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Effects of Oxidized Dietary Oil and Vitamin E Supplementation on Lipid Profile and Oxidation of Muscle and Liver of Juvenile Atlantic Cod (*Gadus morhua*)

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The effects of oxidized dietary lipid and the role of vitamin E on lipid profile, retained tocopherol levels, and lipid oxidation of juvenile Atlantic cod (*Gadus morhua*) were evaluated following a 9-week feeding trial. Four isonitrogenous experimental diets containing fresh or oxidized (peroxide value of 94 mequiv/kg) fish oil with or without added vitamin E (α-tocopherol or mixed tocopherols) were fed to juvenile cod in duplicate tanks. There was no significant ($P > 0.05$) influence on major lipid classes of cod liver and muscle by diet with the exception of sterols. Sterols content was increased in liver but decreased in muscle by oxidized dietary oil in the absence of vitamin E. Dietary vitamin E supplementation decreased the sterols level in cod liver but with no significant ($P > 0.05$) effect on their level in the muscle. Fatty acid composition varied between lipid fractions in muscle tissue and was affected by the diet. Oxidized oil significantly ($P < 0.05$) decreased the deposition of α-tocopherol in liver but not in muscle. γ- and δ-Tocopherols from dietary tocopherol mixtures were retained at very low levels in liver, but higher retention was observed in muscle tissue. 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 dietary vitamin E. The results also indicate 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 relatively high levels.

KEYWORDS: Atlantic cod; *Gadus morhua*; oxidized oil; α-tocopherol; mixed tocopherols

INTRODUCTION

Atlantic cod (*Gadus morhua*) is a cold-water marine fish that has been widely utilized, with cod fillet and cod liver oil being the main products. Cod flesh has a mild flavor and low calories and provides a rich source of proteins and small amounts of essential nutrients such as n-3 fatty acids as well as iodine and some B vitamins (*1*, *2*). In addition to food applications, cod is also known for production of cod liver oil. Atlantic cod has a large fatty liver containing a high proportion of lipid (70% of the liver weight) (*3*). Cod liver oil is rich in polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), which have been shown to promote human health, and serves as an excellent source for fat-soluble vitamins, principally vitamins A and D. Cod liver oil is commercially available and now frequently used in nutrient supplementation and medical treatment.

Due to the increasing market demand for cod products and the dramatic decline of natural cod stocks, Atlantic cod is attracting growing interest from fish farmers, and cod farming appears to be an emerging commercial activity. The quality of farmed fish, however, depends on a number of factors, among which diet is the dominant one. The effect of diet variables on the quality of cultured fish has been extensively studied. Atlantic cod, as a marine fish, is incapable of converting C18 fatty acids into long-chain PUFA through elongation and desaturation, as observed in some freshwater fish and invertebrates. Therefore, dietary n-3 PUFA are required in a sufficient amount for the supply of essential fatty acids in order to support the normal growth and to maintain the high quality of cultured fish. However, these highly unsaturated fatty acids are very susceptible to oxidation and release a number of oxidation products, for instance, hydroperoxides as the primary oxidation products, and aldehydes, ketones, alcohols, and carboxylic acids, among others, as secondary oxidation products. Both primary and secondary oxidation products have detrimental effects on the nutritive value of the diet and thus may potentially lead to

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suppressed growth and modulated body composition of cultured fish (*4*); the latter group also affects the sensory characteristics of products. On the other hand, fish are protected from oxidative damage by endogenous antioxidant systems such as free radical scavenging enzymes and exogenous antioxidants such as vitamin E.

Vitamin E (tocopherols), one of the most extensively used antioxidants in foods, has four naturally occurring homologues (α-, *β*-, *γ*-, and *δ*-tocopherols), among which α-tocopherol has been the focus of vitamin E studies owing to the fact that it has the highest vitamin E activity. In aquaculture, α-tocopherol is added to fish feed to prevent oxidation of dietary lipid and as a supply of vitamin E to satisfy the nutritional requirement of fish. However, *γ*-tocopherol has been found to be 1.4 times as effective as α-tocopherol in inhibiting oxidation of PUFA in vitro (*5*). The antioxidant activity of tocopherols, generally, is in the order of *δ*- > *γ*- > *β*- > α-tocopherol (*6*). The difference among tocopherol homologues in their transportation, deposition, distribution, and retention in animal tissues may account for their varied biological antioxidant efficiency in vivo. Use of mixed tocopherols in aquatic feed has been proposed, as they provide a readily available commercial source of vitamin E, which may help to reduce the cost of fish farming. Large quantities of natural tocopherol mixtures can be obtained as byproducts of vegetable oil production such as deodorizer distillate of soybean oil upon deodorization. This study aimed to evaluate the effect of oxidized dietary lipid and vitamin E supplementation (α-tocopherol and mixed tocopherols) on the quality of juvenile Atlantic cod, namely, lipid profile, retained tocopherol levels, and lipid oxidation of muscle and liver tissues.

MATERIALS AND METHODS

Materials. Feed ingredients were purchased from the following sources: herring meal from Corey Feeds, Fredericton, NB, Canada; krill meal from Special Marine Products Ltd., West Vancouver, BC, Canada; casein from Amersham Pharmacia Canada Ltd., Baie d'Urfé, QC, Canada; wheat middlings from Dover Mills Ltd., Halifax, NS, Canada; vitamins and choline chloride from U.S. Biochemical, Cleveland, OH; minerals from Anachemia Chemicals Inc., Rouses Point, NY; pregelatinized starch from National Starch and Chemical Co., Bridgewater, NJ; marine fish oil (with no antioxidant stabilization) from Ocean Nutrition Canada Ltd., Halifax, NS, Canada; and mixed tocopherols containing 9.4, 1.1, 44.1, and 16.7% of α-, *β*-, *γ*-, and *δ*-tocopherols, respectively, from Archer Daniels Midland Co., Decatur, IL. 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.

Feeding Trial and Fish Sampling. A 9-week feeding trial was carried out at the Ocean Sciences Centre (OSC), Memorial University of Newfoundland, St. John's, NL, Canada. Four hundred juvenile Atlantic cod (average weight of 54.9 g) were allocated among eight tanks (3000 L tank, 50 fish per tank, 2 tanks per diet). All farming conditions were optimized for Atlantic cod. Fish were acclimated for 3 weeks to experimental conditions before the start of the feeding trial. Four isonitrogenous experimental diets (**Table 1**), containing unoxidized oil with α-tocopherol, oxidized oil, oxidized oil with α-tocopherol, and oxidized oil with mixed tocopherols, respectively, were assigned to duplicate tanks. Fresh marine fish oil was oxidized by heating at 55−60 °C with air injection until a peroxide value of 94 mequiv/kg was reached. Feed ingredients were mixed and formulated into dry pellets with a uniform particle size of 3.5 mm. Fish were fed twice daily to apparent satiation.

At the end of the feeding trial, fish were randomly selected from each tank and were sacrificed with an overdose of anaesthetic (Tricaine, methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA). Six fish from each tank (12 from each treatment) were dissected and muscle and liver carefully removed. Muscle and liver samples collected from the same treatment were pooled together and homogenized in a blender. The homogenates of selected fish tissues were stored under nitrogen at −20 °C for further analysis.

Table 1. Composition of Experimental Diets

ingredient (% or as specified)	diet 1 Fr-FO + E[a]	diet 2 Ox-FO[b]	diet 3 Ox-FO + E[c]	diet 4 Ox-FO + COVI[d]
basal diet[e]	89	89	89	89
fresh fish oil	11	0	0	0
oxidized fish oil	0	11	11	11
α-tocopherol (IU of vitamin E equiv)	300	0	300	0
COVI-OX T-70 (IU of vitamin E equiv)	0	0	0	300

[a] Fr-FO + E, fresh fish oil with vitamin E supplementation (from α-tocopherol). [b] Ox-FO, oxidized fish oil (without vitamin E supplementation). [c] Ox-FO + E, oxidized fish oil with vitamin E supplementation (from α-tocopherol). [d] Ox-FO + COVI, oxidized fish oil with vitamin E supplementation (from mixed tocopherols). [e] Basal diet includes herring meal, krill meal, casein, wheat middling, vitamin mixture, mineral mixture, choline chloride, and pregelatinized starch.

Lipid Classification. Lipids were extracted from fish whole body, muscle, and liver samples using the Bligh and Dyer (*7*) method and quantified gravimetrically. 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, Canada) was employed. Separation of lipid classes was carried out following a three-stage development procedure described by Parrish (*8*). Each peak was identified by comparing the retention time with that of known standards, and results were expressed as area percentage of each lipid class.

Lipid Fractionation. Neutral lipids (NL) and phospholipids (PL) were separated from muscle total lipid by column chromatography following a procedure described by Ramadan and Morsel (*9*) and Budge and Parrish (*10*). Lipid extracts were dissolved in chloroform and added to the top of a prepacked column. Neutral lipids and phospholipids were recovered with chloroform and methanol, respectively. Solvents were evaporated under a nitrogen flush and lipid fractions stored at −80 °C for subsequent fatty acid analysis.

Fatty Acid Analysis. Fatty acid composition of muscle neutral lipids and phospholipids was analyzed by gas chromatography (GC). Fatty acids were converted to their corresponding fatty acid methyl esters (FAMEs) by transmethylation prior to GC analysis according to the method of Hamam and Shahidi (*11*). The resultant 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 *μ*m film thickness; Supelco Canada Ltd., Oakville, ON, Canada). The oven temperature increased from 220 to 240 °C at a rate of 30 °C/min. The temperatures of the injector and detector (FID) were both set at 250 °C. Ultrahigh-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). The FAMEs were identified by comparing their retention times with those of authentic standards. Results were expressed as area percentage of each fatty acid.

HPLC-MS Analysis of Tocopherols. Lipid extracts from feed and fish tissue were saponified, and unsaponifiable matters were extracted with hexane according to the method of Maguire et al. (*12*) with minor modifications. Tocopherols were analyzed by normal phase high-performance liquid chromatography (HPLC)−mass spectrometry (MS). The analysis was performed using an Agilent 1100 HPLC system with a UV-diode array detector (UV-DAD). Separation was achieved on a Supelcosil LC-Si column (250 mm × 4.6 mm i.d., 5 *μ*m, Sigma-Aldrich Canada Ltd.) 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 50 μL of sample were injected. Tocopherols were detected at 290 nm by the UV detector and quantified by the area of the corresponding peak in the UV spectrum. LC flow was analyzed on-line by the mass spectrometric detector system (LC-MSD-Trap-SL, Agilent) with a positive ion atmospheric pressure chemical ionization (APCI) for identification of tocopherol homologues. The operating conditions used were 121 V for the fragmentor voltage, 350 °C for the drying temperature, 400 °C for the APCI temperature, 60 psi for the nebulizer pressure, and 7 L/min for the drying gas flow. Tocopherol concentrations in samples were obtained from the standard curve and expressed as milligrams of tocopherol per kilogram feed or fish tissue.

Determination of Lipid Oxidation. Peroxide values (PV) and 2-thiobarbituric acid reactive substances (TBARS) of fresh and oxidized dietary fish oil were determined according to the AOCS (*13*) methods. TBARS of fish liver tissue were determined following the procedure described by Williamson et al. (*14*). Muscle TBARS values were determined according to Lemon's method (*15*). Results were expressed as nanomoles of malondialdehyde (MDA) equivalents per gram of liver or muscle tissue.

Volatile analysis of dietary oils and liver samples was performed using a Perkin-Elmer 8500 gas chromatograph equipped with an HS-6 headspace sampler (Perkin-Elmer Co., Montreal, QC, Canada). A Supelcowax-10 fused-silica capillary column (30 m × 0.32 mm i.d., 0.10 μm film, Supelco Canada Ltd., Mississauga, ON, Canada) 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. The oven temperature was subsequently raised to 200 °C at a rate of 30 °C/min and held there for 5 min. Oil and tissue samples were 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 micrograms of propanal per gram of oil or wet tissue.

Statistical Analysis and Data Interpretation. One-way analysis of variance (ANOVA) with pairwise comparisons (Tukey's HSD) was performed at a $P < 0.05$ level using Sigmastat for Windows version 2.0 (Jandel Corp., San Rafael, CA) 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.

RESULTS AND DISCUSSION

Formulated Diets. The oxidative state of the fresh and oxidized marine fish oil used in feed formulation was determined by measuring the primary and secondary oxidation products, namely, PV, TBARS, and headspace propanal. The oxidized oil had markedly higher PV, TBARS, and headspace propanal values than the fresh oil (**Figure 1**), indicating that the oil was highly oxidized. The fatty acid compositions of dietary lipids are shown in **Figure 2**. The levels of PUFA, especially n-3 PUFA, were slightly but statistically ($P < 0.05$) lower in the oxidized feed than in unoxidized diets. The lowered PUFA content in oxidized diets may result from the consumption of PUFA as the main substrates of oxidation reaction.

Lipid Contents of Fish Tissues. Table 2 presents the lipid content in different tissues of the fish at the beginning and the end of the feeding trial. Muscle and liver lipid contents of fish

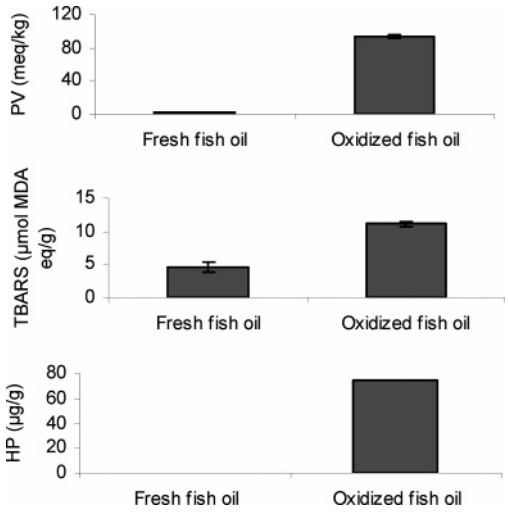

Figure 1. Oxidative state of dietary fish oil as reflected in peroxide value (PV), thiobarbituric acid reactive substances (TBARS), and headspace propanal (HP).

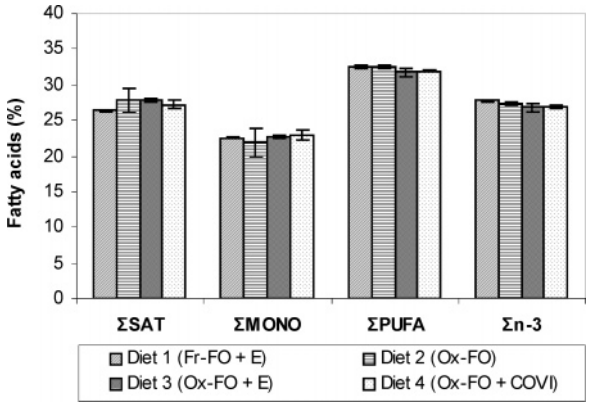

Figure 2. Fatty acid composition of dietary lipid (SAT, saturated fatty acids; MONO, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-3, omega 3 fatty acids; abbreviations for diets are given in footnotes of **Table 1**).

Table 2. Lipid Contents of Initial and Final Fish Tissues[a]

tissue	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 ± 0.01b	1.37 ± 0.02a	1.15 ± 0.02b	1.41 ± 0.05a
liver	72.2 ± 0.74	51.2 ± 0.86c	58.9 ± 0.82a	49.9 ± 0.65c	54.0 ± 0.69b

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

at the end of feeding trial were in the ranges of 1.08−1.41 and 49.9−58.9%, respectively, which are high in comparison with those found in wild Atlantic cod (0.8 and 38.3% lipid in muscle and in liver, respectively) (*16*). There was no major influence on muscle and liver lipid content by oxidized oil ($P > 0.05$). Surprisingly, supplementation of α-tocopherol decreased the lipid content of both muscle and liver tissues ($P < 0.05$). Mixed tocopherols reduced only liver lipid content ($P < 0.05$), but, as expected, had 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. (*17*) showed that

Table 3. Muscle and Liver Lipid Classes (Percent) Determined by Iatroscan[a]

lipid source/ class[b]	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 ± 0.23a	0.82 ± 0.27bc	1.01 ± 0.23b	0.38 ± 0.04c
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 oolar	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 ± 0.15b	2.70 ± 1.27a	1.17 ± 0.55ab	1.45 ± 0.68ab
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

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

both α- and γ-tocopherols in diets did not affect muscle and liver lipid contents of Atlantic salmon.

Lipid Classes of Muscle and Liver Tissues. The lipid class composition of muscle and liver tissue is shown in **Table 3**. Triacylglycerols (TAG), the main neutral lipid class serving as the most concentrated form of energy storage in animals (37.6 kJ/g of lipid) (*18*), comprised the major lipid class of cod liver oil at an average level of 80%. Phospholipids (PL) were present at low levels in cod liver. Fish fed vitamin E supplemented diets had slightly lower TAG and PL contents in their livers. However, no statistical difference ($P > 0.05$) in TAG or PL concentration was shown. Relatively high proportions of free fatty acids (FFA) and diacylglycerols (DAG) were detected in liver oil, 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, which might have occurred during cold storage of lipid or tissue samples. Liver sterols showed significant ($P < 0.05$) difference among diet treatments. 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. At this stage, we cannot offer any reasonable explanation for the change of liver sterols as affected by the diet.

In contrast to liver oil, cod muscle lipid contained a high proportion of PL (77% on average) and a low level of TAG (10% on 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 hydrolyzed or hydrolyzed only to a very limited extent. The only significant ($P < 0.05$) 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 ($P > 0.05$) effect on muscle sterol levels.

Table 4. Fatty Acid Composition (Percent) of Muscle Neutral Lipids[a]

fatty acid	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
C12:0	tr[b]	tr	0.32 ± 0.01	tr
C14:0	1.47 ± 0.02ab	1.39 ± 0.00ab	1.21 ± 0.09b	2.56 ± 0.94a
C15:0	tr	0.23 ± 0.00	0.20 ± 0.02	tr
C16:0	13.7 ± 0.06b	16.7 ± 0.31a	13.7 ± 0.29b	14.3 ± 0.56b
C16:1	2.73 ± 0.01b	2.40 ± 0.03c	2.38 ± 0.05c	2.98 ± 0.11a
C17:0	tr	tr	0.20 ± 0.02	tr
C17:1	4.06 ± 0.56	4.25 ± 1.63	2.72 ± 0.54	3.71 ± 2.27
C18:0	2.63 ± 0.04bc	3.12 ± 0.07a	2.88 ± 0.05ab	2.44 ± 0.19c
C18:1	8.40 ± 0.17a	8.07 ± 0.20ab	8.34 ± 0.27ab	7.59 ± 0.48b
C18:2n-6	3.72 ± 0.04ab	3.48 ± 0.05b	3.82 ± 0.03a	3.92 ± 0.20a
C18:3n-3	tr	0.66 ± 0.00b	0.70 ± 0.02a	0.25 ± 0.00c
C20:1	1.28 ± 0.03a	1.32 ± 0.04a	1.11 ± 0.06b	1.06 ± 0.05b
C20:3n-3	2.24 ± 0.01a	2.00 ± 0.06b	2.22 ± 0.04a	1.91 ± 0.04b
C20:5n-3	26.7 ± 0.06a	22.7 ± 0.34c	24.3 ± 0.57b	24.6 ± 0.47b
C22:5n-3	2.38 ± 0.02ab	2.25 ± 0.11b	2.57 ± 0.08a	2.25 ± 0.08b
C22:6n-3	25.0 ± 0.08a	24.0 ± 0.55b	24.5 ± 0.30ab	21.4 ± 0.07c
ΣSAT	17.8 ± 0.11c	21.4 ± 0.49a	18.2 ± 0.20bc	19.3 ± 0.20b
ΣMONO	15.8 ± 0.10b	16.0 ± 1.37ab	14.6 ± 0.26b	18.2 ± 1.00a
ΣPUFA	59.9 ± 0.08a	54.7 ± 1.43b	58.1 ± 0.14a	54.5 ± 0.20b
Σn-3	56.2 ± 0.04a	51.2 ± 1.38c	54.3 ± 0.17b	50.6 ± 0.00c

[a] 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 letters were significantly different at $P < 0.05$. Rows with no letters are not significantly different at $P > 0.05$. Abbreviations for diets are given in footnotes of **Table 1**. [b] tr, trace.

Table 5. Fatty Acid Composition (Percent) of Muscle Phospholipids[a]

fatty acid	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
C14:0	1.09 ± 0.01c	0.89 ± 0.03c	1.58 ± 0.06a	1.34 ± 0.16b
C15:0	0.20 ± 0.01ab	0.16 ± 0.03b	0.24 ± 0.01a	0.25 ± 0.03a
C16:0	16.9 ± 0.48ab	14.9 ± 0.51b	17.2 ± 0.50a	16.7 ± 1.50ab
C16:1	1.71 ± 0.06b	1.54 ± 0.01b	2.08 ± 0.02a	1.99 ± 0.15a
C17:0	0.28 ± 0.00	0.31 ± 0.02	tr[b]	tr
C17:1	4.54 ± 1.02a	2.07 ± 0.32b	5.56 ± 0.18a	4.40 ± 0.31a
C18:0	5.69 ± 0.06	5.96 ± 1.51	5.69 ± 0.52	5.21 ± 1.97
C18:1	7.39 ± 0.18	7.31 ± 0.03	7.78 ± 0.16	7.66 ± 0.41
C18:2n-6	3.21 ± 0.16ab	3.03 ± 0.14b	3.15 ± 0.02ab	3.33 ± 0.07a
C18:3n-3	0.54 ± 0.03a	0.47 ± 0.00b	0.56 ± 0.02a	0.56 ± 0.03a
C20:1	0.94 ± 0.10	0.92 ± 0.15	0.95 ± 0.08	1.06 ± 0.12
C20:3n-3	2.24 ± 0.10a	2.26 ± 0.00a	1.92 ± 0.02b	2.15 ± 0.06a
C20:5n-3	16.4 ± 1.16	16.5 ± 2.19	14.7 ± 0.61	16.7 ± 2.05
C22:5n-3	2.30 ± 0.04b	2.76 ± 0.10a	2.13 ± 0.03b	2.36 ± 0.15b
C22:6n-3	33.1 ± 0.90b	35.4 ± 0.16a	32.6 ± 0.20b	32.4 ± 1.35b
ΣSAT	24.0 ± 0.78	22.0 ± 1.21	24.7 ± 0.05	23.3 ± 0.19
ΣMONO	14.1 ± 0.31b	11.8 ± 0.13c	15.9 ± 0.13a	14.6 ± 0.28b
ΣPUFA	57.5 ± 1.73ab	60.5 ± 1.8a	54.7 ± 0.52b	57.3 ± 0.84ab
Σn-3	54.3 ± 1.92ab	57.4 ± 1.94a	51.6 ± 0.54b	53.9 ± 0.77ab

[a] 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 letters were significantly different at $P < 0.05$. Rows with no letters are not significantly different at $P > 0.05$. Abbreviations for diets are given in footnotes of **Table 1**. [b] tr, trace.

Fatty Acid Composition of Muscle Neutral Lipids and Phospholipids. Muscle total lipid was fractionated into neutral lipids and phospholipids using column chromatography. Fatty acid composition of each fraction was determined, and results are given in **Tables 4** and **5**, respectively. Both neutral lipids and phospholipids had high percentages of PUFA, mainly EPA and DHA. A dramatically higher proportion of DHA (around 33%) than of EPA (around 16%) was noted in phospholipids with characteristic fatty acid composition of muscle total lipids

Table 6. Tocopherol Concentrations (Milligrams per Kilogram) in Diets, Fish Liver, and Muscle Tissue[a]

tocopherol	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
diet				
α-	95.7 ± 5.07a	8.18 ± 1.03c	98.1 ± 5.26a	31.7 ± 2.14b
γ-	nd[b]	nd	nd	27.1 ± 2.30
δ-	nd	nd	nd	tr[c]
liver				
α-	222 ± 9.10a	170 ± 7.23b	142 ± 5.82c	13 ± 4.89c
γ-	nd	nd	nd	nd
δ-	nd	nd	nd	nd
muscle				
α-	8.59 ± 0.66ab	6.62 ± 0.38c	9.64 ± 0.42a	8.53 ± 0.48b
γ-	nd	nd	nd	10.1 ± 1.04
δ-	nd	nd	nd	6.78 ± 0.54

[a] Values (mean ± SD of three replicates) in the same row with different letters were significantly different at $P < 0.05$. β-Tocopherol was not detected in any of the samples tested. Abbreviations for diets are given in footnotes of **Table 1**. [b] nd, not detected. [c] tr, trace.

(data not shown). In the neutral lipids fraction, however, EPA was present at slightly higher levels than DHA. A similar trend for distribution of EPA and DHA was found in liver total lipids (data not shown). These findings support a previous report on haddock by Nanton et al. (*19*) 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, likely as a 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, 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 (**Figure 2**). Ackman et al. (*20*) have shown that phospholipids are more sensitive to dietary fatty acids than neutral lipids. No significant ($P > 0.05$) effect of oxidized oil or mixed tocopherols on PUFA was observed.

Tocopherol Contents. Tocopherol contents in diets, fish liver, and muscle were determined by HPLC-MS analysis, and results are given in **Table 6**. α-Tocopherol and mixed tocopherols (COVI-OX T-70) were added to diet during feed formulation to supply 300 IU (200 mg/kg) of vitamin E equivalents in treatments of vitamin E supplementation. A marked difference was noted 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 in the lipid extraction and saponification stages, as tocopherols are sensitive to their environment. Loss of tocopherols during feed preparation and storage may also account for the lower 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 in which mixed tocopherols were included; δ-tocopherol was also detected, but in trace amounts; β-tocopherol was not detected. γ- and δ-tocopherols were less abundant in diet, although they were added at higher levels than α-tocopherol. The relatively greater losses of γ-, δ-, and β-tocopherols in the diets may be due to their higher antioxidant activity compared with that of α-tocopherol and thus higher sensitivity to environmental factors.

Dietary vitamin E level significantly affects tissue vitamin E content as evidenced in the literature. 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 (*21*, *22*). Tissue α-tocopherol concentration is proportional to dietary α-tocopherol concentrations, that is, tissue vitamin E content increases when the dietary vitamin E level increases, as reported for many fish species (*23*−*26*). 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 (*27*−*29*). A 10-fold decrease of liver α-tocopherol concentration in African catfish by oxidation of dietary lipids has been demonstrated (*30*). 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 (*31*). Alternatively, Baker and Davis (*30*) 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. (*32*) found that there was no difference in tissue α-tocopherol of Atlantic salmon between fresh oil feeding and oxidized oil feeding. Meanwhile, Huang and Huang (*26*) 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 related to some other, yet to be determined, factors in addition to dietary vitamin E content.

In the present study on Atlantic cod, fish fed oxidized oil had significantly lower liver α-tocopherol concentration than those fed fresh oil ($P < 0.05$), although the same level of dietary vitamin E was included. Oxidized oil caused vitamin E decline in the liver of juvenile Atlantic cod. Unexpectedly, liver α-tocopherol concentration was higher in fish fed vitamin E deficient diet than in those fed vitamin E supplemented diet in treatments of oxidized oil ($P < 0.05$). Vitamin E supplementation in diet containing oxidized oil 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 (*30*). 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 the 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. Meanwhile, fish receiving vitamin E supplementation

Table 7. Oxidative State of Fish Liver and Muscle Tissue[a]

oxidative state	Fr-FO + E	Ox-FO	Ox-FO + E	Ox-FO + COVI
liver				
TBARS[b] (nmol of MDA[c] eq/g of tissue)	34.1 ± 2.69c	76.1 ± 4.72b	127 ± 0.49a	75.2 ± 1.10b
headspace propanal (μg/g of tissue)	73.0 ± 0.02d	96.5 ± 0.69c	228 ± 1.37a	173 ± 1.14b
muscle				
TBARS (nmol of MDA eq/g of tissue)	1.13 ± 0.32b	4.27 ± 0.98a	1.26 ± 0.34b	1.10 ± 0.20b
headspace propanal (μg/g of tissue)	tr[d]	tr	tr	tr

[a] Values are mean ± SD of six replicates. Mean values in the same row with different letters were significantly different at $P < 0.05$. Abbreviations for diets are given in footnotes of **Table 1**. [b] TBARS, thiobarbituric acid reactive substances. [c] MDA, malondialdehyde. [d] tr, trace.

had a remarkably lower lipid content in their liver than those fed a diet without vitamin E (**Table 2**), which may account for their lower liver α-tocopherol concentration. In contrast to liver α-tocopherol, the 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 in 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 ($P > 0.05$) affect muscle α-tocopherol levels. The fact that a big difference between groups 1 and 3 was found for liver tocopherol but not for muscle tocopherol 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 (*30*).

In addition to α-tocopherol, γ- and δ-tocopherols 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-tocopherols, respectively, was used to provide vitamin E in treatment of mixed tocopherols. Among them, β-tocopherol appears to be less important as a minor form of tocopherol in nature compared to other tocopherol homologues. Its concentration was below the measurable level both in the diet and in fish tissues and is not discussed here. Retained levels of dietary γ- and δ-tocopherols in liver and muscle tissues were studied. No γ- or δ-tocopherol was detected in the fish liver in all treatments. Supplementation of the diet with mixed tocopherols either did not lead to deposition or resulted in poor deposition of γ- and δ-tocopherols in the liver. However, α-tocopherol was present at a level of 137 mg/kg of tissue in the liver of fish receiving mixed tocopherols in the diet, although a lower concentration of α-tocopherol was present in the diet compared to γ- and δ-tocopherols. This gives further support to the hypothesis that biodiscrimination exists between the tocopherols in their uptake in fish liver. The discrimination is thought to be mediated through a competition for a hepatic tocopherol-binding protein that preferentially transports α-tocopherol to the site of very low-density lipoprotein in the liver (*33*). The tocopherol-binding protein has been purified from rat liver cytosol (*34*). 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 tocopherols (*35*). The variable binding capacity of this protein to tocopherol homologues gives rise to the varied biological activity and antioxidant efficiency of tocopherols in vivo. α-Tocopherol is considered to be a more potent antioxidant in vivo because its competitive power far exceeds that of other tocopherols. It has been found that the retention of γ- and δ-tocopherols in animal tissues was elevated when α-tocopherol was deficient (*36*). 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 retention levels in fish liver (*37*). The 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 receive 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 (*38*). Deposition of γ- and δ-tocopherols was found to be more efficient in perivisceral fat and adipose tissue than in liver (*17*). Fish muscle appears to be supplied with tocopherols from chylomicrons that have not been exposed to the hepatic discrimination mechanism (*33*). Therefore, muscle tissue can be enriched in all tocopherols through a lipoprotein lipase-mediated catabolism of chylomicrons (*33*). In the current study, 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. Deposition of tocopherols in cod muscle was roughly in response to the concentrations of dietary tocopherols. The level of γ-tocopherol in muscle was only slightly higher than that of α-tocopherol, possibly because of its higher antioxidant efficiency and thus greater consumption for antioxidant defense either in vivo or post-mortem.

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 an undesirable aroma that adversely affects the sensory quality of food and, furthermore, some products are found to be toxic to animals and humans. Therefore, lipid oxidation is regarded as one of the major concerns in quality preservation of fish products. In the present study, TBARS and headspace propanal in liver and muscle tissue were measured as indicators of oxidative state. However, headspace propanal of muscle tissue was detected in only trace amounts in all diet treatments, probably because of the low lipid level and/or low extent of lipid oxidation in cod fillets. Additionally, the muscle phospholipids may be protected because 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 7**. A strong correlation existed 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 an elevated concentration of oxidation products in liver regardless of vitamin E supplementation. A similar trend was observed in Atlantic salmon (*32*). This may be interpreted as an increased uptake of oxidation products,

including TBARS and propanal, from the oxidized diet through the intestine, or an uptake of lipid hydroperoxides that are later decomposed to aldehydes, as demonstrated by Hamre et al. (*32*). 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 cause of formation and the origin of lipid oxidation products in fish tissues. Both TBARS and headspace propanal contents 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 level of 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. γ- and δ-tocopherols were not detected in cod liver because they were utilized as effective antioxidants and due to their inferiority in the competition for the tocopherol-binding protein, as discussed earlier. This finding is in agreement with that of Ruff et al. (*25*), who reported that lipid oxidation was solely related to the concentration of tocopherols in fish. Furthermore, Tocher et al. (*39*) 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 defense of fish, as observed in Arctic char (*22*). In this work, vitamin E deficiency in cod liver occurred in the treatments for which 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 of vitamin E in the diet was not sufficient to protect the liver from oxidation when fish were fed a highly oxidized oil.

Muscle TBARS values also showed a negative correlation with the tissue tocopherol levels. The highest TBARS value was accompanied by 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 slightly lower TBARS values than those containing only α-tocopherol. Cod muscle probably has mechanisms similar to those in liver in preventing the absorption of oxidation products from dietary sources.

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