the ability to habituate oxidized dietary lipid *in vivo*. Fish are protected from oxidative stress by physiological antioxidant systems such as endogenous free radical scavenging enzymes, including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX), and exogenous antioxidants such as vitamin E (Tocher *et al.*, 2003). The antioxidant defence system of fish varies from species to species, giving rise to varied sensitivity of fish to oxidized dietary lipid. For instance, highly oxidized herring oil or canola oil are not toxic to aquaculture salmonoids as long as dietary α-tocopherol is adequate (Ackman *et al.*, 1997). Meanwhile, Horrobin (1991) has hypothesized that secondary oxidation products released by lipid oxidation may not seriously damage cells. More research has to be carried out in order to answer the following questions: what fish species are affected by oxidized dietary lipid, to what extent fish are affected, and how fish are protected by antioxidants?

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Cultured Atlantic cod (*Gadus morhua*), reared from eggs to juveniles in the Aquaculture Research and Development Facility (ARDF), Ocean Sciences Centre (OSC), Memorial University of Newfoundland, St. John's, Newfoundland, were used in this experiment. Feed ingredients were purchased from the following sources: herring meal from Corey Feeds, Fredericton, New Brunswick, Canada; krill meal from Special Marine Products Ltd., West Vancouver, Canada; casein from Amersham Pharmacia Canada Ltd., Baie d'Urfé, Quebec, Canada; wheat middlings from Dover Mills Ltd., Halifax, Canada; vitamins and choline chloride from US Biochemical, Cleveland, OH, USA; minerals from Anachemia Chemicals Inc., Rouses Point, NY, USA; pre-gelatinized starch from National Starch and Chemical Co., Bridgewater, NJ, USA; marine fish oil from Ocean Nutrition Canada Ltd., Halifax, NS, Canada; and mixed tocopherols from Archer Daniels Midland Company, Decatur, IL, USA. All chemicals used were obtained from Fisher Scientific Ltd., Ottawa, ON, Canada, or Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. The solvents were of ACS grade, pesticide grade or HPLC grade.

2.2 Methods

2.2.1 Feeding trial

A 9-week feeding trial was carried out at the Ocean Sciences Centre (OSC), Memorial University of Newfoundland, St. John's, Newfoundland. Four hundred juvenile Atlantic

cod (average weight of 54.9g) were allocated among 8 tanks (tank size: 3000L, 50 fish per tank, 2 tanks per diet) at 10-11°C. Filtered and UV treated seawater was supplied to each tank at a flow rate of 10 L/min. Water temperature and dissolved oxygen were monitored daily and adjusted to the optimal levels for Atlantic cod. A 12 h light/ 12 h dark photoperiod was employed with a light intensity of 60 Lux. Fish were acclimated for 3 weeks to experimental conditions before the feeding trial. Fresh marine fish oil, for provision of oxidized dietary lipid, was oxidized by heating at 55-60 °C with air-bubbling until a peroxide value of 94 meq/kg was reached. Feed ingredients were mixed and formulated into dry pellets with a uniform particle size of 3.5 mm. Feed were stored at -20°C until use. Four experimental diets (Table 2.1), containing unoxidized oil, oxidized oil, oxidized oil with α-tocopherol, and oxidized oil with mixed tocopherols, respectively, were assigned to duplicate tanks. Fish were fed twice daily at 0900 and 1600 hours to apparent satiation for 9 weeks. Feed consumption and mortality were recorded on a daily basis. Fish were weighed at 3-week intervals, and fasted for 24 hours before each weighing. On completion of the feeding period, growth and feed utilization parameters were calculated as follows:

a. Survival = 100 × (initial number - dead number) / initial number

b. Weight gain = 100 × (final weight - initial weight) / initial weight

c. Specific growth rate (% day^{-1}) = 100 × [ln (final weight) - ln (initial weight)] / days

d. Feed conversion efficiency = weight gain / feed consumed

e. Net protein utilization = 100 × (final body protein - initial body protein) / protein consumed

f. Protein efficiency ratio = weight gain/protein consumed.

Table 2.1 Formulation of experimental diets.

Ingredients	Diet 1 Fr-FO + E[1]	Diet 2 Ox-FO[2]	Diet 3 Ox-FO + E[3]	Diet 4 Ox-FO + COVI[4]
Herring meal (%)	25	25	25	25
Krill meal (%)	2	2	2	2
Casein (%)	35.5	35.5	35.5	35.5
Wheat middlings (%)	19.8	19.8	19.8	19.8
Fresh Fish oil (%)	11	0	0	0
Oxidized Fish oil (%)	0	11	11	11
Vitamin mixture (%)	1.5	1.5	1.5	1.5
Mineral mixture (%)	1	1	1	1
Choline chloride (%)	0.2	0.2	0.2	0.2
Pre-gelatinized starch (%)	4	4	4	4
α-tocopherol (IU vitamin E eq.)	300	0	300	0
COVI-OX T-70) (IU vitamin E eq.)	0	0	0	300

[1] Fr-FO + E: fresh fish oil with vitamin E supplementation (from α-tocopherol);
[2] Ox-FO: oxidized fish oil (without vitamin E supplementation);
[3] Ox-FO + E: oxidized fish oil with vitamin E supplementation (from α-tocopherol);
[4] Ox-FO + COVI: oxidized fish oil with vitamin E supplementation (from mixed tocopherols).

2.2.2 Fish sampling

Before starting the feeding trial, 12 representative fish were sacrificed with an overdose of anaesthetic (MS 222) and the whole body homogenized. At the end of the feeding trial, fish were fasted for 24 hours and weighed individually to obtain the final body weight. Fish for chemical analyses were sacrificed in the same manner as the initial fish. Three fish from each tank (6 from each treatment) were minced together for proximate composition of whole body. Another 6 fish from each tank (12 from each treatment) were dissected and muscle, liver and viscera carefully removed. The whole body weight, liver and viscera weight were recorded for calculation of hepatosomatic index (HSI) and viscerosomatic index (VSI) (HSI = 100 × liver weight / total body weight; VSI = 100 × viscera weight / total body weight). Muscle and liver samples collected from the same treatment were pooled together. The homogenates of fish whole body and selected tissues were stored under nitrogen at -20 °C prior to analysis.

2.2.3 Analytical methods

2.2.3.1 Blood tests

2.2.3.1.1 Hemotocrit

Fish were randomly selected from each tank and anaesthetized. Blood samples were collected from the caudal vein with heparinized syringes and transferred into 1.5 ml heparinized Eppendorf tubes. All samples were stored on ice and analyzed within 3 hours of collection. Samples with apparent clots were discarded. Hematocrit was determined by centrifuging the whole blood at 14000 x g for 5 min with a Micro Hematocrit Centrifuge (Damon International Equipment Co., Needham Heights, MA). The height of the whole

blood and the precipitated red blood cell (RBC) in the Micro-Hematocrit capillary tubes was recorded; hematocrit was expressed as percentage of RBC in the whole blood.

2.2.3.1.2 Erythrocyte osmotic fragility (EOF)

Erythrocyte osmotic fragility (EOF) was measured as hemolysis in saline solution with varying concentrations, as described by Miale, (1982). Briefly, a saline stock solution (10%) was prepared by dissolving 9g of NaCl, 1.366g of $Na_2HPO_4 \cdot H_2O$ and 0.243g of $NaH_2PO_4 \cdot 2H_2O$ in 100 ml of deionized water. The stock solution was diluted to a series of 12 concentrations from 0.1 to 0.85%. Aliquots of the whole blood sample (30 μl) were added to 1 ml of each saline series and incubated at room temperature for 30 min. Samples were then centrifuged at 750 xg for 5 min, and the absorbance of resultant supernatant measured at 545 nm using a Hewlett-Packard model 8452A diode array spectrophotometer (Agilent, Palo Alto, CA). Hemolysis was calculated using 0.85% saline tube as blank (0% hemolysis) and 0.1% saline tube as 100% hemolysis, according to the equation given below:

$$\%\ \text{hemolysis}_x = 100 \times (Abs_x - Abs_{0.85\%}) / (Abs_{0.1\%} - Abs_{0.85\%})$$

An osmotic fragility curve (% hemolysis versus saline concentration) was plotted.

2.2.3.2 Proximate composition

2.2.3.2.1 Moisture content

Moisture content of feed and fish whole body was determined by an oven-drying method (AOAC, 1990). Approximately 2-4 g of each homogenized sample was accurately weighed, and placed in a pre-weighed aluminum dish. Samples were dried in a forced-air

convection oven (Fisher Isotemp 300, Fair Lawn, NJ) at 103 °C for 24 hours to a constant weight. Samples and dishes were cooled in a desiccator and weighed. Moisture content was calculated as the percentage of weight loss of the sample during drying.

2.2.3.2.2 Ash content

Ash content of feed and fish whole body was measured by incineration in a muffle furnace (AOAC, 1990). Homogenized samples (2-4 g for feed samples and 8-10 g for fish samples) were accurately weighed into pre-weighed porcelain crucibles and charred. Samples were then incinerated in a muffle furnace (Thermolyne, F 62700, Dubuque, IA) at 550 °C for 24 hours until a white creamy powder without black particles was observed. Samples and crucibles were cooled in desiccator and weighed. Ash content was expressed as the weight percentage of the residue in each sample.

2.2.3.2.3 Crude protein content

Crude protein content of feed and fish whole body was determined by the Kjeldahl method (AOAC, 1990). Homogenized samples (200-300 mg for feed samples and 800-900 mg for fish sample) were accurately weighed into digestion tubes, to which 20 ml of concentrated sulfuric acid (Fisher Scientific Co., Fair Lawn, NJ) and two catalyst tablets (Kjeltabs, Profamo Analytical Service Inc., Dorval, QC) were added. Samples and blank were heated in a digestion unit (Büchi 430 Digester, Flawil, Switzerland) at low temperature setting for 20 min and then at high temperature setting for an additional 40 min until the solution appeared clear or pale yellow. The digested samples were cooled and transferred to a distillation unit (Büchi 321 Distillation unit, Flawil, Switzerland).

The digestion tubes were filled with distilled water to the 50 ml mark, and then with 25% (w/v) sodium hydroxide solution to the 200 ml mark. The samples were then steam distilled and the distillate collected into a 50 ml boric acid (4%, w/v) solution containing 12 drops of methyl red/methyl blue indicator (EM Science, Gibbstown, NJ). Distillation was ended when 150 ml of condensate were collected. The condensate was titrated with 0.1N sulfuric acid to a pink end point. The nitrogen content was calculated using the following equation:

$$\%N = 100 \times (V_{sample}-V_{blank}) \times N \times 14.0067 / W$$

where, V_{sample} and V_{blank} stand for the volume (ml) of titrant for sample and blank, respectively, N is the normality of sulfuric acid solution used in titration, and W is the weight of the sample in mg. The crude protein content was calculated by multiplying the nitrogen content by a factor of 6.25 (crude protein = N% × 6.25).

2.2.3.2.4 Total lipid content

Lipids were extracted from feed, fish whole body, muscle and liver samples using the Bligh and Dyer (1959) method, and quantified gravimetrically. Approximately 50 g of each sample were homogenized in 100 ml of methanol and 50 ml of chloroform for 2 min with a Polytron PT 3000 (Brinkmann Instruments, Rexdale, ON) homogenizer. A small amount of tert-butylhydroquinone (TBHQ) was added to prevent oxidation during extraction. Another 50 ml of chloroform was added to the mixture, followed by an additional blending for 30s. The homogenate was filtered through a Whatman No.1 filter paper using a Buchner funnel coupled with air suction. The residue was re-extracted with 50 ml of chloroform for 30s and filtered as described above. The filtrates were combined,

and 100 ml of distilled water were added and mixed well. The mixture was transferred to a separatory funnel and allowed to stand in a cold room for separation. The separatory funnel was flushed with nitrogen and wrapped with aluminum foil to avoid oxidation. After separation, the chloroform layer containing lipid was collected in a pre-weighed round bottom flask after passing through a layer of anhydrous sodium sulfate. The solvent was evaporated in a rotary evaporator (Büchi Rotavapor, Flawil, Switzerland) at 40 °C (Büchi 461 water bath, Flawil, Switzerland). The flask with the oil was weighed, and total lipid content calculated gravimetrically. For determination of lipid content of the feed, 10 g of ground feed samples were used instead of 50 g. However, 40 ml of distilled water were added to make a net weight of 50 g.

2.2.3.3 Identification and quantification of major lipid classes

Determination of muscle and liver lipid classes was performed by thin layer chromatography-flame ionization detection (TLC-FID). An Iatroscan MK-5 TLC-FID Analyzer System (Iatroscan Laboratories Inc., Tokyo, Japan) equipped with TSCAN data handling software (Scientific Products and Equipment, Concord, ON) was employed. The hydrogen flow rate was 190 ml/min and air flow rate was 2000 ml/min. The scanning speed was 30s/rod for all samples. Silica gel coated Chromarods-S III were treated with nitric acid overnight prior to the operation. Rods were then washed with distilled water and acetone and impregnated with 3% (w/v) boric acid solution for 5 min in order to improve separation. Separation of lipid classes was carried out following a three-stage development procedure described by Christopher (1999). Samples (10 mg/ml) were prepared by dissolving 100 mg of lipid extracts in 10 ml of hexane. Lipid standards (1

mg/ml), including triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MONO), free fatty acid (FFA), sterol ester (SE), sterols (ST), acetone mobile polar lipids (AMPL), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SPH) and lysophosphatidylcholine (LPC) were prepared by dissolving each standard in hexane. The rods were blank-scanned three times in the Iatroscan to burn any remaining impurities. A 1 µl aliquot of samples and standards was spotted on the rods with a syringe. The bands were focused twice in acetone to produce a narrow band of lipid material near the lower end of the rods. The rods were dried and conditioned in a constant-humidity chamber (over saturated $CaCl_2$) for 5 min. They were then developed twice in hexane/diethyl ether/formic acid (99:1:0.05, v/v/v). The first development was for 25 min; the rods were then dried in a constant-humidity chamber for 5 min and redeveloped for 20 min. They were dried in a drying oven at 110 °C for 3 min and partially scanned (partial pyrolysis selection, pps 25). The rods were then conditioned for 5 min and developed for 40 min in hexane/diethyl ether/formic acid (79: 20:1, v/v/v). They were dried at 110 °C for 3 min and partially scanned (pps 11) to the lowest point after the DAG peak. The rods were conditioned for another 5 min, and developed twice for 15 min in 100% acetone. After conditioning again for 5 min, they were developed twice for 10 min in chloroform/methanol/distilled water (5:4:1, v/v/v). The rods were dried at 110 °C for 3 min and fully scanned to obtain the polar lipid peaks. On completion of the scanning, the three chromatograms were combined, and each peak identified by comparing the retention time with that of known standards. Quantification of each lipid class was achieved by calculating the area percentage of a specific peak to the total peak area.

2.2.3.4 Lipid fractionation

Neutral lipids (NL) and phospholipids (PL) were separated from muscle total lipid by column chromatography as described below, and following a procedure described by Ramadan and Morsel (2002) and Budge and Parrish (2003). The column was prepared using a Pasteur pipette packed with silica gel. A small amount of glass wool was placed in the tapered end of a Pasteur pipette. The pipette was packed with 0.8 g of activated silica gel and rinsed with 6 ml of methanol followed by 6 ml of chloroform. Approximately 15 mg of lipid extract were dissolved in chloroform and added to the top of the column. Neutral lipids were recovered with 20 ml of chloroform at a flow rate of 1 ml/min. Phospholipids were eluted with 20 ml of methanol at the same flow rate. Solvents were evaporated under a nitrogen flush and lipid fractions stored at -20 °C for subsequent fatty acid analysis.

2.2.3.5 Fatty acid composition

2.2.3.5.1 Preparation of fatty acid methyl esters (FAMEs)

Fatty acid composition of dietary oil, muscle and liver total lipids, muscle neutral lipids and phospholipids was determined by gas chromatography (GC). Fatty acids were converted to their corresponding fatty acid methyl esters (FAMEs) by transmethylation prior to GC analysis. Two different transmethylation methods, namely sulfuric acid/methanol method and boron trifloride/methanol method, were employed for preparation of FAMEs, and results were compared.

2.2.3.5.1.1 Sulfuric acid/methanol method

Transmethylation reagent (TMR) containing sulfuric acid and methanol was used to prepare FAMEs according to Hamam and Shahidi (2005). TMR (6%) was prepared by mixing 6 ml of concentrated sulfuric acid with 94 ml of methanol. Fifteen milligrams of tert-butylhydroquinone (TBHQ) were added as antioxidant. Approximately 1 mg of lipid was added to 2 ml of freshly prepared TMR followed by vortexing. The mixture was incubated at 60°C overnight and subsequently cooled down to room temperature. Distilled water (1 ml) and a small amount of TBHQ were added and mixed well. Samples were extracted three times with 1.5 ml of hexane each. Hexane layers containing FAMEs were combined and then washed with 1.5 ml of distilled water. Solvent was removed by evaporation under a stream of nitrogen. The resultant FAMEs were then dissolved in 1 ml of carbon disulfide for subsequent GC analysis.

2.2.3.5.1.2 Boron trifluoride/methanol method

Fatty acids were derivatized to FAMEs following the procedure of (Budge and Parrish, 2003) with minor modifications. Approximately 1 mg of lipid extract was mixed with 0.5 ml of hexane and 1.5 ml of BF_3/methanol (14%) in a transmethylation vial. The vial was then topped with nitrogen, sealed with Teflon tape and vortexed. The mixture was heated at 85°C for 1.5 hour and then cooled down to room temperature. Distilled water (0.5 ml) was added to the vial and mixed well. Samples were then extracted with 1.5 ml of hexane three times. Hexane layers containing FAMEs were combined and then washed with 1.5 ml of distilled water. Hexane layers were then flushed with nitrogen to dryness to remove

the solvent. The resultant FAMEs were then dissolved in 1 ml of carbon disulfide for subsequent GC analysis.

2.2.3.5.2 GC analysis of FAMEs

The FAMEs were analyzed using a Hewlett-Packard 5890 Series II gas chromatograph (Agilent, Palo Alto, CA) equipped with a fused capillary column (Supelcowax-10, 30 m length, 0.25 mm diameter, 0.25 μm film thickness; Supelco Canada Ltd., Oakville, ON). The oven temperature was first set at 220°C for 10.25 min and then increased to 240°C at a rate of 30°C/min and then held there for 15 min. The samples and standards (GLC-461, Nu-Check-Prep Inc., Elsyian, MN, USA) were injected onto the GC using a Hewlett-Packard 7673 autoinjector (Hewlett-Packard, Toronto, ON). The temperature of the injector and detector (FID) was both set at 250°C. Ultra high purity (UHP) helium was used as the carrier gas at a flow rate of 15 ml/min. Data were analyzed with Hewlett-Packard 3365 Series II Chem Station Software (Agilent, Palo Alto, CA). The FAMEs were identified by comparing their retention times with those of authentic standards. Results were expressed as area percentage of each fatty acid.

2.2.3.6 Determination of lipid oxidation

2.2.3.6.1 Determination of peroxide value (PV)

Peroxide values of fresh and oxidized dietary fish oil were determined according to the AOCS (1990) method. Approximately 1.5 g of oil samples were dissolved in 30 ml of acetic acid/chloroform (3:2, v/v) mixture in a 125 ml conical flask. Saturated potassium iodide (0.5 ml) were then added and each flask was shaken for 1 min to facilitate the

reaction. Thirty milliliters of distilled water was then added and the liberated iodine was titrated with 0.01 N sodium thiosulfate solution. Two milliliters of starch indicator (1%, w/v) were added when the yellow color of iodine had almost disappeared. Titration was continued to the end point, where the blue color of the solution disappeared. A blank was conducted each time. Peroxide value was expressed as the uptake of milliequivalents of active oxygen per kilogram of oil and calculated as follows:

$$PV = (V_{sample}-V_{blank}) \times N \text{ of } Na_2S_2O_3 \times 1000 / \text{Sample weight (g)}$$

where, V_{sample} and V_{blank} stand for the volume of sodium thiosulfate solution (ml) consumed for samples and blank, respectively. N is the normality of sodium thiosulfate used.

2.2.3.6.2 Determination of 2-thiobarbituric acid reactive substances (TBARS)

2.2.3.6.2.1 TBA test of dietary oils

TBARS values of fresh and oxidized dietary oils were measured according to the AOCS (1990) method. A standard stock solution (10 μmol/ml) of malondialdehyde (MDA) precursor, namely 1,1,3,3-tetramethoxypropane (TMP), was prepared in 0.1 N HCl and diluted with HCl into a series of concentrations. An aliquot (0.1 ml) of each serial solution was transferred into a borosilicate-glass test tube with a Teflon cap. To each tube 4.9 ml of 1-butanol and 5 ml of TBA reagent (2 mg/ml of TBA in 1-butanol) were added. A tube containing 0.1 ml of HCl, 5 ml of 1-butanol and 5 ml of TBA reagent was used as a blank. All tubes were heated in a water bath at 95-100°C for 120 min and cooled down to room temperature under running tap water. The absorbance of the resultant solution was measured at 532 nm using a Hewlett-Packard 8452A diode array spectrophotometer

(Agilent, Palo Alto, CA). A standard curve (absorbance versus TMP concentration) was constructed for future analysis of oil samples.

Approximately 0.2 g of oil samples were weighed into 50 ml volumetric flasks and dissolved in a small volume of 1-butanol and then made up to the mark with the same solvent. Five milliliters of above solutions were transferred into dry test tubes and 5 ml of TBA reagent were added into each tube. A tube containing 5 ml of 1-butanol and 5 ml of TBA reagent was used as a blank. The contents were heated in a water bath at 95-100°C for 120 min and cooled down to room temperature under running tap water. The absorbance of the resultant solution was measured at 532 nm. The concentrations of MDA equivalents in oil samples were read from the standard curve and TBARS values expressed as the total μmol of MDA equivalents per gram of oil.

2.2.3.6.2.2 TBA test of muscle tissue

Muscle TBARS values were determined following the procedure described by Lemon (1975). A standard solution (1 mM) of TMP in deionized water was prepared and diluted into a series of concentrations. Two milliliters of each solution were transferred into dry screw capped tubes and 2 ml of TBA reagent (0.02 M TBA in deionized water) were added to each tube. Contents were heated in a water bath at 100°C for 40 min and cooled down on ice. The absorbance of resultant solutions was measured at 532 nm. A standard curve (absorbance versus TMP concentration) was plotted for future analysis of muscle tissue samples.

An extraction solution containing 7.5% TCA (trichloroacetic acid), 0.1% propyl gallate and 0.1% EDTA was prepared in deionized water. Approximately 4 g of fish muscle

samples were accurately weighed into 50 ml centrifuge tubes. Eight milliliters of the extraction solution were added and the mixture was homogenized for 1 min using a Polytron homogenizer. The Polytron was rinsed with 8 ml of extraction solution. The mixture was then centrifuged at 2000 xg for 10 min and 2 ml of the resultant supernatant were transferred into dry screw capped tubes. To each tube 2 ml of TBA reagent were added and the tubes were then capped under nitrogen and vortexed for 15 sec. A mixture of 2 ml of extraction solution and 2 ml of TBA reagent was used as a blank. All tubes were heated in a water bath at 100°C for 40 min and cooled on ice. The absorbance of the resultant solutions was recorded at 532 nm. The concentrations of MDA equivalents (μmolMDA/ml) were read from the standard curve and TBARS values of muscle tissue were calculated as follows:

TBARS =
μmolMDA/ml x (volume of extracting solution + sample weight x muscle moisture content) / sample weight (g)

2.2.3.6.2.3 TBA test of liver tissue

TBARS values of fish liver tissue were determined according to (Williamson *et al.*, 2003). A standard TMP solution (0.1 mM) was prepared in HPLC grade ethanol, and diluted into a series of concentrations. A phosphate buffer solution (PBS) was prepared by dissolving 3.6 g of NaCl, 0.74 g of Na_2HPO_4 and 0.215 g of NaH_2PO_4 in 500 ml of deionized water with pH adjusted to 7.2. Approximately 1 g of liver samples were homogenized in 10 ml of PBS. One milliliter of homogenates and 1 ml of each serial standard TMP solution were transferred into individual 15 ml centrifuge tubes, respectively. One milliliter of PBS was used as blank. To each tube the following solutions were added: 0.5 ml of TCA (15% trichloroacetic acid in deionized water), 0.5

ml of HCl (0.25 N), 0.5 ml of BHT (2.5 mM butylated hydroxytoluene in absolute ethanol), 0.5 ml of TBA (0.375% TBA in deionized water) and 0.2 ml of SDS (8.1% of sodium dodecyl sulfate in deionized water). All tubes were vortexed for 15 sec and heated in a water bath at 95-100°C for 60 min. Samples and standards were then cooled in ice to room temperature and centrifuged at 1060 xg for 20 min. The supernatants were filtered through 0.45 um syringe filters and the absorbance of the resultant filtrates was measured at 532 nm. A standard curve was constructed by plotting the absorbance against TMP concentrations. The contents of TBARS were then determined and expressed as nmol MDA equivalents per gram of liver tissue using the standard curve.

2.2.3.6.3 Analysis of headspace propanal

A Perkin-Elmer 8500 gas chromatograph equipped with an HS-6 headspace sampler (Perkin-Elmer Co., Montreal, QC) was used for volatile analysis of dietary oils and liver samples. A Supelcowax-10 fused-silica capillary column (30 m X 0.32 mm i.d., 0.10 μm film, Supelco Canada Ltd., Mississauga, ON) was used to separate the volatiles. Helium was the carrier gas employed at an inlet column pressure of 20 psig with a split ratio of 7:1. The temperature for the injector and flame ionization detector (FID) was 280°C. The oven temperature was maintained at 40 °C for 5 min, and then increased to 115 °C at a rate of 10 °C/min. Subsequently, the oven temperature was raised to 200 °C at a rate of 30 °C/min and held there for 5 min.

Oil and tissue samples were transferred into special glass vials and the vials were capped with special butyl septa and preheated in the HS-6 magazine assembly at 90 °C for a 30 min equilibrium period. Pressurization time was 6 s and the vapor phase was drawn at an

approximate volume of 1.5 ml. Propanal was identified by comparing the retention time of GC peak with a commercial propanal standard. 2-Heptanone was used as the internal standard for quantification of the headspace propanal in the samples. Results were expressed as μg propanal per gram of oil or wet tissue.

2.2.3.7 HPLC-MS analysis of tocopherols

2.2.3.7.1 Sample and standards preparation

Lipid extracts from fish feed and fish tissue were saponified according to Maguire *et al.* (2004) with some modifications. Briefly, 0.5g lipid extract was mixed thoroughly with 1 ml of 60% KOH (w/v) and 4 ml of ethanolic pyrogallol (w/v) in screw-top tubes fitted with Teflon lined screw-caps. The tubes were heated at 70 °C in a water bath for 45 min. The tubes were then cooled on ice and 2 ml of deionized water were added. The solution was mixed well on a vortex and unsaponifiable matters were extracted three times with 2 ml of hexane. The combined hexane extracts were dried under a nitrogen flush and redissolved in 1 ml of hexane. Tocopherol standards were prepared by dissolving a certain amount of known standards in hexane and then diluting into a series of concentrations. Both sample and standard solutions were filtered using a 0.45 μm syringe-filter and then used for HPLC analysis. Care was taken throughout the procedure to avoid exposure of tocopherols to light.

2.2.3.7.2 Normal phase HPLC-MS analysis of tocopherols

Tocopherol contents in fish feed and fish tissue were determined by normal phase high performance liquid chromatography (HPLC) - mass spectrometry (MS). The analysis was

performed using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) with a UV-diode array detector (UV-DAD). Separation was achieved on a Supelcosil LC-Si column (250 mm X 4.6 mm i.d., 5 μm, Sigma-Aldrich Canada Ltd., Oakville, ON) coupled with a Supelcosil LC-Si guard column. Tocopherols were eluted using an isocratic solvent system containing hexane/2-propanol (99:1, v/v) at a flow rate of 1.2 ml/min. Fifty microliters of each tocopherol standard and sample was injected. Tocopherols were detected at 290 nm by the UV detector. LC flow was analyzed on-line by a mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA) with a positive ion APCI (Atmospheric pressure chemical ionization). The operating conditions used were 121V for the fragmentor voltage, 350°C for drying temperature, 400°C for APCI temperature, 60 psi for the nebulizer pressure, and 7 L/min for the drying gas flow. A standard curve was constructed for each tocopherol homologue standard (peak area versus concentration). Tocopherol concentrations in samples were obtained from the standard curve, and expressed as mg tocopherol per kg feed or fish tissue.

2.2.4 Statistical analysis and data interpretation

One way ANOVA (analysis of variance) with pairwise comparisons (Tukeys HSD) were performed at a $P<0.05$ level using Sigmastat for Windows Version 2.0 (Jandel Corporation, San Rafael, CA, USA) to determine the significant differences. All parameters in fish group 3 (treatment of oxidized oil with α-tocopherol supplementation) were compared with those in group 1 (treatment of fresh oil with α-tocopherol supplementation) to obtain the effect of oxidized oil on fish. Similarly, fish in group 3 (treatment of oxidized oil with α-tocopherol supplementation) and group 4 (treatment of

oxidized oil and tocopherol mixture supplementation) were compared with those in group 2 (treatment of oxidized oil without vitamin E supplementation) for the effect of α-tocopherol and mixed tocopherols, respectively, on Atlantic cod.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Formulated diets

3.1.1 Proximate composition

Proximate compositions of the experimental diets are shown in Table 3.1. The results confirmed the intended values of nutrients in feed formulation. All treatments had the identical feed composition in terms of dry matter, ash content and macronutrients including protein and lipid. The approximate protein and lipid contents in all diets were about 58% and 15%, respectively. These values are considered optimal for cod diet. A good protein/lipid ratio was obtained in all diets that are expected to satisfy the requirement of nutrition and energy for normal growth.

3.1.2 Oxidative state of dietary oils

Fresh and oxidized marine fish oil were used in feed formulation to provide dietary lipid. Oxidative state of both oils was determined by measuring the primary and secondary oxidation products, namely, peroxide value, thiobarbituric acid reactive substances (TBARS) and headspace propanal. Table 3.2 shows the oxidative state of fresh and oxidized dietary oils.

The oxidized fish oil had a peroxide value of 94.0 meq/kg, which was significantly higher than that of the fresh oil (1.22 meq/kg). The TBARS values and headspace propanal correlated strongly with peroxide values. TBARS value in oxidized oil was 11.1 μmol MDA eq/g, which was high in comparison to that of fresh oil (4.47 μmol MDA eq/g).

Table 3.1 Proximate composition (%) of formulated diets [1]

Diets	Moisture	Protein	Lipid	Ash
Fr-FO + E	8.60 ± 0.16	58.2 ± 1.10	15.7 ± 0.78	3.77 ± 0.06
Ox-FO	8.75 ± 0.03	58.6 ± 0.67	14.8 ± 2.96	3.93 ± 0.03
Ox-FO + E	7.92 ± 0.22	58.0 ± 0.69	15.5 ± 0.98	4.06 ± 0.04
Ox-FO + COVI	9.25 ± 0.19	59.9 ± 1.06	14.3 ± 0.85	3.86 ± 0.01

[1] Values are mean ± SD of three replicates.
Abbreviations for diets are given in footnotes to Table 2.1.

Table 3.2 Oxidative state of fresh and oxidized dietary oils [1]

Oxidation products	Fresh fish oil	Oxidized fish oil
Peroxide value (meq/kg)	1.22 ± 0.31	94.0 ± 1.82
TBARS value (μmol MDA eq/g)	4.47 ± 0.75	11.1 ± 0.38
Headspace propanal (μg/g)	tr [2]	74.0 ± 0.00

[1] Values are mean ± SD of three replicates.
[2] tr: trace.
Abbreviations for diets are given in footnotes to Table 2.1.

Propanal concentration was 74.0 μg/g in oxidized oil, whereas only trace amount of headspace propanal was detected in fresh oil. The large amounts of both primary oxidation products, namely the hydroperoxides, and secondary oxidation products, including malondialdehyde and propanal, present in oxidized fish oil indicate that the oil was highly oxidized.

3.1.3 Fatty acid profiles of dietary lipid

Both sulfuric acid/methanol and boron trifluoride/methanol method were used for preparation of FAMEs, and the results from GC analysis were compared (Table 3.3 and 3.4). The sulfuric acid/methanol method, with which more fatty acids were identified and a lower standard deviation was obtained compared to the boron trifluoride/methanol method, was found to be a better method of transmethylation in this specific case. Results of fatty acid analysis given after Tables 3.3 and 3.4 are from the sulfuric acid/methanol method; subsequent data from the boron trifluoride/methanol method are not shown. It should be noted that although the results are in favor of methanolysis with sulfuric acid, condemnation of methanolysis assisted by boron trifluoride would be premature (Ackman, 2005).

Fatty acid composition of dietary lipid is shown in Table 3.4 and Figure 3.1. All diets had high proportions of PUFA (about 32%), especially n-3 PUFA (about 27%). The fish oil used in feed formulation provided a good source of EFA such as EPA (about 14%) and DHA (about 9.5%), which are of great importance in the maintenance of optimal fish health and growth performance (Howell *et al.*, 1998). The fatty acid compositions of dietary lipid were compared among the four experimental diets. The results were fairly

Table 3.3 Fatty acid composition of dietary lipid [1] (boron trifluoride/methanol method)

FA (%)	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
C 10:0	n.d.[2]	n.d.	n.d.	n.d.
C 12:0	n.d.	n.d.	n.d.	n.d.
C 14:0	6.02 ± 0.14^{b}	5.65 ± 0.12^{c}	5.99 ± 0.02^{b}	6.34 ± 0.06^{a}
C 14:1	n.d.	n.d.	n.d.	n.d.
C 15:0	tr[3]	0.46 ± 0.01^{b}	0.48 ± 0.02^{ab}	0.50 ± 0.01^{a}
C 16:0	18.6 ± 1.37	18.2 ± 0.11	17.7 ± 0.32	16.9 ± 2.23
C 16:1	6.80 ± 0.51	6.36 ± 0.10	6.76 ± 0.04	6.79 ± 0.01
C 17:0	n.d.	n.d.	n.d.	n.d.
C 17:1	n.d.	n.d.	n.d.	n.d.
C 18:0	2.99 ± 0.20^{ab}	3.03 ± 0.01^{ab}	2.97 ± 0.03^{b}	3.40 ± 0.25^{a}
C 18:1	10.2 ± 0.37	10.1 ± 0.10	10.1 ± 0.11	10.1 ± 0.10
C 18:2	9.06 ± 1.08	9.39 ± 0.23	8.83 ± 0.20	8.48 ± 0.24
C 18:3n-6	n.d.	n.d.	n.d.	n.d.
C 18:3n-3	1.36 ± 0.05	1.32 ± 0.02	1.34 ± 0.03	1.29 ± 0.03
C 20:0	n.d.	n.d.	n.d.	n.d.
C 20:1	2.98 ± 0.06^{d}	4.22 ± 0.06^{a}	3.80 ± 0.16^{b}	3.29 ± 0.07^{c}
C 20:3n-6	n.d.	n.d.	n.d.	n.d.
C 20:3n-3	tr	0.75 ± 0.01^{a}	0.77 ± 0.04^{a}	0.78 ± 0.02^{a}
C 20:5n-3	15.4 ± 0.56^{a}	13.5 ± 0.12^{b}	14.2 ± 0.17^{b}	13.8 ± 0.03^{b}
C 22:1	n.d.	n.d.	n.d.	n.d.
C 22:5n-3	1.74 ± 0.04^{a}	1.48 ± 0.01^{c}	1.55 ± 0.03^{b}	1.52 ± 0.01^{bc}
C 22:6n-3	11.5 ± 0.29^{a}	10.8 ± 0.10^{b}	10.8 ± 0.15^{b}	10.2 ± 0.11^{c}
ΣSAT[4]	27.6 ± 1.58	27.3 ± 0.13	27.1 ± 0.97	27.1 ± 1.87
ΣMONO	20.0 ± 1.57	20.7 ± 0.14	20.7 ± 0.99	20.2 ± 1.65
ΣPUFA	39.1 ± 0.61^{a}	37.2 ± 1.36^{ab}	34.5 ± 0.93^{c}	36.1 ± 0.76^{bc}
Σn-3	30.0 ± 0.59^{a}	27.9 ± 1.02^{ab}	28.7 ± 0.89^{ab}	27.6 ± 0.74^{b}

[1] Results are expressed as area percentage of the fatty acid in total fatty acids. Values in the same row with different superscripts were significantly different at $P<0.05$. Rows with no superscript letters are not significantly different at $P>0.05$.

[2] n.d.: not detected.

[3] tr: trace.

[4] SAT: saturated, MONO: monounsaturated, PUFA: polyunsaturated, n-3: n-3 polyunsaturated.

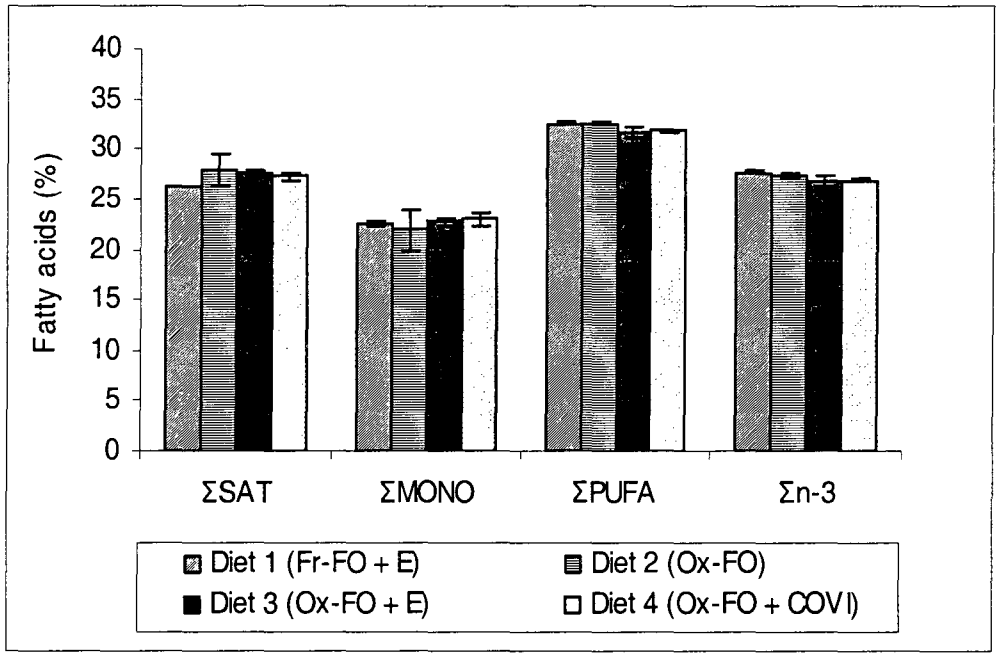
Abbreviations for diets are given in footnotes to Table 2.1.

Table 3.4 Fatty acid composition of dietary lipid [1] (sulfuric acid/methanol method)

FA (%)	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
C 10:0	0.05 ± 0.00^{b}	0.05 ± 0.00^{b}	0.08 ± 0.00^{a}	0.08 ± 0.00^{a}
C 12:0	0.16 ± 0.00^{b}	0.17 ± 0.00^{a}	0.17 ± 0.00^{a}	0.17 ± 0.00^{a}
C 14:0	6.17 ± 0.08	6.33 ± 0.07	6.36 ± 0.15	6.34 ± 0.07
C 14:1	0.19 ± 0.00	0.21 ± 0.03	0.19 ± 0.01	0.19 ± 0.00
C 15:0	0.48 ± 0.01	0.49 ± 0.01	0.49 ± 0.01	0.50 ± 0.00
C 16:0	16.0 ± 0.12^{c}	16.3 ± 0.18^{bc}	17.0 ± 0.11^{a}	16.6 ± 0.36^{ab}
C 16:1	7.61 ± 0.05^{b}	7.78 ± 0.03^{ab}	7.86 ± 0.12^{a}	7.81 ± 0.04^{a}
C 17:0	0.37 ± 0.00^{c}	0.37 ± 0.00^{c}	0.40 ± 0.00^{a}	0.39 ± 0.00^{b}
C 17:1	0.75 ± 0.01	1.05 ± 0.84	tr [2]	0.59 ± 0.84
C 18:0	3.05 ± 0.02^{b}	3.03 ± 0.05^{b}	3.30 ± 0.02^{a}	3.18 ± 0.08^{a}
C 18:1	9.61 ± 0.08^{b}	9.83 ± 0.07^{b}	10.2 ± 0.17^{a}	10.0 ± 0.09^{ab}
C 18:2	4.51 ± 0.10^{c}	5.07 ± 0.09^{a}	4.85 ± 0.06^{b}	4.89 ± 0.04^{ab}
C 18:3n-6	0.18 ± 0.00	0.18 ± 0.00	tr	0.19 ± 0.00
C 18:3n-3	1.03 ± 0.01	1.08 ± 1.01	1.04 ± 0.00	1.05 ± 0.01
C 20:0	0.06 ± 0.08	tr	tr	tr
C 20:1	3.65 ± 0.07^{a}	3.57 ± 0.07^{b}	3.77 ± 0.04^{ab}	3.74 ± 0.09^{a}
C 20:3n-6	0.08 ± 0.12	tr	tr	tr
C 20:3n-3	0.74 ± 0.00^{b}	0.72 ± 0.00^{c}	0.75 ± 0.00^{a}	0.75 ± 0.00^{a}
C 20:5n-3	14.3 ± 0.13^{a}	14.2 ± 0.12^{ab}	13.9 ± 0.23^{b}	13.9 ± 0.09^{b}
C 22:1	0.63 ± 0.02^{b}	0.60 ± 0.00^{b}	0.63 ± 0.01^{a}	0.63 ± 0.01^{a}
C 22:5n-3	2.02 ± 0.02	1.94 ± 0.03	1.83 ± 0.12	1.88 ± 0.10
C 22:6n-3	9.66 ± 0.00^{a}	9.46 ± 0.02^{b}	9.39 ± 0.24^{bc}	9.35 ± 0.00^{c}
ΣSAT	26.3 ± 0.11	27.9 ± 1.58	27.8 ± 0.27	27.3 ± 0.50
ΣMONO	22.6 ± 0.19	21.9 ± 1.89	22.7 ± 0.33	23.0 ± 0.65
ΣPUFA	32.5 ± 0.15^{a}	32.5 ± 0.17^{a}	31.7 ± 0.51^{b}	31.8 ± 0.13^{ab}
Σn-3	27.7 ± 0.13^{a}	27.3 ± 0.18^{ab}	26.9 ± 0.57^{b}	26.9 ± 0.14^{b}

[1] Some minor fatty acids have not been identified because of their absence in the GC standard; results are expressed as area percentage of the fatty acid in total fatty acids.
Values in the same row with different superscripts were significantly different at $P<0.05$.
Rows with no superscript letters are not significantly different at $P>0.05$.
[2] tr: trace.
Abbreviations for diets are given as footnotes to Table 2.1.
Note: some minor fatty acids might not be identified because of their absence in the GC standard.

Figure 3.1 Fatty acid profile of dietary lipids (Abbreviations for diets are given in footnotes to Table 2.1)


40
35
30
25
20
15
10
5
0
Fatty acids (%)
ΣSAT
ΣMONO
ΣPUFA
Σn-3
Diet 1 (Fr-FO + E)
Diet 2 (Ox-FO)
Diet 3 (Ox-FO + E)
Diet 4 (Ox-FO + COVI)

close to each other, although the numbers were statistically different. The levels of PUFA especially n-3 PUFA were slightly lower in the oxidized feed than in unoxidized diets. The reduced PUFA content in oxidized diets may be explained by the consumption of PUFA as the main substrates of oxidation reaction.

3.2 Fish performance and feed utilization

The overall results for fish growth and feed utilization parameters during the 9-week feeding trial are shown in Table 3.5.

The specific growth rate (SGR) of juvenile cod appeared quite low, as a result of low feed intake. The SGR has been related to the environmental temperature which affects feed consumption through fish metabolism. Generally, the higher the temperature, the greater the biological activity, and hence the larger the feed consumption (Hinshaw, 1999). In this respect, cold water species, as observed in Atlantic cod, display a slower growth than warm water species. Growth of juvenile cod was compared among the four treatments used in this experiment. The survival and specific growth rate (SGR) in all treatments were similar over the course of the feeding trial. Oxidized dietary oil did not have any significant effect on growth performance of juvenile cod ($P>0.05$). This is in agreement with the findings in sea bream. Tocher *et al.* (2003) found that oxidized dietary oil had no deleterious effects on the overall growth of sea bream, and unexpectedly, the growth was slightly stimulated by rancid oil. However, other studies have demonstrated the reduced growth of fish by oxidized dietary oils. Lipid oxidation in diets adversely affected the survival and SGR of Atlantic salmon (Koshio *et al.*, 1994), carp (Hashimoto *et al.*, 1966; Stephan *et al.*, 1991), sea bass (Stephan *et al.*, 1991), channel catfish (Murai and

Table 3.5 Growth performance of juvenile cod [1]

	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
Survival [2] (%)	47.4 ± 0.56	51.5 ± 11.0	54.7 ± 5.74	55.6 ± 0.44
Weight gain [3] (%)	75.6 ± 11.8	73.7 ± 2.01	73.3 ± 13.4	78.9 ± 1.07
Specific growth rate [4] (%/day)	0.87 ± 0.11	0.85 ± 0.02	0.84 ± 0.12	0.90 ± 0.01
Feed consumption (g/fish)	34.1 ± 4.30	33.3 ± 2.24	31.2 ± 3.84	34.6 ± 0.35
Feed conversion efficiency [5]	1.22 ± 0.04	1.23 ± 0.12	1.28 ± 0.08	1.26 ± 0.01
Hepatosomatic index [6] (%)	6.85 ± 0.6	6.72 ± 0.92	7.14 ± 1.92	7.11 ± 1.56
Vicerosomatic index [7] (%)	12.2 ± 1.74	11.7 ± 0.74	14.3 ± 5.33	12.7 ± 2.08
Net protein utilization [8] (%)	28.5 ± 1.12	28.8 ± 2.75	28.8 ± 2.24	29.5 ± 0.11
Protein efficiency ratio [9]	2.09 ± 0.07	2.09 ± 0.20	2.21 ± 0.14	2.09 ± 0.01

[1] Values are mean ± SD of three replicates. There is no significant difference among treatments ($P>0.05$).
[2] Survival (%) = 100 × (initial fish number - dead fish number) / initial fish number.
[3] Weight gain (%) = 100 × (final weight - initial weight) / initial weight.
[4] Specific growth rate (% day^{-1}) = 100 × [ln (final weight) - ln (initial weight)] / days.
[5] Feed conversion efficiency = weight gain / feed consumed.
[6] Hepatosomatic index (%) = 100 × liver weight / total body weight.
[7] Vicerosomatic index (%) = 100 × viscera weight / total body weight.
[8] Net protein utilization (%) = 100 × (final body protein - initial body protein) / protein consumed.
[9] Protein efficiency ratio = weight gain / protein consumed.
Abbreviations for diets are given in footnotes to Table 2.1.

Andrews, 1974), African catfish (Baker and Davis, 1996a; Baker and Davis, 1997a), turbot and halibut (Tocher *et al.*, 2003). Feed conversion efficiency (FCE) and protein efficiency ratio followed the same trends in these studies. In the present experiment, no significant ($P>0.05$) impact of oxidized oil on FCE or PER was observed. A high FCE (>1) was obtained because the weight of dry feed pellets was used for calculation, which resulted in underestimation of real feed consumption when moisture absorption was not considered. Feed consumption was not affected by lipid oxidation in the diets, suggesting that cod did not discriminate against feed containing oxidized oil. Oxidized oil had no remarkable effect ($P>0.05$) on hepatosomatic index (HSI) or viscerosomatic index (VSI), which contradicts the findings of Sakaguchi and Hamaguchi (1969) for yellowtail, where oxidized dietary oil decreased HSI. These results indicate that Atlantic cod may have an adaptation to accepting oxidized diets, as the sensitivity of fish to oxidized lipid varies among species. A similar phenomenon was observed in Eurasian perch (Kestemont *et al.*, 2001).

With regard to dietary vitamin E supplementation, neither α-tocopherol nor mixed tocopherols had any significant effect ($P>0.05$) on cod growth performance such as survival and SGR, or feed utilization such as FCE, PER and NPU (net protein utilization). Similar results were reported in previous studies on several fish species, including turbot (Stephan *et al.*, 1995; Tocher *et al.*, 2003; Ruff *et al.*, 2003), halibut (Tocher *et al.*, 2003), rainbow trout (Kiron *et al.*, 2004), Atlantic salmon (Parazo *et al.*, 1998; Lygren *et al.*, 2000; Scaife *et al.*, 2000), African catfish (Baker and Davis, 1996b) and channel catfish (Bai and Gatlin, 1993), where dietary vitamin E did not show any significant effect on growth or feed utilization. Nevertheless, improvement of growth by dietary vitamin E

supplementation was observed in some other species. Increasing dietary vitamin E level resulted in appreciably improved growth and feed utilization in hybrid tilapia (Huang and Huang, 2004), sea bream (Tocher *et al.*, 2003), amago salmon (Taveekijakarn *et al.*, 1996) and Korean rockfish (Bai and Lee, 1998). Vitamin E supplementation was also found to prevent decreased HSI caused by oxidized dietary oil (Sakaguchi and Hamaguchi, 1969). Baker and Davis (1996b) showed that elevated dietary vitamin E levels resulted in enlarged livers of African catfish. Contradictory findings were, however, reported that higher vitamin E dose led to reduced liver size of African catfish (Baker and Davis, 1996a). In the present investigation in Atlantic cod, neither HSI nor VSI was significantly ($P>0.05$) affected by vitamin E supplementation.

The growth performance of Atlantic cod on various feeds in this experiment was not distinctly different. Fish were not influenced by lipid oxidation in diets or vitamin E deficiency to any significant ($P>0.05$) extent. One possible reason could be the species dependent capability of antioxidant system *in vivo*. Tocher *et al.* (2003) found that the activities of the primary radical scavenging enzymes, catalase and superoxide dismutase (SOD), were increased by feeding oxidized oil and decreased by dietary vitamin E, which gives rise to a progressive adaptation of fish to oxidized diets and varied vitamin E levels.

3.3 Blood parameters

Blood parameters of fish were determined in terms of hematocrit and erythrocyte osmotic fragility (EOF). Hematocrit and EOF are useful indicators for environmental and chemical stress such as oxidative stress in fish. Red blood cells (RBC) are one of the major production sites of free radicals, which can trigger oxidation of unsaturated fatty

acids in their membrane phospholipids and thus altering their quantity (referred to as hematocrit) and quality (referred to as EOF) (Kiron *et al.*, 2004). Owing to the high concentration of PUFA in their membrane and intracellular oxygen and hemoglobin content, erythrocytes are sensitive to oxidative stress (Dariyerli *et al.*, 2004). Oxidation of unsaturated membrane lipids increases the erythrocyte osmotic fragility and lysis of cell; however, RBC are protected from oxidative damage by various enzymatic and non-enzymatic radical scavengers with vitamin E making a great contribution to the latter group (Kiron *et al.*, 2004). Vitamin E is generally known for its protective role against oxidative damage of biomembranes.

3.3.1 Hematocrit

Decreased hematocrit of fish by oxidized dietary oil and vitamin E deficiency has been reported (Murai and Andrews, 1974; Hung and Slinger, 1980), because of the increased level of dietary lipid peroxides and/or the absence of adequate vitamin E. Nevertheless, Sakai *et al.* (1992) observed increased hematocrit in yellowtail fed on oxidized oil. The hematocrit values of Atlantic cod fed various diets in the current study are given in Table 3.6. Hematocrit ranged from 22.2 to 24.1% with the hematocrit of fish fed oxidized oil being slightly lower than those fed on fresh oil. No statistical variation was observed among treatments ($P>0.05$). Dietary lipid oxidation and vitamin E supplementation had no major influence on the hematocrit.

Table 3.6 Hemacocrit values of final fish after 9-week feeding trial [1]

Hc. (%)	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
Mean	24.1	22.6	22.2	22.7
SD	2.06	2.27	2.29	1.99

[1] Values are mean and SD of three replicates. There is no significant difference among treatments ($P>0.05$).
Abbreviations for diets are given in footnotes to Table 2.1.

3.3.2 Erythrocyte osmotic fragility

EOF, measured as hemolysis in saline solution with varying concentrations, is also used to indicate oxidative damage in erythrocytes. Hemolysis could be affected by both oxidative state of dietary lipid and antioxidant status. Hemolysis was raised in fish fed either a diet containing oxidized oil or a diet deficient in vitamin E, as reported in the literature (Cowey *et al.*, 1981; Cowey *et al.*, 1983; Obach *et al.*, 1993; Sau *et al.*, 2004; Kiron *et al.*, 2004). The results for hemolysis of Atlantic cod in this experiment are shown in Table 3.7 and Figure 3.2. Hemolysis of fish in all treatments decreased as the saline concentration increased. No significant difference ($P>0.05$) was found among the four treatments when saline concentration was in the range of 0.1 - 0.55%. At higher saline concentrations (0.6 - 0.85%), fish fed on oxidized oil had a higher hemolysis compared to those fed fresh oil ($P<0.05$). Supplementation with α-tocopherol reduced the hemolysis, while mixed tocopherols had no effect on hemolysis. It is indicated that oxidized dietary oil increased EOF by increasing the oxidative stress on red blood cells, and that α-tocopherol enhanced the resistibility of erythrocyte membranes to hemolysis, which is in accordance with literature reports (Hung *et al.*, 1981). Mixed tocopherols did not show remarkable capability in improving the strength of the RBC, which might be due to the low level of α-tocopherol in the tocopherol mixture and the poor absorption of γ- and δ-tocopherols and/or transfer into fish blood.

3.4 Proximate composition of fish whole body

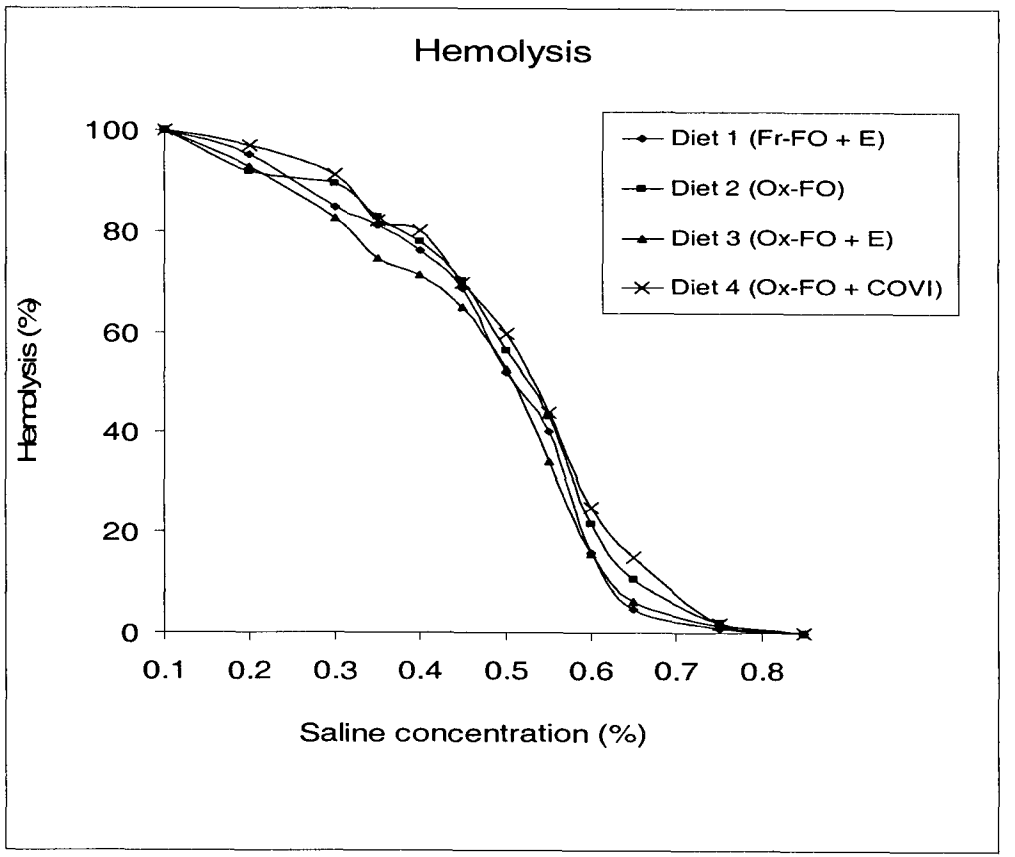
Diet is one of the major factors affecting the proximate composition of cultured fish, which has been correlated with the quality and nutritional value of fish products.

Table 3.7 Hemolysis (%) of cod juveniles in varing saline concentrations [1]

Saline (%)		0.20	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.65	0.75
Fr-FO+E	*Mean*	95.2	84.7	81.4	76.1	68.8	52.1	40.1	15.8 ^{b}	4.66 ^{c}	0.97 ^{b}
	SD	1.68	6.04	7.58	4.36	6.14	6.58	7.55	2.55	1.24	0.02
Ox-FO	*Mean*	91.8	89.4	82.8	78.1	70.2	56.3	43.2	21.7 ^{ab}	10.6 ^{ab}	1.93 ^{a}
	SD	6.15	6.04	7.34	15.6	13.4	1.52	5.53	4.48	5.14	0.04
Ox-FO+E	*Mean*	92.8	82.6	74.7	71.4	64.9	52.7	34.1	15.6 ^{b}	6.15 ^{bc}	1.34 ^{ab}
	SD	3.58	9.14	6.47	4.61	7.07	7.52	12.9	2.02	2.69	0.88
Ox-FO+COVI	*Mean*	96.8	91.2	82.1	80.2	69.9	59.8	43.8	24.8 ^{a}	14.9 ^{a}	1.79 ^{a}
	SD	1.57	7.26	10.0	8.29	8.68	13.7	13.4	7.00	4.20	0.27

[1] Values are mean and SD of six replicates. Mean values in the same column with different superscripts were significantly different at $P<0.05$. Columns with no superscript letters are not significantly different at $P>0.05$.
Abbreviations for diets are given in footnotes to Table 2.1.

Figure 3.2 Hemolysis of cod juveniles in varying saline concentrations (Abbreviations for diets are given in footnotes to Table 2.1)


Hemolysis
Diet 1 (Fr-FO + E)
Diet 2 (Ox-FO)
Diet 3 (Ox-FO + E)
Diet 4 (Ox-FO + COVI)
Hemolysis (%)
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Saline concentration (%)

Investigations into the effect of oxidized dietary oil and vitamin E supplementation on fish proximate composition has received attention, but different results have been obtained. Huang and Huang (2004) demonstrated that dietary vitamin E did not affect whole body composition of tilapia fed on oxidized oil. Similar results were found in sea bass (Gatta *et al.*, 2000), turbot (Ruff *et al.*, 2003) and Atlantic salmon (Waagbo *et al.*, 1993). However, a difference in proximate composition of Atlantic salmon caused by vitamin E deficiency was reported by Hamre and Lie (1995).

In the current study, the whole body proximate composition of fish over the 9-week feeding experiment was determined initially and at the end, and results are shown in Table 3.8. The fish after 9 weeks of feeding had slightly higher moisture and lipid contents and lower protein and ash contents than the fish at the start of the experiment. Body composition of fish at the end varied among treatments. Fish fed oxidized oil had a lower ash content than those fed fresh oil. A reduction in moisture content by supplemental α-tocopherol and mixed tocopherols was observed ($P<0.05$), while oxidized oil did not show any significant effect on moisture content ($P>0.05$). Lipid contents ranged from 5.48 to 6.09%, with no statistical variation among treatment ($P>0.05$). A similar trend was detected in whole body protein. Neither oxidized oil nor vitamin E supplementation exerted any distinct effect on crude protein or lipid contents.

3.5 Muscle and liver lipids

3.5.1 Lipid contents

Atlantic cod is a lean fish that stores fat in its liver. Muscle contains only a low level of lipid (about 1%). Table 3.9 shows the lipid content in different tissues of the fish at the

Table 3.8 Proximate composition (%) of fish whole body [1]

Components	Initial	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
Moisture	74.7 ± 0.36	75.6 ± 0.16^{ab}	76.3 ± 0.53^{a}	75.2 ± 0.28^{b}	75.2 ± 0.49^{b}
Protein	15.0 ± 0.21	14.5 ± 0.00	14.5 ± 0.04	14.2 ± 0.23	14.6 ± 0.12
Lipid	5.28 ± 0.19	5.61 ± 0.64	6.09 ± 0.31	5.86 ± 0.36	5.48 ± 0.45
Ash	3.68 ± 0.14	3.19 ± 0.23^{a}	2.87 ± 0.25^{ab}	2.64 ± 0.06^{b}	2.85 ± 0.15^{ab}

[1] Values are mean ± SD of three replicates from 6 fish in each group. Values in the same row with different superscripts were significantly different at $P<0.05$. Rows with no superscript letters are not significantly different at $P>0.05$.
Abbreviations for diets are given in footnotes to Table 2.1.

Table 3.9 Lipid contents of initial and final fish tissues [1]

	Initial	Fr-FO+E	Ox-FO	Ox-FO+E	Ox-FO+COVI
Whole body	5.28 ± 0.19	5.61 ± 0.64	6.09 ± 0.31	5.86 ± 0.36	5.48 ± 0.45
Muscle	0.87 ± 0.05	1.08 ± 0.01^{b}	1.37 ± 0.02^{a}	1.15 ± 0.02^{b}	1.41 ± 0.05^{a}
Liver	72.2 ± 0.74	51.2 ± 0.86^{c}	58.9 ± 0.82^{a}	49.9 ± 0.65^{c}	54.0 ± 0.69^{b}

[1] Lipid content is expressed as weight percentage of lipid in wet tissues. Values are mean ± SD of three replicates from 6 fish in each group. Values in the same row with different superscripts were significantly different at $P<0.05$. Rows with no superscript letters are not significantly different at $P>0.05$.
Abbreviations for diets are given in footnotes to Table 2.1.

beginning and the end of a 9-week feeding trial. The whole body and muscle lipid levels of fish at the end were slightly higher than those in the fish at the beginning, whereas liver lipid content dropped significantly from 72% to about 50% at the end of the feeding experiment. Muscle and liver lipid content of final fish was in the range of 1.08 – 1.41% and 49.9 – 58.9%, respectively. These values are high in comparison with those found in wild Atlantic cod (0.8% and 38.3% lipid in muscle and in liver, respectively) (Copeman and Parrish, 2004). There was no major influence on muscle and liver lipid content by oxidized oil ($P>0.05$). Supplementation of α-tocopherol decreased the lipid content of both muscle and liver tissues ($P<0.05$). Mixed tocopherols reduced only liver lipid content ($P<0.05$) with no influence on muscle lipid ($P>0.05$). These findings are different from the literature reports in other fish species such as Atlantic salmon. Parazo *et al.* (1998) showed that both α- and γ-tocopherol in diets did not affect muscle and liver lipid contents of Atlantic salmon.

3.5.2 Lipid classes

The lipid class composition of muscle and liver tissue was determined and results are shown in Table 3.10. Triacylglycerols (TAG), the main neutral lipid class serving as the most concentrated form of energy storage in animals (37.6 kJ/g lipid) (Holmer, 1989), comprised the major lipid class of cod liver at an average level of 80%. The TAG level in juvenile cod liver lipids ranged from 78.0% to 83.1% with the liver of fish fed vitamin E supplemented diets containing slightly lower content of TAG. Nevertheless, no statistical variation in TAG content was observed among treatments ($P>0.05$). Phospholipids (PL) were present at low levels (ranging from 1.77 to 4.49%) in cod liver, and their

Table 3.10 Muscle and liver lipid classes determined by Iatroscan [1]

	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
Muscle lipid				
SE	0.79 ± 0.05	0.49 ± 0.02	0.80 ± 0.37	0.58 ± 0.27
TAG	8.88 ± 0.63	10.1 ± 0.98	13.2 ± 6.25	7.97 ± 1.04
ST	1.57 ± 0.23^{a}	0.82 ± 0.27^{bc}	1.01 ± 0.23 ^{b}	0.38 ± 0.04^{c}
AMPL	4.50 ± 0.75	4.13 ± 0.44	5.49 ± 1.5	3.67 ± 0.29
PL	77.4 ± 2.23	80.4 ± 2.97	74.1 ± 4.47	75.7 ± 2.00
Σ Neutral	11.3 ± 0.53	11.4 ± 0.87	15.0 ± 6.33	8.93 ± 1.17
Σ Polar	81.9 ± 2.05	84.6 ± 3.89	79.5 ± 4.75	79.4 ± 2.16
Liver lipid				
SE	0.48 ± 0.18	0.38 ± 0.18	0.49 ± 0.02	0.73 ± 0.21
TAG	81.0 ± 6.03	83.1 ± 2.51	78.1 ± 0.21	78.0 ± 0.04
FFA	1.15 ± 0.62	2.70 ± 1.34	2.60 ± 2.47	1.78 ± 0.84
ST	0.51 ± 0.15^{b}	2.70 ± 1.27^{a}	1.17 ± 0.55^{ab}	1.45 ± 0.68^{ab}
DAG	8.88 ± 2.69	7.30 ± 1.15	8.36 ± 0.91	9.60 ± 1.16
AMPL	3.75 ± 0.55	5.37 ± 1.22	6.05 ± 1.48	5.41 ± 2.25
PL	4.49 ± 2.12	2.08 ± 1.13	1.77 ± 0.97	1.85 ± 1.21
Σ Neutral	92.1 ± 7.39	96.2 ± 4.21	90.7 ± 3.54	91.6 ± 2.16
Σ Polar	8.24 ± 2.16	7.45 ± 1.20	7.82 ± 1.05	7.26 ± 2.06

[1] Values are mean ± SD of three replicates from 12 fish in each group. Values in the same row with different superscripts were significantly different at $P<0.05$. Rows with no superscript letters are not significantly different at $P>0.05$.
SE: sterol esters; TAG: triacylglycerols; FFA: free fatty acids; ST: sterols;
DAG: diacylglycerols; AMPL: acetone mobile polar lipids; PL: phospholipids.
Abbreviations for diets are given in footnotes to Table 2.1.

distribution among treatments followed the same trend as that of TAG. Fish fed vitamin E supplemented diets had slightly lowered PL contents in their livers. However, no statistical difference in PL concentration was shown ($P>0.05$). A relatively high proportions of free fatty acids (FFA) and diacylglycerols (DAG) were detected in liver lipids, and their distribution among treatments negatively correlated with that of TAG and PL. This may possibly be due to the hydrolysis of TAG and PL. Hydrolysis of TAG and PL may have occurred during cold storage of lipid or tissue samples, giving rise to the formation of FFA and DAG. It has been found that TAG had higher stability against hydrolysis than PL (Copeman and Parrish, 2004). There was no significant difference in FFA or DAG contents among treatments although both FFA and DAG were present at slightly higher levels in liver of fish fed vitamin E supplemented diets. Liver sterols showed significant differences among diet treatments ($P<0.05$). The highest liver sterol content was found in fish fed oxidized oil without vitamin E supplementation. Dietary α-tocopherol decreased the sterol level in cod liver; mixed tocopherols, to a lesser extent, also reduced sterol content. No significant ($P>0.05$) effect of oxidized oil and vitamin E on sterol esters (SE) or acetone mobile polar lipids (AMPL) fractions was observed.

In contrast to liver oil, cod muscle lipid contained a high proportion of PL (77% in average) and a low level of TAG (10% in average). PL are one of the major components of cell membranes and act both structurally and functionally in animals. Their use as an energy source is limited. The contents of FFA and DAG in cod muscle lipid were below the measurable level, suggesting that TAG and PL were not hydrolysed or they were hydrolysed to a small extent. The only significant difference in lipid class composition of muscle tissue caused by diet variables was in sterols. Oxidized dietary oil reduced the

sterol content in muscle lipid ($P<0.05$), whereas the reverse was true in liver sterol concentration, which was increased by oxidized oil. Neither α-tocopherol nor mixed tocopherols showed any significant effect on muscle sterol levels ($P>0.05$).

3.5.3 Fatty acid composition of muscle and liver total lipids

Fatty acid composition of liver and muscle total lipid is presented in Tables 3.11 and 3.12 as well as Figures 3.3 and 3.4. Although statistical analysis was performed for each individual fatty acid, effect of diet treatment on fatty acid composition of fish tissue was obtained mainly by observing the changes in the major fatty acid groups, namely saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids (PUFA) and n-3 polyunsaturated fatty acids.

Cod liver lipid had a high proportion of PUFA (30.7 – 32.1%), in particular n-3 PUFA (24.8 – 26.3%), and its fatty acid composition reflected that of dietary lipid, which contained 31.7 – 32.5% and 26.9 – 27.7% of PUFA and n-3 PUFA, respectively. Palmitic acid (C16:0) was the major saturated fatty acid in liver lipid, followed by stearic acid (C18:0) and myristic acid (C14:0). Monounsaturated fatty acids were high in liver, with oleic acid (C18:1) being predominant. Liver lipid was rich in n-3 fatty acids. EPA (C20:5n-3) was the major n-3 PUFA at an average level of 13% in total liver fatty acids. DHA (C22:6n-3), although present at lower levels compared with EPA, also had high concentrations in cod liver, ranging from 8.40 to 9.26%. The influence of diet treatment on liver fatty acid composition was investigated. A marginal increase in saturated fatty acids and decrease in PUFA especially n-3 PUFA by oxidized dietary oil was observed ($P<0.05$). This is in accordance with the findings in sea bream (Tocher *et al.*, 2003) and

Table 3.11 Fatty acid composition of liver total lipid [1]

FA (%)	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
C 12:0	tr [2]	tr	tr	0.09 ± 0.00
C 14:0	3.68 ± 0.01^{a}	3.72 ± 0.03^{a}	3.52 ± 0.10^{b}	3.59 ± 0.06^{b}
C 15:0	0.32 ± 0.00	0.33 ± 0.01	0.33 ± 0.01	0.34 ± 0.01
C 16:0	13.6 ± 0.03^{c}	13.8 ± 0.04^{b}	13.8 ± 0.08^{b}	14.0 ± 0.01^{a}
C 16:1	7.39 ± 0.01^{a}	7.42 ± 0.03^{a}	6.55 ± 0.33^{b}	7.29 ± 0.08^{a}
C 17:0	0.27 ± 0.00^{b}	0.30 ± 0.00^{a}	0.30 ± 0.00^{a}	0.30 ± 0.00^{a}
C 17:1	0.71 ± 0.00^{a}	0.68 ± 0.01^{b}	0.72 ± 0.00^{a}	tr
C 18:0	4.47 ± 0.03^{c}	4.66 ± 0.05^{b}	4.96 ± 0.08^{a}	4.80 ± 0.06^{b}
C 18:1	19.8 ± 0.07^{b}	19.7 ± 0.09^{b}	20.0 ± 0.03^{a}	20.1 ± 0.07^{a}
C 18:2	5.86 ± 0.03^{a}	5.90 ± 0.03^{a}	5.61 ± 0.07^{c}	5.73 ± 0.01^{b}
C 18:3n-6	tr	tr	0.21 ± 0.00^{a}	tr
C 18:3n-3	1.23 ± 0.01^{a}	1.24 ± 0.01^{a}	1.13 ± 0.04^{c}	1.19 ± 0.02^{ab}
C 20:1	2.73 ± 0.03	2.66 ± 0.03	2.68 ± 0.09	2.62 ± 0.03
C 20:2	tr	tr	0.31 ± 0.00	0.30 ± 0.00
C 20:3n-3	0.73 ± 0.00^{a}	0.71 ± 0.01^{b}	0.70 ± 0.01^{b}	0.70 ± 0.00^{b}
C 20:5n-3	13.0 ± 0.03	12.9 ± 0.07	12.5 ± 0.38	12.7 ± 0.16
C 22:1	tr	tr	0.33 ± 0.00	tr
C 22:5n-3	2.02 ± 0.01^{a}	1.91 ± 0.03^{b}	1.89 ± 0.00^{b}	1.85 ± 0.00^{b}
C 22:6n-3	9.26 ± 0.05^{a}	8.71 ± 0.04^{b}	8.68 ± 0.11^{b}	8.40 ± 0.05^{c}
ΣSAT	22.4 ± 0.05^{b}	22.8 ± 0.05^{a}	22.9 ± 0.10^{a}	23.1 ± 0.03^{a}
ΣMONO	30.3 ± 0.27	30.5 ± 0.09	30.3 ± 0.25	30.0 ± 0.02
ΣPUFA	32.1 ± 0.04^{a}	31.3 ± 0.08^{b}	30.8 ± 0.33^{b}	30.7 ± 0.09^{b}
Σn-3	26.3 ± 0.08^{a}	25.4 ± 0.12^{ab}	24.9 ± 0.52^{b}	24.8 ± 0.23^{b}

[1] Results are expressed as area percentage of the fatty acid in total fatty acids. Values (mean ± SD of three replicates) in the same row with different superscripts were significantly different at $P<0.05$. Rows with no superscript letters are not significantly different at $P>0.05$.

[2] tr: trace.

Abbreviations for diets are given in footnotes to Table 2.1.

Figure 3.3 Fatty acid profile of liver total lipid (Abbreviations for diets are given in footnotes to Table 2.1)

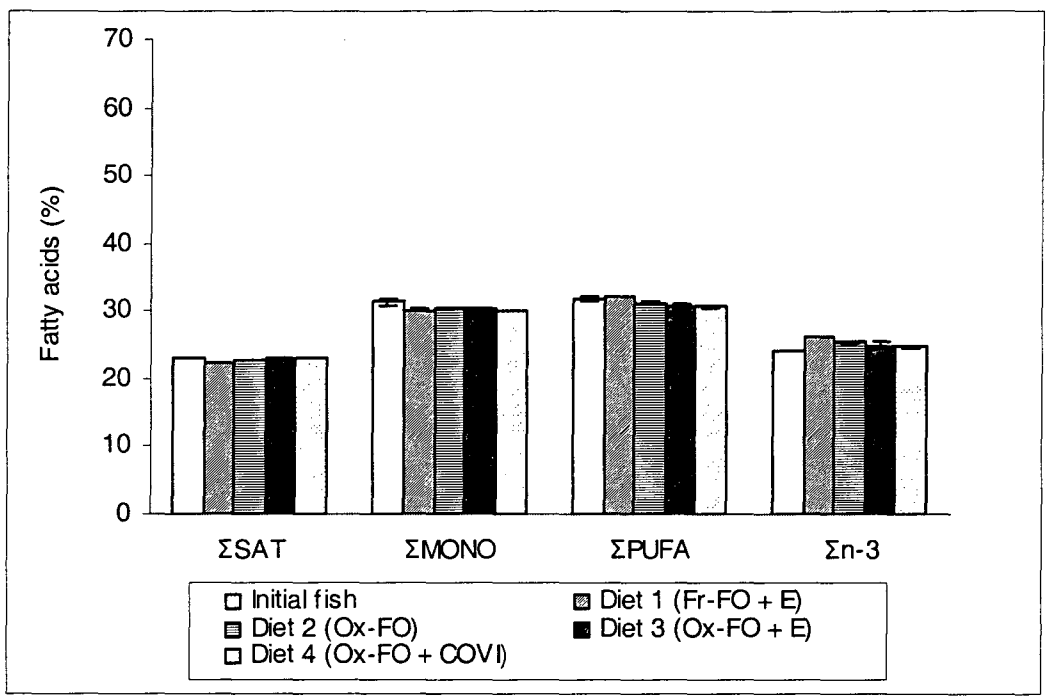

70
60
50
40
30
20
10
0
Fatty acids (%)
ΣSAT
ΣMONO
ΣPUFA
Σn-3
Initial fish
Diet 1 (Fr-FO + E)
Diet 2 (Ox-FO)
Diet 3 (Ox-FO + E)
Diet 4 (Ox-FO + COVI)

Table 3.12 Fatty acid composition of muscle total lipid [1]

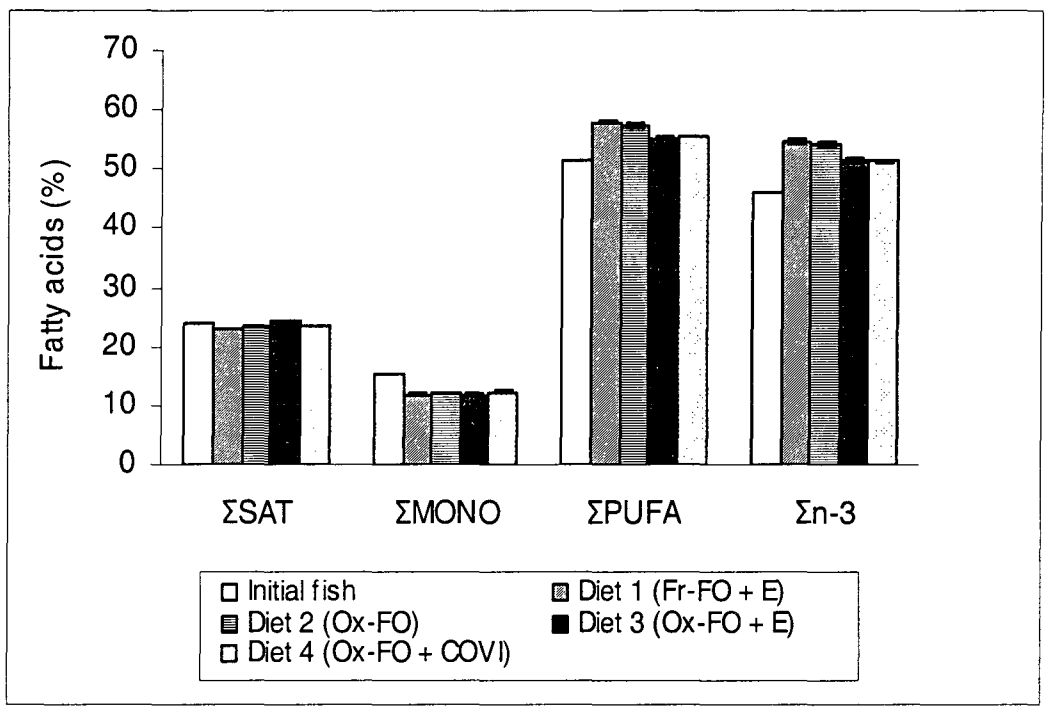
FA (%)	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
C 14:0	1.09 ± 0.03^{c}	1.07 ± 0.01^{c}	1.23 ± 0.01^{a}	1.18 ± 0.01^{b}
C 15:0	0.19 ± 0.00^{b}	0.21 ± 0.00^{a}	0.22 ± 0.00^{a}	0.21 ± 0.01^{a}
C 16:0	16.6 ± 0.05^{d}	17.0 ± 0.05^{b}	17.6 ± 0.04^{a}	16.8 ± 0.01^{c}
C 16:1	1.91 ± 0.03^{b}	1.80 ± 0.02^{c}	2.01 ± 0.02^{a}	2.01 ± 0.01^{a}
C 17:0	0.25 ± 0.00^{b}	0.27 ± 0.00^{a}	0.28 ± 0.01^{a}	0.27 ± 0.00^{a}
C 17:1	0.65 ± 0.24^{ab}	0.98 ± 0.01^{a}	0.24 ± 0.00^{b}	0.74 ± 0.28^{a}
C 18:0	4.96 ± 0.01^{a}	4.98 ± 0.02^{a}	4.86 ± 0.01^{b}	4.95 ± 0.00^{a}
C 18:1	8.45 ± 0.01^{b}	8.44 ± 0.03^{b}	8.47 ± 0.05^{b}	8.84 ± 0.01^{a}
C 18:2	3.42 ± 0.02^{b}	3.25 ± 0.02^{c}	3.40 ± 0.00^{b}	3.64 ± 0.01^{a}
C 18:3n-3	0.55 ± 0.01^{b}	0.50 ± 0.01^{c}	0.55 ± 0.01^{b}	0.58 ± 0.00^{a}
C 20:1	1.19 ± 0.01	1.12 ± 0.01	1.03 ± 0.09	1.11 ± 0.10
C 20:2	tr [2]	tr	0.24 ± 0.01	0.25 ± 0.01
C 20:3n-6	tr	tr	0.23 ± 0.01	0.21 ± 0.02
C 20:3n-3	2.26 ± 0.01^{a}	2.16 ± 0.01^{b}	2.03 ± 0.02^{d}	2.07 ± 0.00^{c}
C 20:5n-3	17.9 ± 0.17^{a}	17.3 ± 0.15^{bc}	17.6 ± 0.15^{ab}	17.0 ± 0.05^{c}
C 22:1	tr	tr	0.21 ± 0.00	tr
C 22:5n-3	2.65 ± 0.02^{b}	2.66 ± 0.01^{b}	2.72 ± 0.02^{a}	2.68 ± 0.02^{ab}
C 22:6n-3	31.2 ± 0.10^{a}	31.5 ± 0.22^{a}	28.6 ± 0.19^{b}	29.0 ± 0.07^{b}
ΣSAT	23.0 ± 0.07^{c}	23.6 ± 0.08^{b}	24.2 ± 0.03^{a}	23.5 ± 0.03^{b}
ΣMONO	12.0 ± 0.03^{b}	12.3 ± 0.06^{a}	11.9 ± 0.13^{b}	12.4 ± 0.10^{a}
ΣPUFA	58.0 ± 0.31^{a}	57.4 ± 0.40^{a}	55.3 ± 0.37^{b}	55.4 ± 0.08^{b}
Σn-3	54.6 ± 0.30^{a}	54.1 ± 0.39^{a}	51.4 ± 0.36^{b}	51.3 ± 0.10^{b}

[1]Results are expressed as area percentage of the fatty acid in total fatty acids. Values (mean ± SD of three replicates) in the same row with different superscripts were significantly different at $P<0.05$. Rows with no superscript letters are not significantly different at $P>0.05$.

[2] tr: trace.

Abbreviations for diets are given in footnotes to Table 2.1.

Figure 3.4 Fatty acid composition of muscle total lipid (Abbreviations for diets are given in footnotes to Table 2.1)


70
60
50
40
30
20
10
0
Fatty acids (%)
ΣSAT
ΣMONO
ΣPUFA
Σn-3
Initial fish
Diet 1 (Fr-FO + E)
Diet 2 (Ox-FO)
Diet 3 (Ox-FO + E)
Diet 4 (Ox-FO + COVI)

African catfish (Baker and Davis, 1997a). The dietary fatty acid patterns may account for the variation of liver fatty acid profiles of fish receiving the diets. Fatty acids distribution in diets varied among treatments with the fresh diet containing larger amount of PUFA than oxidized diets when vitamin E was added at the same level. In addition to the lower PUFA available in oxidized diets, loss of liver PUFA in fish fed oxidized oil may also, at least partially, be due to the reduction of n-3 PUFA including EPA, DPA and DHA as the major substrates of oxidation reaction in fish liver. The relatively increased saturated fatty acids may have resulted from decreased abundance of PUFA in the liver lipids. Neither dietary α-tocopherol nor mixed tocopherols had any marked effect on fatty acid composition of cod liver lipids ($P>0.05$), which lends support to literature findings for turbot (Tocher *et al.*, 2003) and Arctic char (Olsen *et al.*, 1999).

The muscle lipid contained a higher concentration of PUFA than liver lipid. The PUFA content in muscle lipid ranged from 55.3 to 58.0% with most being n-3 PUFA (51.3 – 54.6% in total fatty acids). DHA, in addition to EPA in liver oil, was the predominant n-3 PUFA in cod muscle lipid having an average level of 30%. EPA was less abundant than DHA and accounted for 17.0 – 17.9% of total muscle fatty acids. This is consistent with the fact that the membranes of the muscle cells of marine fish are almost always rich in DHA, with lesser amounts of EPA, as described by Ackman *et al.* (1997). The trend among treatments of muscle fatty acid composition mirrored that of dietary fatty acids. Fish fed oxidized oil had a lower level of PUFA in muscle than those fed fresh oil. However, the lowest PUFA content was found in muscle of fish fed oxidized oil with vitamin E supplementation. Suppression of PUFA by tocopherols may not be due to their antioxidant role, but simply because of the lesser amount of PUFA available from dietary

source in vitamin E treatments (Table 3.4). Lower levels of dietary PUFA may have led to lower levels of muscle PUFA.

3.5.4 Fatty acid composition of muscle neutral lipids and phospholipids

Muscle total lipid was fractionated using column chromatography into neutral lipids and phsopholipids. Fatty acid composition of each fraction was determined and results are given in Tables 3.13 and 3.14, respectively.

Both neutral lipids and phospholipids had high percentages of PUFA (mainly EPA and DHA), ranging from 54.5 to 60.0%, and 54.7 to 60.5%, respectively. Containing a dramatically higher proportion of DHA (around 33%) than of EPA (around 16%), phospholipids had the characteristic fatty acid composition of muscle total lipids. In the neutral lipids fraction, however, EPA was incorporated at slightly higher levels than DHA. A similar trend for distribution of EPA and DHA was found in liver lipids. In addition, muscle neutral lipids had higher levels of monounsaturated fatty acids, including C16:1, C18:1 and C20:1, and lower levels of PUFA than phosopholipids, and appeared more closely related to liver lipid. These findings support a previous report on haddock by Nanton *et al.* (2001) that muscle neutral lipids had a fatty acid composition more closely resembling the storage lipid in the liver compared to phospholipids. Nevertheless, the total amounts of EPA and DHA were similar in both neutral lipids and phospholipids regardless of their varying distributions.

Fatty acid composition of muscle neutral lipids and phospholipids varied among dietary treatments. Fish fed oxidized oil without supplemental vitamin E had the highest concentration of saturated fatty acids and lowest PUFA content in neutral lipids, as a

Table 3.13 Fatty acid composition of muscle neutral lipids [1]

FA (%)	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
C 12:0	tr [2]	tr	0.32 ± 0.01	tr
C 14:0	1.47 ± 0.02^{ab}	1.39 ± 0.00^{ab}	1.21 ± 0.09^{b}	2.56 ± 0.94^{a}
C 15:0	tr	0.23 ± 0.00	0.20 ± 0.02	tr
C 16:0	13.7 ± 0.06^{b}	16.7 ± 0.31^{a}	13.7 ± 0.29^{b}	14.3 ± 0.56^{b}
C 16:1	2.73 ± 0.01^{b}	2.40 ± 0.03^{c}	2.38 ± 0.05^{c}	2.98 ± 0.11^{a}
C 17:0	tr	tr	0.20 ± 0.02	tr
C 17:1	4.06 ± 0.56	4.25 ± 1.63	2.72 ± 0.54	3.71 ± 2.27
C 18:0	2.63 ± 0.04^{bc}	3.12 ± 0.07^{a}	2.88 ± 0.05^{ab}	2.44 ± 0.19^{c}
C 18:1	8.40 ± 0.17^{a}	8.07 ± 0.20^{ab}	8.34 ± 0.27^{ab}	7.59 ± 0.48^{b}
C 18:2	3.72 ± 0.04^{ab}	3.48 ± 0.05^{b}	3.82 ± 0.03^{a}	3.92 ± 0.20^{a}
C 18:3n-3	tr	0.66 ± 0.00^{b}	0.70 ± 0.02^{a}	0.25 ± 0.00^{c}
C 20:1	1.28 ± 0.03^{a}	1.32 ± 0.04^{a}	1.11 ± 0.06^{b}	1.06 ± 0.05^{b}
C 20:3n-3	2.24 ± 0.01^{a}	2.00 ± 0.06^{b}	2.22 ± 0.04^{a}	1.91 ± 0.04^{b}
C 20:5n-3	26.7 ± 0.06^{a}	22.7 ± 0.34^{c}	24.3 ± 0.57^{b}	24.6 ± 0.47^{b}
C 22:5n-3	2.38 ± 0.02^{ab}	2.25 ± 0.11^{b}	2.57 ± 0.08^{a}	2.25 ± 0.08^{b}
C 22:6n-3	25.0 ± 0.08^{a}	24.0 ± 0.55^{b}	24.5 ± 0.30^{ab}	21.4 ± 0.07^{c}
ΣSAT	17.8 ± 0.11^{c}	21.4 ± 0.49^{a}	18.2 ± 0.20^{bc}	19.3 ± 0.20^{b}
ΣMONO	15.8 ± 0.10^{b}	16.0 ± 1.37^{ab}	14.6 ± 0.26^{b}	18.2 ± 1.00^{a}
ΣPUFA	59.9 ± 0.08^{a}	54.7 ± 1.43^{b}	58.1 ± 0.14^{a}	54.5 ± 0.20^{b}
Σn-3	56.2 ± 0.04^{a}	51.2 ± 1.38^{c}	54.3 ± 0.17^{b}	50.6 ± 0.00^{c}

[1]Results are expressed as area percentage of the fatty acid in total fatty acids. Values (mean ± SD of three replicates) in the same row with different superscripts were significantly different at $P<0.05$. Rows with no superscript letters are not significantly different at $P>0.05$.

[2] tr: trace.

Abbreviations for diets are given in footnotes to Table 2.1.

Table 3.14 Fatty acid composition of muscle phospholipids [1]

FA (%)	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
C 14:0	1.09 ± 0.01^{c}	0.89 ± 0.03^{c}	1.58 ± 0.06^{a}	1.34 ± 0.16^{b}
C 15:0	0.20 ± 0.01^{ab}	0.16 ± 0.03^{b}	0.24 ± 0.01^{a}	0.25 ± 0.03^{a}
C 16:0	16.9 ± 0.48^{ab}	14.9 ± 0.51^{b}	17.2 ± 0.50^{a}	16.7 ± 1.50^{ab}
C 16:1	1.71 ± 0.06^{b}	1.54 ± 0.01^{b}	2.08 ± 0.02^{a}	1.99 ± 0.15^{a}
C 17:0	0.28 ± 0.00	0.31 ± 0.02	tr [2]	tr
C 17:1	4.54 ± 1.02^{a}	2.07 ± 0.32^{b}	5.56 ± 0.18^{a}	4.40 ± 0.31^{a}
C 18:0	5.69 ± 0.06	5.96 ± 1.51	5.69 ± 0.52	5.21 ± 1.97
C 18:1	7.39 ± 0.18	7.31 ± 0.03	7.78 ± 0.16	7.66 ± 0.41
C 18:2	3.21 ± 0.16^{ab}	3.03 ± 0.14^{b}	3.15 ± 0.02^{ab}	3.33 ± 0.07^{a}
C 18:3n-3	0.54 ± 0.03^{a}	0.47 ± 0.00^{b}	0.56 ± 0.02^{a}	0.56 ± 0.03^{a}
C 20:1	0.94 ± 0.10	0.92 ± 0.15	0.95 ± 0.08	1.06 ± 0.12
C 20:3n-3	2.24 ± 0.10^{a}	2.26 ± 0.00^{a}	1.92 ± 0.02^{b}	2.15 ± 0.06^{a}
C 20:5n-3	16.4 ± 1.16	16.5 ± 2.19	14.7 ± 0.61	16.7 ± 2.05
C 22:5n-3	2.30 ± 0.04^{b}	2.76 ± 0.10^{a}	2.13 ± 0.03^{b}	2.36 ± 0.15^{b}
C 22:6n-3	33.1 ± 0.90^{b}	35.4 ± 0.16^{a}	32.6 ± 0.20^{b}	32.4 ± 1.35^{b}
ΣSAT	24.0 ± 0.78	22.0 ± 1.21	24.7 ± 0.05	23.3 ± 0.19
ΣMONO	14.1 ± 0.31^{b}	11.8 ± 0.13^{c}	15.9 ± 0.13^{a}	14.6 ± 0.28^{b}
ΣPUFA	57.5 ± 1.73^{ab}	60.5 ± 1.8^{a}	54.7 ± 0.52^{b}	57.3 ± 0.84^{ab}
Σn-3	54.3 ± 1.92^{ab}	57.4 ± 1.94^{a}	51.6 ± 0.54^{b}	53.9 ± 0.77^{ab}

[1]Results are expressed as area percentage of the fatty acid in total fatty acids. Values (mean ± SD of three replicates) in the same row with different superscripts were significantly different at $P<0.05$. Rows with no superscript letters are not significantly different at $P>0.05$.

[2] tr: trace.

Abbreviations for diets are given in footnotes to Table 2.1.

result of oxidative stress. Dietary α-tocopherol was able to reduce the loss of PUFA ($P<0.05$); mixed tocopherols, although less pronounced, also reduced the loss of PUFA caused by oxidized oil ($P<0.05$). However, the PUFA content in muscle phospholipids of fish fed oxidized oil was decreased by α-tocopherol supplementation ($P<0.05$). This was probably because less PUFA provision was available in the diet in which oxidized oil and α-tocopherol were used (Table 3.4). Ackman *et al.* (1997) have shown that phospholipids are more sensitive to dietary fatty acids than neutral lipids. No significant effect of oxidized oil or mixed tocopherols on PUFA was observed ($P>0.05$).

3.6 Tocopherol analysis

Tocopherol concentrations in diets, fish liver and muscle were determined by HPLC-MS analysis. Results were expressed as mg tocopherol per kg diet or wet tissue (Table 3.15).

3.6.1 Dietary vitamin E

The dietary requirement of vitamin E is variable among fish species, varying from 3.5 mg/kg diet in Atlantic salmon, to 500 mg/kg feed in carp (Kaewsrithong *et al.*, 2001). In the present study, α-tocopherol and mixed tocopherols (COVI-OX T 70) were added to diet during feed formulation to supply 300 IU (200 mg/kg) vitamin E equivalent in treatments of vitamin E supplementation. A marked difference was noticed between the analyzed values of tocopherol concentration and the amounts of tocopherols added to feed. This may be caused by an underestimation of analyzed tocopherol concentration. Loss of tocopherols may have occurred during the analysis especially during lipid extraction and saponification, as tocopherols are sensitive to their environment sensitive.

Table 3.15 Tocopherol concentrations in diets, fish liver and muscle tissue [1]

Tocopherols (mg/kg)	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
Diet				
α-	95.7 ± 5.07[a]	8.18 ± 1.03[c]	98.1 ± 5.26[a]	31.7 ± 2.14[b]
γ-	n.d.[2]	n.d.	n.d.	27.1 ±2.30
δ-	n.d.	n.d.	n.d.	tr [3]
β-	n.d.	n.d.	n.d.	n.d.
Liver				
α-	222 ± 9.10[a]	170 ± 7.23[b]	142± 5.82[c]	137± 4.89[c]
γ-	n.d.	n.d.	n.d.	n.d.
δ-	n.d.	n.d.	n.d.	n.d.
β-	n.d.	n.d.	n.d.	n.d.
Muscle				
α-	8.59 ± 0.66[ab]	6.62 ± 0.38[c]	9.64 ± 0.42[a]	8.53 ± 0.48[b]
γ-	n.d.	n.d.	n.d.	10.1 ± 1.04
δ-	n.d.	n.d.	n.d.	6.78 ± 0.54
β-	n.d.	n.d.	n.d.	n.d.

[1] Values (mean ± SD of three replicates) in the same row with different superscripts were significantly different at $P<0.05$.
[2] n.d.: not detected.
[3] tr: trace
Abbreviations for diets are given in footnotes to Table 2.1.

The internal standard could have been used to avoid this underestimation by involving a recovery factor. The loss of tocopherols during feed preparation and storage may also account for the lowered values of analyzed tocopherol concentrations. However, an apparent trend of dietary tocopherol levels among treatments was obtained, which can be considered identical with the original trend in diets as the recovery factor for α-tocopherol would perhaps be identical in all treatments. Feed supplemented with vitamin E had significantly higher α-tocopherol levels than those without vitamin E. γ-Tocopherol was found only in the diet where mixed tocopherols were included; δ-tocopherol was also detected but in trace amount; β-tocopherol was not detected. The relatively greater loss of γ-, δ- and β-tocopherol in diet may be owing to their higher antioxidant activity compared to α-tocopherol and thus higher sensitivity to environmental factors.

3.6.2 Alpha-tocopherol in liver and muscle tissues

Retention of tocopherols in fish tissue has been studied. Alpha-tocopherol has been the focus of most of previous studies because it has the highest biological activity. Alpha-tocopherol status has important health and product quality implications(Baker and Davis, 1997b). Fish incorporate α-tocopherol at different levels in different organs, and generally in the order of liver > kidney > heart > muscle (Ruff *et al.*, 2003). Only liver and muscle were investigated in this experiment for tocopherol concentration, and liver was found to contain remarkably higher concentrations of tocopherols than muscle both on lipid basis (data not shown) and on tissue basis. Liver and muscle vitamin E contents are known to be highly dependent on several factors such as species, size and age of fish, rearing conditions and diet including concentrations and forms of dietary tocopherols,

dietary lipid concentrations, fatty acid composition of the diet, and presence of other antioxidants in the diet (Chaiyapechara *et al.*, 2003; Ruff *et al.*, 2003). Dietary vitamin E level significantly affects tissue vitamin E content. There appears to be a dose response relationship between the dietary level of α-tocopherol and its deposition in fish tissue, and this correlation is more pronounced in the liver than in the muscle (Stephan *et al.*, 1995; Olsen *et al.*, 1999). Tissue α-tocopherol concentration is proportional to dietary α-tocopherol concentrations, i.e. tissue vitamin E content increases when dietary vitamin E level increases, as reported for many fish species (Baker and Davis, 1997a; Chaiyapechara *et al.*, 2003; Ruff *et al.*, 2003; Huang and Huang, 2004). Thus, tissue α-tocopherol levels may be modulated by supplementation of vitamin E into diets in order to increase tissue resistance to oxidative stress. Nevertheless, this is only the case when fresh oil is used in the diet. Decrease of tissue α-tocopherol in fish consuming oxidized oil has been observed in a number of studies (Hung *et al.*, 1980; Stephan *et al.*, 1991; Sakai *et al.*, 1992). A ten-fold decrease of liver α-tocopherol concentration in African catfish by oxidation of dietary lipids has been demonstrated (Baker and Davis, 1997b). Depletion of vitamin E has been associated with its antioxidant property in quenching the free radicals initiated by lipid peroxyl radicals and other assorted products of lipid oxidation from dietary sources. Consequently, α-tocopherol is utilized in fish tissue where oxidative stress is imposed (McDowell, 1989). Alternatively, Baker and Davis (1997b) speculated that oxidation products from rancid dietary oil may hinder intestinal absorption of free alcohols, or merely oxidize the tocopherols prior to digestive assimilation. However, studies in some fish species have provided different results. Hamre *et al.* (2001) found that there was no difference in tissue α-tocopherol of Atlantic

salmon between fresh oil feeding and oxidized oil feeding; Huang and Huang (2004) reported that oxidized oil in the diet did not increase the dietary vitamin E requirement of hybrid tilapia. One could assume that the effect of oxidized dietary oil on tissue tocopherol level may be species dependent and/or be related to some other factors in addition to dietary vitamin E content, which require more research.

In the present study, fish fed oxidized oil had significantly lower liver α-tocopherol concentration than those fed fresh oil ($P<0.05$) though the same level of dietary vitamin E was included. Unexpectedly, liver α-tocopherol concentration was higher in fish fed vitamin E deficient diet than those fed vitamin E supplemented diet in treatments of oxidized oil ($P<0.05$). Inclusion of additional dietary vitamin E did not increase the liver vitamin E content. A possible explanation could be that the supplemental vitamin E was not absorbed by fish because of the lipid oxidation products in the diet, as hypothesized by Baker and Davis (1997b). The hepatic α-tocopherol present was probably from those accumulated in the liver prior to the feeding trial. Alternatively, dietary vitamin E may have been absorbed by fish but was utilized to alleviate the oxidative stress caused by oxidized dietary oil. The hepatic α-tocopherol concentration detected in both treatments may be the minimal amount of vitamin E that cod liver always reserves for maintenance of normal growth. Therefore, dietary vitamin E did not exert any positive effect on liver vitamin E when oxidized oil was used in the diet. Simultaneously, fish receiving vitamin E supplementation had a remarkably lower lipid content in their liver than those fed diet without vitamin E (Table 3.9), which may account for their lower liver α-tocopherol concentration.

In contrast to liver α-tocopherol, concentration of muscle α-tocopherol reflected that of dietary α-tocopherol. Muscle α-tocopherol concentration was enhanced by dietary vitamin E supplementation ($P<0.05$). Greater enhancement was observed in the treatment containing α-tocopherol than that containing mixed tocopherols as mixed tocopherols contained a lesser amount of α-tocopherol that was introduced in the diet. Oxidized dietary oil did not significantly affect muscle α-tocopherol levels ($P>0.05$). This finding supports the hypothesis that muscle tocopherol may not be depleted by lipid oxidation due to reserves of hepatic vitamin E. α-Tocopherol tends to accumulate in the liver and then be mobilized and redistributed to other tissues (such as muscle) that are oxidatively stressed (Baker and Davis, 1997b).

3.6.3 Other tocopherol homologues in liver and muscle tissues

In addition to α-tocopherol, other forms of tocopherol such as γ- and δ-tocopherol also act as powerful antioxidants *in vitro*. However, the retention of dietary tocopherols in animal tissues has been the bottleneck of *in vivo* utilization of these vitamin E homologues. In this experiment, COVI-OX T 70 containing 9.4, 1.1, 44.1 and 16.7% of α-, β-, γ- and δ-tocopherol, respectively, was used to provide vitamin E in treatment of mixed tocopherols. Among them, β-tocopherol appears less important as a minor form of tocopherol in nature compared to other tocopherol homologues. Its concentration was below the measurable level both in diet and in fish tissues and is not discussed here.

Retained levels of dietary γ- and δ-tocopherols in liver and muscle tissue were studied. No γ- or δ-tocopherol was detected in the fish liver in all treatments. Supplementation of mixed tocopherols in diet did not result in deposition or resulted in poor deposition of γ-

and δ-tocopherol in liver. However, α-tocopherol was present at a level of 137 mg/kg tissue in the liver of fish receiving mixed tocopherols in the diet although a lower concentration of α-tocopherol was added to the diet compared to γ- and δ-tocopherols. This gives further support to the hypothesis that biodiscrimination exists between the tocopherols in their uptakes in fish liver. The discrimination is thought to be mediated through a competition for a hepatic tocopherol-binding protein, a very low density lipoprotein in the liver that preferentially secretes α-tocopherol (Hamre and Lie, 1997). The tocopherol-binding protein has been purified from rat liver cytosol (Yoshida *et al.*, 1992). This protein easily differentiates tocopherol homologues by the number of methyl groups on the chromanol ring and has higher affinity for α-tocopherol than for other forms of tocopherols (Hughes *et al.*, 1992). The variable binding capacity of tocopherol homologues to this protein gives rise to the varied biological activity and antioxidant efficiency of tocopherols *in vivo*. α-Tocopherol is considered a more potent antioxidant *in vivo* because its competitive power far exceeds that of other tocopherols. It has been found that retention of γ- and δ-tocopherols in animal tissues was elevated when α-tocopherol was deficient, as demonstrated by (Behrens and Madre, 1987). In addition to the preference for α-tocopherol over γ- and δ-tocopherols to be selected by the tocopherol-binding protein, the faster disappearance of γ- and δ-tocopherols also partially accounts for their lower retained levels in fish liver (Tran and Chan, 1992). This latter effect might arise from better antioxidant efficiency of γ- and δ-tocopherols that may lead to sparing of α-tocopherol in fish tissues.

Other tissues obtain tocopherols mainly from the liver, and thus have a relatively lower transfer of γ- and δ-tocopherols than α-tocopherol. Muscle and adipose tissue are

exceptions, which retain γ-tocopherol at a similar rate as α-tocopherol, and δ-tocopherol at a rate of approximately 50% that of α-tocopherol (Hamre *et al.*, 1998). Deposition of γ- and δ-tocopherols was found more efficient in perivisceral fat and adipose tissue than in liver (Parazo *et al.*, 1998). Fish muscle appears to be supplied with tocopherols from chylomicrons that have not been exposed to the hepatic discrimination mechanism (Hamre and Lie, 1997). Therefore, muscle tissue can be enriched in all tocopherols through a lipoprotein lipase-mediated catabolism of chylomicrons (Hamre and Lie, 1997). In the current experiment, γ- and δ-tocopherols were detected in the muscle of fish fed mixed tocopherols, and the concentrations of α-, γ- and δ-tocopherols were 8.53, 10.1 and 6.78 mg/kg, respectively (Table 3.15). Deposition of tocopherols in cod muscle was in response to the concentrations of dietary tocopherols.

3.7 Oxidative state of liver and muscle tissues

Oxidative state is an important indicator of food quality. Marine fish products are rich in PUFA, which are very prone to oxidation and consequently release a number of primary and secondary oxidation products. Some of these oxidation products impart a rancid aroma that adversely affects the sensory quality of food, and furthermore, some products are found toxic to animals and humans. Therefore, lipid oxidation is regarded as a major concern in quality preservation of fish products. Factors that influence the oxidative stability of fish products have been studied. These include environmental conditions during storage and processing of post-mortem fish as well as the intrinsic chemical composition of fish or tissues. Diets play an important role in oxidative stability of fish product by modulating the body composition of farmed fish. In the present study on

Atlantic cod, TBARS and headspace propanal in liver and muscle tissue were measured as indicators of oxidative state. However, headspace propanal of muscle tissue was only detected in trace in all diet treatments, probably because of the low lipid level in cod fillets. Additionally, the muscle phospholipids may be protected since it is suspected that α-tocopherol fits into the biomembranes structure, giving effective protection *in situ*.

The oxidative state of liver and muscle is given in Table 3.16. A strong correlation was observed between liver TBARS and headspace propanal levels. Fish fed fresh oil had the lowest level of TBARS and propanal in their liver. Oxidized dietary oil resulted in elevated concentration of oxidation products in liver regardless of vitamin E supplementation. A similar trend was observed in Atlantic salmon (Hamre *et al.*, 2001). This may be interpreted as an increased uptake of oxidation products, including TBARS and propanal, from the oxidized diet through the intestine, or uptake of lipid hydroperoxides that later decomposed to aldehydes, as demonstrated by Hamre *et al.* (2001). Alternatively, the appearance of TBARS and propanal may be the result of oxidation of tissue fatty acids, which were exposed to oxidative stress caused by oxidized diet or vitamin E deficiency. More research is required to unravel the source of lipid oxidation product in fish tissues. Both TBARS and headspace propanal values in cod liver negatively correlated with liver tocopherol levels, which can be explained by the antioxidant property of tocopherols. The higher the tocopherol content, the better the protection of liver lipid from oxidation, and hence a lower lipid oxidation in the liver. The liver of fish fed mixed tocopherols had a lower concentration of TBARS and propanal than those fed α-tocopherol alone, although α-tocopherol was retained at the same level, probably due to the antioxidant role of γ- and δ-tocopherols from mixed tocopherols.

Table 3.16 Oxidative state of fish liver and muscle tissue [1]

	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
Liver				
TBARS (nmol MDA/g tissue)	34.1 ± 2.69^{c}	76.1 ± 4.72^{b}	127 ± 0.49^{a}	75.2 ± 1.10^{b}
Headspace propanal (μg/g tissue)	73.0 ± 0.02^{d}	96.5 ± 0.69^{c}	228 ± 1.37^{a}	173 ± 1.14^{b}
Muscle				
TBARS (nmol MDA/g tissue)	1.13 ± 0.32^{b}	4.27 ± 0.98^{a}	1.26 ± 0.34^{b}	1.10 ± 0.20^{b}
Headspace propanal (μg/g tissue)	tr[2]	tr	tr	tr

[1]Values are mean and SD of six replicates. Mean values in the same row with different superscripts were significantly different at $P<0.05$.
[2] tr: trace.
Abbreviations for diets are given in footnotes to Table 2.1.

Gamma- and δ-tocopherols were not detected in cod liver because of being utilized as effective antioxidants, and because of their inferiority in the competition for the tocopherol-binding protein, as discussed in the previous section. This finding is in agreement with that of Ruff *et al.* (2003), who found that lipid oxidation was solely related to the concentration of tocopherols in fish. Furthermore, Tocher *et al.* (2003) reported that feeding oxidized oil did not generally increase lipid oxidation products in the liver of sea bream and turbot but they were generally reduced by a decreased level of tissue vitamin E. If this is correct, one can assume that fish does not absorb or has only limited absorption of lipid oxidation products from dietary sources, and that the oxidative stability of liver will not be affected by oxidized diet provided that there is a sufficient level of vitamin E in the liver. However, oxidized dietary oil may lead to increased vitamin E requirement and cause vitamin E deficiency in fish, and thus may exert an adverse effect on tissue oxidative state. Dietary tocopherols above a minimum requirement do not significantly improve the antioxidant defence of fish, as observed in Arctic char (Olsen *et al.*, 1999). In this experiment, vitamin E deficiency in cod liver occurred in the treatments where oxidized oil was used, and dietary vitamin E supplementation did not result in increased deposition of tocopherols in the liver. In other words, 300 IU vitamin E in the diet was not sufficient to protect the liver from oxidation when fish were fed highly oxidized oil.

Muscle TBARS values also showed a negative correlation with the tissue tocopherol levels. The highest TBARS value was accompanied with the lowest tocopherol concentration in the muscle tissue. Oxidized oil did not influence TBARS in cod fillet in the presence of vitamin E. Fillets containing γ- and δ-tocopherols had a slightly lower

TBARS values than those containing only α-tocopherol. Cod muscle probably has similar mechanisms as liver in preventing the absorption of oxidation products from dietary source.

CHAPTER 4

SUMMARY AND RECOMMENDATIONS

In this study, highly oxidized marine fish oil with different levels of tocopherol supplementation was fed to juvenile Atlantic cod for 9 weeks. Although an oil with a high peroxide value of 94 meq/kg is considered toxic for human consumption, cod seemed to have the ability to tolerate such oil, at least in the short term. Neither growth performance nor feed utilization was significantly affected by oxidized dietary lipid ($P>0.05$). It was assumed that fish may have similar mechanisms as mammals to prevent absorption of lipid oxidation products from the diet (Hamre *et al.*, 2001). In rats, ^{14}C-labelled MDA was recovered as $^{14}CO_2$ in excretion including urinary metabolites and feces (Hamre *et al.*, 2001). It is possible that cod did not absorb or had only limited absorption of oxidation products from dietary sources. Furthermore, endogenous antioxidant defence systems *in vivo* such as free radical scavenging enzymes, catalase and superoxide dismutase (SOD), protect the fish from oxidative damage. No significant ($P>0.05$) change in growth was caused by supplemental α-tocopherol or mixed tocopherols.

Erythrocyte osmotic fragility (EOF) showed significant variation among diet treatments. Fish fed oxidized oil had a higher hemolysis than those fed fresh oil ($P<0.05$). This may be due to the depletion or hindered absorption of vitamin E in the blood caused by oxidized dietary oil. Supplementation of α-tocopherol improved the EOF. However, mixed tocopherols had no significant ($P>0.05$) effect on EOF, probably because of the poor retention and deposition of γ- and δ-tocopherols in fish blood.

With regard to proximate composition of fish whole body, a reduction of ash content by oxidized oil was observed. Oxidized oil might have interfered with the absorption of minerals or caused mineral loss. There was no significant ($P>0.05$) difference among treatments in whole body protein or lipid content. However, lipid content of both liver and muscle tissue was decreased by supplemental α-tocopherol. Mixed tocopherols reduced only liver lipid content ($P<0.05$) with no effect on muscle lipid ($P>0.05$).

The liver oil of Atlantic cod was composed of a high proportion of neutral lipid (>90%, mainly triacylglycerols), whereas phospholipids predominated in muscle lipid. There was no significant ($P>0.05$) influence on major lipid classes of cod liver and muscle by diet with the exception of sterols. The highest concentration of liver sterols was found in fish fed oxidized oil without vitamin E supplementation. Dietary α-tocopherol was able to decrease the sterol level in cod liver; mixed tocopherols, to a lesser extent, also reduced the liver sterol content. Nevertheless, opposite results were obtained in muscle sterols. Oxidized oil reduced the sterol content in muscle lipid. Neither α-tocopherol nor mixed tocopherols showed any significant ($P>0.05$) effect on muscle sterol levels.

Fatty acid composition of cod liver lipids reflected that of dietary lipid, with a high proportion of EPA followed by DHA as the major PUFA. Fish fed oxidized oil had a lower level of PUFA and a higher level of saturated fatty acids in their livers compared to those fed fresh oil regardless of vitamin E supplementation. Cod muscle typically contained a higher concentration of PUFA than liver, with DHA being the predominant fatty acid. The trend among treatments of muscle fatty acid composition mirrored that of dietary fatty acids. Oxidized oil decreased muscle PUFA content in the presence of supplemental dietary vitamin E. Fatty acid composition varied between lipid fractions in

muscle tissue. Oxidized oil in the absence of dietary vitamin E decreased PUFA content and increased saturated fatty acid content in muscle neutral lipids. Dietary α-tocopherol reduced the loss of PUFA. A similar effect was observed for mixed tocopherols, although less pronounced. However, PUFA content in muscle phospholipids was decreased by supplemental α-tocopherol when oxidized oil was used.

Oxidized oil elevated lipid oxidation in liver tissue, probably because of the loss of tissue vitamin E caused by oxidized diet rather than through direct absorption and accumulation of oxidation products from dietary sources. In both liver and muscle tissue, levels of oxidation products, including TBARS and headspace propanal, were negatively correlated with tissue vitamin E levels. Atlantic cod may discriminate against lipid oxidation products in their absorption due to an unknown protection mechanism. The oxidative stability of liver and muscle was solely dependent on tissue tocopherol concentration. However, the oxidative state of dietary lipid was found to affect liver tocopherols and consequently influence the oxidative stability of liver tissue. Retention of dietary α-tocopherol in liver dropped significantly when oxidized oil was included in the feed. Supplementation of 300IU α-tocopherol did not show any improvement in liver α-tocopherol level, and hence exerted no additional protection to liver tissue. This indicates that oxidized dietary oil increased the vitamin E requirement and 300IU vitamin E was not sufficient to protect liver tissue from oxidative damage. Gamma- and δ-tocopherols from dietary tocopherol mixture were retained at very low levels in the liver compared to α-tocopherol, probably owing to their inferiority in the competition for the tocopherol-binding protein and/or the fast disappearance. Nevertheless, muscle tocopherol concentration was in response to dietary tocopherol content irrespective of the use of

rancid oil. As a result, lipid oxidation in muscle was not affected by oxidized dietary oil. Dietary α-tocopherol improved the oxidative stability of muscle lipid by deposition in the muscle tissue and some γ- and δ-tocopherols were also retained, thus providing further protection to the muscle tissue against lipid oxidation.

The overall results from this study suggest that oxidized dietary oil affected juvenile Atlantic cod by causing vitamin E deficiency in certain tissues and that these effects could be alleviated by supplementation of sufficient amounts of vitamin E in the diet. In addition, mixed tocopherols were good antioxidants for Atlantic cod, but less effective than α-tocopherol alone in many tissues, except for muscle, where γ- and δ-tocopherols were deposited at high levels. Nevertheless, because muscles are the major edible portion of fish, these results may be of use to fish farmers for producing products with better oxidative stability and hence extended shelf life.

The results of this study were based on short-term effects of diet on fish. The duration of feeding trial (9 weeks) may not be long enough to show significant difference in some parameters amongst treatments. Therefore, it is recommended that a longer period of feeding be employed to ascertain the long-term effects of oxidized oil and vitamin E on fish growth parameters, health and quality. Carbon ^{14} isotope technique can be adopted to visualize the absorption, degradation and excretion of oxidation products from dietary sources in fish. In addition, further research may be directed to establish vitamin E requirements of Atlantic cod when oxidized oil is used in the diet. Similar studies may also be conducted in larger size fish due to its potential interest to the user industry.

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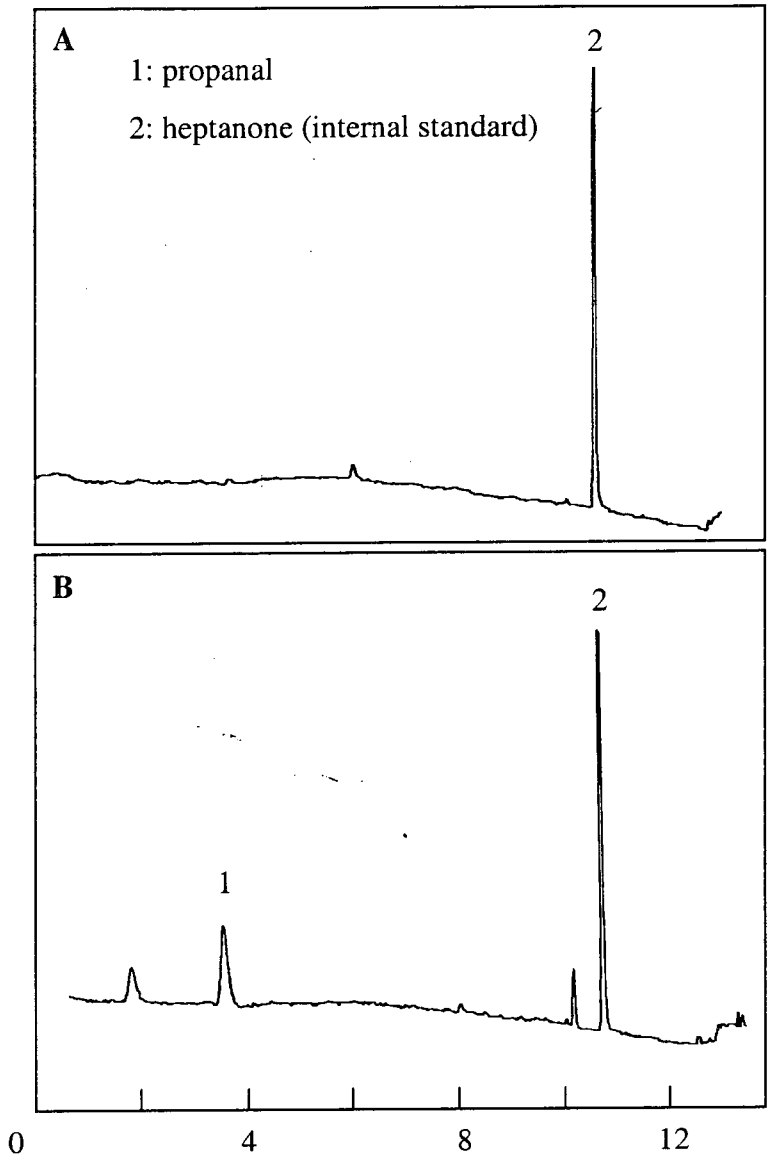
APPENDIX

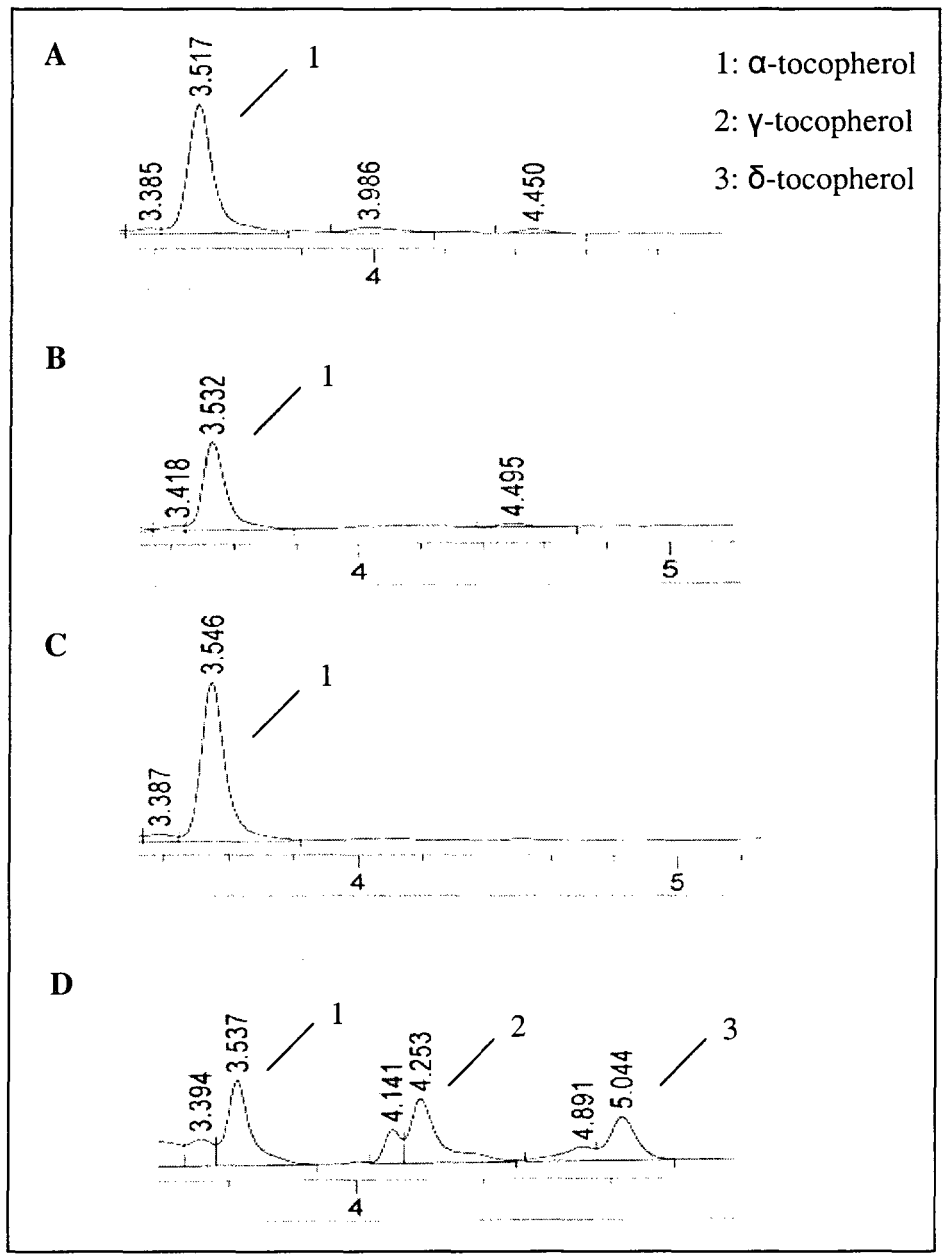
Figure A.1 Gas chromatogram of headspace volatiles of fresh fish oil (A) and oxidized fish oil (B)



A: fresh fish oil

B: oxidized fish oil

Figure A.2 HPLC chromatogram of tocopherols in muscle tissue



A: diet 1 (Fr-FO + E) C: diet 3 (Ox-FO + E)

B: diet 2 (Ox-FO) D: diet 4 (Ox-FO + COVI)

(Abbreviations are given as footnotes to Table 3)

Figure A.3 Mass spectrum of α-tocopherol

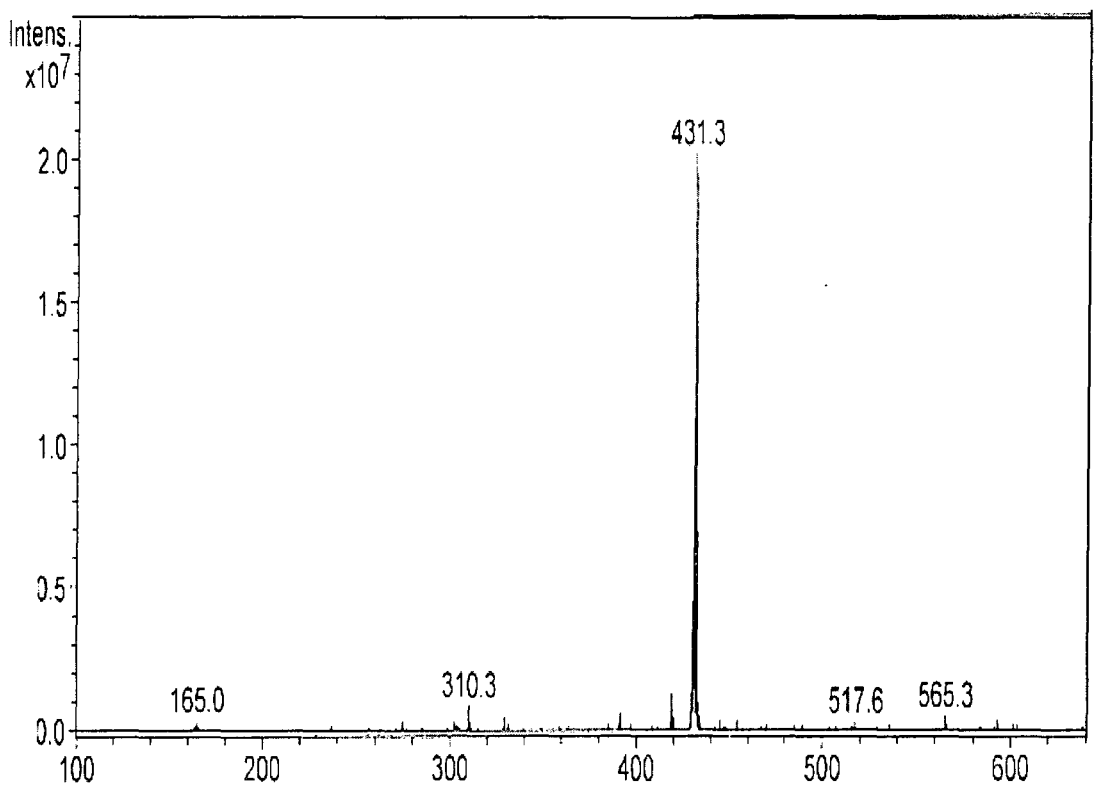

Intens.
x10^7
2.0
1.5
1.0
0.5
0.0
431.3
310.3
165.0
517.6
565.3
100
200
300
400
500
600

Figure A.4 Mass spectrum of γ-tocopherol

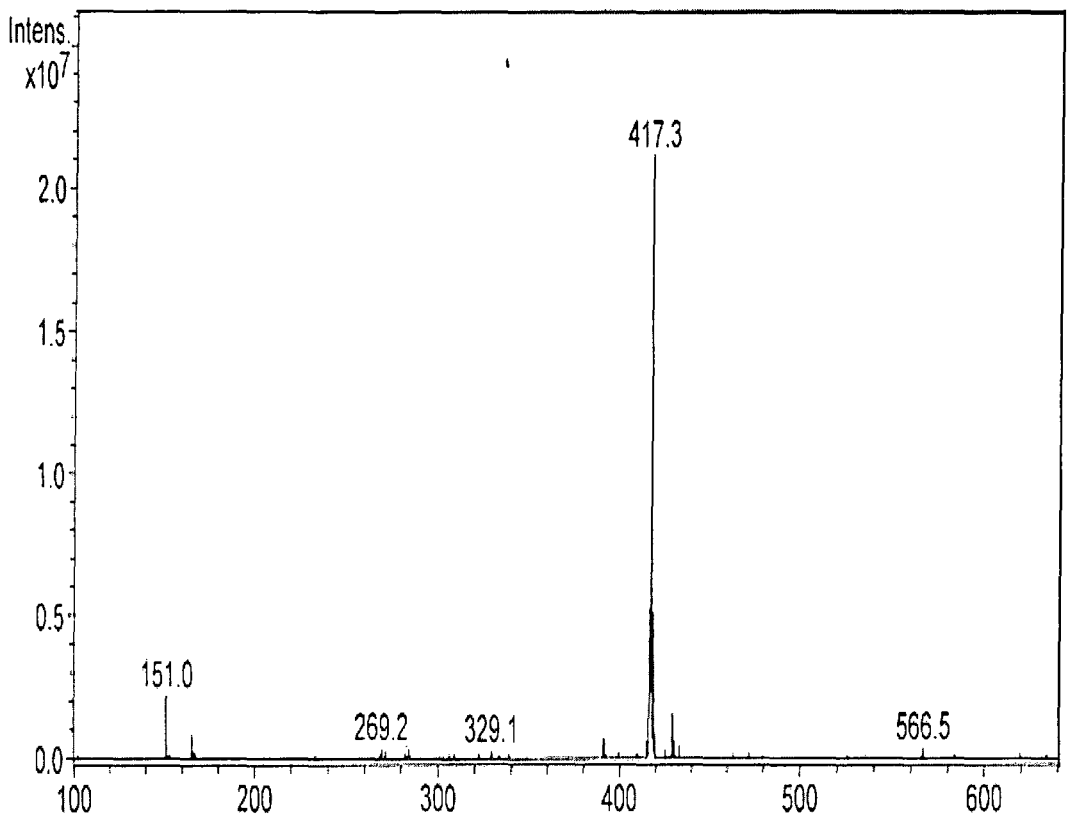
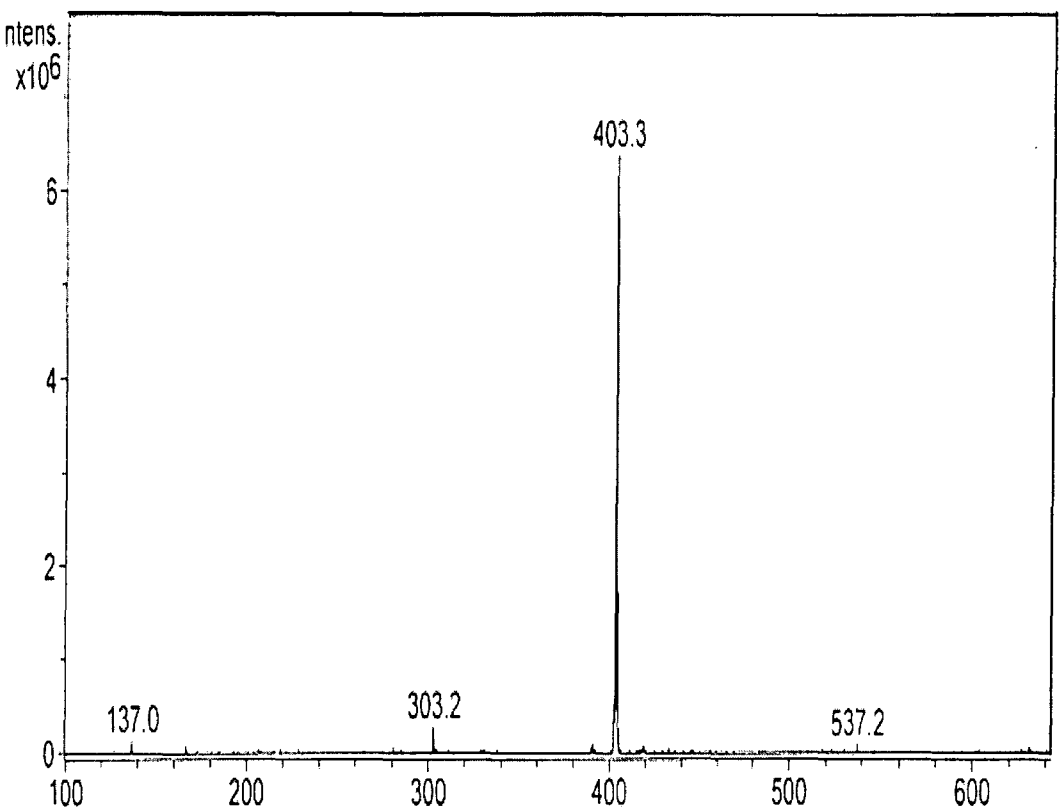

Intens.
x10^7
2.0
1.5
1.0
0.5
0.0
417.3
151.0
269.2
329.1
566.5
100
200
300
400
500
600

Figure A.5 Mass spectrum of δ-tocopherol


ntens.
x10^6
6
4
2
0
403.3
137.0
303.2
537.2
100
200
300
400
500
600