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Does Feed Composition Affect Oxidation of Rainbow Trout (*Oncorhynchus mykiss*) during Frozen Storage?

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Rainbow trout (*Oncorhynchus mykiss*) were fed a diet containing either fish oil or rapeseed oil and with or without 200 mg/kg carotenoid (either astaxanthin or canthaxanthin). A total of six diets were obtained: (1) fish oil/astaxanthin; (2) vegetable oil/astaxanthin; (3) fish oil/canthaxanthin; (4) vegetable oil/canthaxanthin; (5) fish oil/no pigment; and (6) vegetable oil/no pigment. The fish were slaughtered and stored in polyethylene bags individually as butterfly fillets for up to 22 months at −20 °C. The composition of the fish muscle at slaughter and during frozen storage was evaluated by sampling after 4, 8, 13, 18, and 22 months. The carotenoid content in the muscle was found to be approximately 9−10 mg/kg of fish for both carotenoids. Primary oxidation lipid products (peroxides) as well as secondary oxidation products (volatiles) were measured. In addition, the level of protein carbonyl groups and the content of tocopherols and carotenoids in the muscle were also measured. To estimate the overall changes in sensory properties of the different samples during storage, a trained sensory panel also evaluated the samples. Both the sensory panel and the chemical analysis revealed that in this investigation fish fed fish oil were slightly more oxidized than fish fed vegetable oil. Results showed that canthaxanthin effectively protected both protein and lipid against oxidation during frozen storage. In contrast, astaxanthin did not seem to have a clear and systematic effect. Results indicated that the feed composition influenced the fish muscle composition and subsequently the oxidative stability of the fish during frozen storage. Besides, other constituents in the feed might influence deposition of antioxidants in the tissue and consequently affect the oxidative stability of the muscle.

KEYWORDS: Trout; feed composition; lipid oxidation; frozen storage; protein oxidation

INTRODUCTION

Fatty fish species such as salmon and trout are good sources of omega-3 fatty acids, which have been recognized for their important nutritional properties (*1*, *2*). A high intake of omega-3 fatty acids has been associated with the prevention of cardiovascular diseases and neurodegenerative diseases such as Alzheimer's (*3*, *4*). In the Western world, the consumption of fatty fish species is too low and, therefore, highly encouraged. During frozen storage of fatty fish there may be a significant loss in nutritional value due to deterioration of the long-chain omega-3 fatty acids, which are very susceptible to oxidative damage (*5*). Oxidation leads to unpleasant flavor development, reduced nutritional value, and, in general, low consumer appeal. Different strategies have been proposed to prevent oxidative deterioration in muscle during frozen storage, for example, using packaging material with a high oxygen barrier, adding antioxidants, or even reducing storage temperature and light exposure (*6*). Depletion of antioxidant in the muscle tissue post-mortem has been reported to correlate with the decrease in oxidative stability of fish and fish products (*7*). It has also been proposed that the quality of the fish muscle was linked to the fish feeding regimen as well as its physiological conditions (*8*). Manipulation of the fish diet might therefore be an effective strategy for the aquaculture industry to produce fish with an optimal oxidative stability. Earlier investigations showed that the addition of the liposoluble antioxidant tocopherol in the feed could increase the eating quality of beef, pork, poultry, and fish (*9*−*12*). Others have reported that the carotenoids astaxanthin and canthaxanthin, which give salmonoids their characteristic color, could impart protection against oxidative degradation in fish muscle tissue during frozen storage (*12*, *13*). However, some studies indicated that the carotenoids were not able to protect fish muscle from oxidative damage (*14*), and the impact of carotenoids on the oxidative stability of fish muscle during frozen storage is today still unclear. To date, the limits set by EU legislation for the addition of carotenoids in the feed for salmon and trout are 25 mg/kg for canthaxanthin and 100 mg/kg for astaxanthin either alone or in combination with canthaxanthin. Another interesting aspect of manipulating the feeding regimen for aquaculture fish is the increasing demand for alternative oils to replace fish oil in the fish diet. Increasing demands for fish oil for aquaculture in combination with poor raw material supply make fish oil a limited and costly feed ingredient. In contrast, vegetable oils such as rapeseed oil are a

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sustainable resource and easily available commercially. Several investigations have been performed to evaluate the replacement of fish oil by vegetable oil in salmonoid feeds (*15*, *16*). Most of these studies have focused on, for example, feed digestibility, fish growth, and the fatty acid profile of the fish tissues and have demonstrated that the fatty acid profile of the diet had a direct impact on the fatty acid composition of the flesh (*17*). However, fewer studies have investigated the impact of the type of oil in the feed on the oxidative stability of fish muscle during storage, and fewer, if any, have studied the effect on protein oxidation. As a matter a fact, little is known about the kinetics of protein oxidation and its importance on fish quality, and it is yet unclear if protein and lipid oxidation are concomitant processes or if one event precedes the other (*5*). The objective of this study was to investigate the impact of feed composition on both lipid and protein oxidation in rainbow trout (*Oncorhynchus mykiss*) during frozen storage. Not only was the effect of the oil type (vegetable vs fish oil) investigated, but also the effect of the presence of carotenoids (astaxanthin or canthaxanthin) in the feed was studied. The effect of feed type on muscle composition, oxidative stability, and sensory quality of the fish muscle was investigated during frozen storage at −20 °C for up to 22 months.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents used were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma (St. Louis, MO). Tocopherol standards were purchased from Calbiochem (San Diego, CA).

Feed Composition. The feed was obtained from Biomar A/S, Brande, Denmark, and contained 32.6% fishmeal, 25% soy protein, 20.4% wheat protein, 21.3% oil, and 0.7% vitamins. A total of six diets, fish oil/astaxanthin, fish oil/canthaxanthin, fish oil/no pigments, vegetable oil/astaxanthin, vegetable oil/canthaxanthin, and vegetable oil/no pigments, were obtained. The vegetable oil used for the feed preparation was rapeseed oil, and the fish oil was from unidentified fish species. CAROPHYLL Pink (9.3%) and CAROPHYLL Red (8%) (Hoffmann-la Roche, Basel, Switzerland) were used as synthetic sources of astaxanthin and canthaxanthin, respectively. The level of carotenoid pigment added to the feed was calculated to match 200 mg/kg of feed. The feeds were kept under appropriate storage conditions (at 5 °C, in the dark) during the entire feeding experiment.

Feeding Experiment, Slaughtering, and Storage. Rainbow trout (*O. mykiss*) were fed the experimental diets for 64 days and had then reached a weight of approximately 350 g before slaughter. The fish were slaughtered by electrocution, filleted as butterfly fillets with skin on using an industrial filleting machine, frozen at −30 °C on an industrial steel belt freezer with a freezing time of 20 min, and stored at −30 °C for 1 week ($t = 0$) before they were transferred to −20 °C. Fish were packed individually in standard air-permeable polyethylene bags with a thickness of 70 μm (Tobiplast A/S, Greve, Denmark), heat-sealed, and stored frozen for 22 months. Fish were taken for chemical ($n = 3$) and sensory analysis ($n = 10$) after 0, 4, 8, 13, 18, and 22 months of frozen storage. $t = 0$ samples corresponded to frozen samples before they were placed at −20 °C. No freezer burn or dehydration was observed during the entire storage period of the fish. All chemical analyses were conducted independently on a minimum of three fish for each feeding conditions. Individual fish samples were minced prior to chemical analysis and a portion of the mince was used immediately.

Analyses of Feed and Fish. *Oil Content*. Fish muscle samples or feed samples (approximately 50 g) were ground, and 10 g in duplicate was used for determination of the oil content, which was determined gravimetrically after extraction using chloroform and methanol according to the protocol of Bligh and Dyer, but using a reduced amount of solvent (*18*). The obtained extract is referred to as lipid extract in the following.

Fatty Acid Composition. Fatty acids in lipid extracts from feed and fish were transesterified to methyl esters using a base-catalyzed transesterification followed by a boron trifluoride catalyzed esterification according to the AOCS method (*19*). The methyl esters were dissolved in *n*-heptane to a concentration of about 20 mg/mL. A HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector was used for separation of the fatty acid methyl esters. The column was an Omegawax 320 fused silica capillary (0.32 mm × 30 m × 0.25 μm) (Supelco, Bellefonte, PA). The injection volume was 0.2 μL injected in the split mode with a split ratio of 1:50. The injection and detection temperatures were 250 and 240 °C, respectively. The initial oven temperature was 160 °C, immediately raised at 3 °C min^{-1} to 200 °C, held for 1 min, further raised at 3 °C min^{-1} to 220 °C, and held for 12 min. The helium carrier gas flow was 21 cm s^{-1}. Methyl tricosanoate (C23:0) was used as a qualitative internal standard. Fatty acids were identified with commercial standards and quantified by the peak area expressed as percentage of total fatty acids. The unsaturation index of the feed and fish muscle was calculated as the moles of a given polyunsaturated fatty acid per 100 mol of total fatty acids multiplied by the number of double bounds in that fatty acid. The values for all fatty acids were then summed to obtain the unsaturation index.

Antioxidants. Tocopherols (α, β, γ, and δ) and carotenoids (astaxanthin and canthaxanthin) contents were determined in duplicate from the lipid extracts from both feed and fish using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA), equipped with a fluorescence detector or a UV diode array detector, respectively. A fraction of the lipid extract was evaporated under nitrogen and redissolved in 2 mL of *n*-heptane before injection. For tocopherol analysis an aliquot (40 μL) of the *n*-heptane fraction was injected on a Spherisorb s5w column (250 mm × 4.6 mm) (Phase Separation Ltd., Deeside, U.K.) and eluted with an isocratic mixture of *n*-heptane/2-propanol (100: 0.4, v/v) at a flow of 1 mL min^{-1}. Detection was performed using a fluorescence detector with excitation at 290 nm and emission at 330 nm and according to the AOCS method (*19*). Concentrations of the (α, β, γ, and δ) tocopherol isomers were obtained using authentic standards from Merck Chemicals Ltd. (Darmstadt, Germany). Astaxanthin and canthaxanthin contents were determined after injection of an aliquot (50 μL) of the *n*-heptane fraction onto a LiChrosorb S160 column (100 mm × 3 mm, 5 μm) equipped with a Crompsep silica (S2) guard column (Chrompack, Middleburg, The Netherlands) and eluted with a flow of 1.2 mL min^{-1} using *n*-heptane/acetone (86:14, v/v) and detection at 470 nm. Concentrations of astaxanthin and canthaxanthin were calculated using authentic standards from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Peroxide Values (PV). PV were measured in duplicate on the lipid extract from fish by colorimetric determination using the ferric thiocyanate assay as previously described (*20*). Briefly, the lipid extract was evaporated to dryness and redissolved in 10 mL of chloroform/methanol (7:3). Ammonium thiocyanate solution (50 μL) and iron Fe(II) solution (50 μL) were added, the sample was mixed and left to react for 5 min at room temperature, and subsequently the absorbance was read at 500 nm.

Volatiles. Volatiles were collected from the rainbow trout muscle by dynamic headspace sampling. The samples were prepared according to the protocol described by Refsgaard et al. (*21*). Briefly, 50 g of fish pieces was frozen in liquid nitrogen and comminuted into a fine powder. The powder (20 g in duplicate) was slurried in water, and the sample was purged with nitrogen at 340 mL min^{-1} for 10 min at 37 °C. The volatiles were trapped on Tenax-GR traps column (Varian Chrompack International, Bergen op Zoom, The Netherlands), and volatiles were released from the column by thermal desorption (ATD-400, Perkin-Elmer, Boston, MA) and analyzed by GC-MS on a 30 m DB 1701 capillary column (J&W Scientific, Folsom, CA). The temperature program used was 40 °C for 10 min, with ramping at 3 °C min^{-1} to 140 °C, at 5 °C min^{-1} to 170 °C, and at 10 °C min^{-1} to 240 °C final temperature. The ionization energy was set to 70 eV in the IE and the scan range from 30 to 250 atomic mass units with repetition rate at 3.4 scans s^{-1}. For quantification, calibration curves were made by injecting standards directly to the Tenax-GR traps column and released as described above for the samples.

Protein Carbonyls. Protein carbonyls were measured on fish samples as described by Levine et al. (*22*). A fish sample (0.5 g) was homogenized in 10 mL of Tris buffer (pH 7.4, 50 mM, 1 mM EDTA) containing 0.01% BHT. For spectroscopic determination of carbonyl groups 100 μL of the homogenate was precipitated with TCA to a final concentration of 20% (w/v). After centrifugation (13000*g*, 3 min), the

pellet was incubated with dinitrophenyl hydrazine (DNPH) in 2 M HCl in the dark for 1 h. For each sample a blank incubated in 2 M HCl and without DNPH was run in parallel. The samples were precipitated with TCA, and the pellets were washed three times with 1 mL of ethanol/ethyl acetate (1:1, v/v). The pellet was redissolved in 6 M guanidine chloride in 20 mM KH_2PO_4. The carbonyl content was calculated from the absorbance measured at 370 and 280 nm.

Color Measurement. Changes in a^* value (redness), b^* value (yellowness), and L^* value (lightness) were followed during storage of rainbow trout. Measurements were performed on a minimum of three fish with three measurements per fillet using a Minolta Chromameter (Cr-200, Osaka, Japan). The results are given as the mean of at least 18 measurements ± standard deviation.

Sensory Profiling. The sensory panel consisted of 10 assessors. They were all selected, tested, and specifically trained in descriptive analysis ISO 11035 (*23*) of rainbow trout. Before evaluation of the samples from the storage experiment, a sensory vocabulary was developed using samples from the different diets. Subsequently, the assessors were trained in three sessions to evaluate the sensory attributes on an unstructured 15 cm line scale with anchor points. Retraining was carried out continuously during the evaluation period. The sensory attributes and the definitions are shown in **Table 3**. The evaluations were performed in separated booths under normal daylight and at ambient temperature according to ISO 8589 (*24*). The assessors used water and flatbread to clean the palate between samples. Data were collected using a computer system (FIZZ Network version 2.0, Biosystems, France). The samples were placed in individual porcelain bowls and covered with porcelain lids with three-digit codes. The amount of fish prepared for each assessor was approximately 75 g. The fish samples were heated in a prewarmed convection oven (RATIONAL Combi-Dämpfer CCM, Germany) with air circulation for 15 min at 100 °C. After heat treatment, the samples were immediately served for the panel. The samples were served one by one, and each panelist evaluated in total six samples in each session, served randomly.

Statistical Analysis. The chemical analyses were carried out on at least three fish per treatments ($n = 3$) for each time point and were performed at least in triplicate. The results are given as mean values of triplicates with indication of the standard deviation. Sensory analyses were performed on 10 fish per treatment for each time point. Chemical analysis and sensory data were correlated using a principal component analysis (PCA), which was performed on the entire data set including all sampling time points using Simca P (Umetric, Umeå, Sweden). Variables were centered and scaled to "unit variance"; that is, the base weight was computed as 1/SD, where SD is the standard deviation of the mean of the variable. For sensory data, sensory properties were correlated to assessors and treatments by partial least-squares regression (PLSR) and PCA using The Unscrambler, version 9.1 (Camo ASA, Oslo, Norway). Data from the sensory profiling were corrected for the "level effect" (i.e., assessors using different parts of the line scale) as previously described (*25*). Furthermore, ANOVA was performed using GraphPad Prism (GraphPad Software, Inc.) to evaluate the difference between the feed and the fish (according to the feed composition) and also the effect of time.

RESULTS

Feed Type and Fish Muscle Composition. Rainbow trout were fed for 64 days with diets containing either fish oil or vegetable oil in combination with astaxanthin or canthaxanthin or without any pigment, giving a total of six different diets (**Table 1**). The feeds were analyzed for oil, carotenoids, and tocopherols content at the beginning, in the middle, and at the end of the feeding period, and obtained results were similar, indicating that the composition of the feed did not change over time. In addition, oxidation in the feed during storage was found to be negligible during the feeding period with an average peroxide value of 4.0 mequiv/kg of oil (with SD ±2.2; min 1.8−max 7.7) for all feed types (data not shown). All feed types contained approximately 25% oil. The oil content in the fish muscle tissue was similar for all samples irrespective of the feed type and reached an average value of 8% (data not shown). The feeding period of 64 days was found to be acceptable for addressing our objectives as the fatty acid profile and the carotenoid content of the muscle did reflect the feeding regime of the fish (as explained below).

The fatty acid composition of the fish muscle reflected the fatty acid profile of the feed as reported in **Table 2**. The feed prepared with fish oil contained a high proportion of saturated fat (29%) and of omega-3 fatty acids (19%) for EPA (20:5 n-3) plus DHA (22:6 n-3). The feed prepared with vegetable oil contained a lower level of saturated fat (8%) but high levels of linoleic acid (C18:2 n-6)(18%) and linolenic acid (C18:3 n-3) (8.55%). As expected, the muscle of the fish fed fish oil contained a higher amount of long-chain omega-3 fatty acids with EPA and DHA representing 23% of the total fatty

Table 2. Fatty Acid Composition (in Percent of Total Fatty Acids) of the Feed and of Rainbow Trout Muscle from Trout Fed a Diet Containing either Fish Oil or Vegetable Oil and 200 mg/kg Astaxanthin

	fish oil		vegetable oil	
fatty acid	feed	muscle	feed	muscle
C14:0	7.15 (±0.35)	4.13 (±0.18)	0.48 (±0.01)	1.49 (±0.11)
C16:0	18.88 (±1.15)	14.95 (±0.32)	6.30 (±0.07)	10.33 (±0.31)
C18:0	3.34 (±0.2)	3.08 (±0.10)	1.89 (±0.02)	2.80 (±0.10)
Σ saturated	**29.37**	**22.16**	**8.67**	**14.62**
C16:1 n-7	6.97 (±0.19)	5.57 (±0.26)	0.78(±0.01)	2.5 (±0.20)
C18:1 n-7	2.62 (±0.11)	2.79 (±0.13)	3.61 (±0.21)	2.93 (±0.05)
C18:1 n-9	10.61 (±0.40)	13.76 (±0.24)	53.26 (±0.44)	36.74 (±1.30)
C20:1 n-9	5.68(±0.38)	5.23 (±0.17)	2.46(±0.02)	4.43 (±0.25)
C22:1 n-11	7.12 (±1.02)	4.82 (±0.11)	0.74 (±0.24)	2.62 (±0.18)
Σ MUFA	**33.00**	**32.17**	**60.85**	**49.23**
C18:2 n-6	3.98 (±0.23)	5.69 (±0.80)	18.46 (±0.17)	12.20 (±0.27)
C18:3 n-3	1.31 (±0.04)	1.20 (±0.09)	8.55 (±0.07)	3.70 (±0.15)
C18:4 n-3	3.08 (±0.23)	1.48 (±0.10)	0.21 (±0.01)	0.82 (±0.14)
C20:4 n-6	0.56 (±0.01)	1.24 (±0.04)	0	0.59 (0.01)
C20:5 n-3	9.73 (±0.10)	6.12 (±0.24)	0.97 (±0.02)	2.11 (±0.8)
C21:5 n-3	0.4 (±0.02)	0.40 (±0.02)	0	0.14 (±0.01)
C22:5 n-3	1.13 (±0.10)	2.28 (±0.16)	0	0.81 (±0.04)
C22:6 n-3	9.64 (±0.63)	17.27 (±1.36)	1.10 (±0.20)	7.97 (±0.87)
Σ PUFA	**29.83**	**35.68**	**29.29**	**28.34**
others	**7.8**	**9.99**	**1.19**	**7.81**

Table 1. Oil Content and Antioxidant Content (α-Tocopherol, γ-Tocopherol, and Carotenoids) in the Feed Used in the Feeding Trial[a]

feed type	oil %	α-tocopherol (mg/kg of feed)	γ-tocopherol (mg/kg of feed)	carotenoids (mg/kg of feed)
fish oil/astaxanthin	26.18 a (±3.29)	21.74 a (±2.38)	4.29 a (±0.45)	110.80 a (±3.84)
vegetable oil/astaxanthin	24.09 a (±0.72)	36.63 b (±1.38)	69.57 b (±1.70)	116.96 a (±1.22)
fish oil/canthaxanthin	23.49 a (±1.75)	19.76 a (±1.22)	4.88 a (±0.40)	160.95 b (±1.46)
vegetable oil/canthaxanthin	24.31 a (±0.02)	34.70 b (±1.37)	68.90 b (±0.72)	183.14 b (±5.65)
fish oil/no pigment	25.10 a (±0.44)	20.13 a (±1.21)	4.13 a (±0.18)	
vegetable oil/no pigment	24.75 a (±0.07)	34.83 b (±0.33)	71.33 b (±0.93)	

[a] Numbers in the same column with different letters (a, b) are significantly different with $P < 0.001$.

Table 3. Sensory Attributes, Their Description, and Their Corresponding Abbreviation

	attribute	abbreviation	description
odor	earthy	EO	characteristic for trout
	muddy	MDO	geosmin and/or methylisoborneol
	sourish	SO	sourish like in fruit
	cooked potato	CP	cooked potato
	rancid	RO	rancid fish, paint, varnish
flavor	sweet	SWF	sweet
	mushroom	MF	fresh mushroom
	sourish	SOF	sourish like in fruit
	cooked potato	CF	cooked potato
	green	GF	green flavor like in plants
	muddy	MDF	geosmin and/or methylisoborneol
	rancid	RF	rancid fish, paint, varnish
	sour	SF	sour dishcloth, sour socks
texture	firm	FT	force required to compress the sample between the molar teeth
	juicy	JT	amount of juice released during mastication
	fibrousnesses	FIT	impression of fibers in the sample during chewing
	graininess	GT	heterogeneity; amount of particles in the sample after three chews
	flaky	FLT	tissue parts into flakes by pressing with a fork

acids, whereas fish fed vegetable oil contained only 10% of these two fatty acids. Conversely, fish muscle from fish fed vegetable oil contained a higher level of n-6 fatty acids such as linoleic acid (12%) when compared with fish fed fish oil feed (5%), respectively. The unsaturation indices for the feed were 173 for fish oil and 135 for vegetable oil. Similarly, the unsaturation index was higher in fish fed fish oil than in fish fed vegetable oil, with 207 for fish oil and 149 for vegetable oil, respectively.

The feeds containing fish oil were lower in tocopherols than the feeds prepared with vegetable oil with values of 20 and 35 mg/kg for α-tocopherol, respectively, and 4.5 and 70 mg/kg, respectively, for γ-tocopherol (**Table 1**). Both δ-tocopherol and β-tocopherol were present in the feed, but below 2 mg/kg and not detected at all in the fish muscle. The α-tocopherol content in the fish muscle at $t = 0$ ranged from 20 to 35 mg/kg, with significantly higher values for fish fed vegetable oil (**Figure 1**) as a direct reflection of their diet containing a higher level of α-tocoperol (**Table 1**). It is noteworthy that α-tocopherol content in the fish muscle at $t = 0$ was significantly higher in fish fed a diet containing no pigments. The γ-tocopherol content was below 1 mg/kg for fish fed fish oil and around 5 mg/kg for fish fed vegetable oil. In contrast to α-tocopherol, the γ-tocopherol levels in the muscle did not reflect the level in the feed to the same extent. However, the level of γ-tocopherol was indeed lower in fish fed fish oil compared with the level in fish fed vegetable oil, and this was in agreement with the lower level of γ-tocopherol in the fish oil diet (**Table 1**).

The initial amount of pigment added during feed preparation was set to 200 mg/kg for both canthaxanthin and astaxanthin. This was chosen well above the EU legislation limits as a clear impact of the carotenoid pigments in the diet and subsequently in the fish muscle was desired. However, the carotenoid contents analyzed in the feeds were 110 mg/kg for feeds prepared with astaxanthin and 170 mg/kg for feeds prepared with canthaxanthin. Differences between the formulation (200 mg/kg) and the analysis (170 and 110 mg/kg for

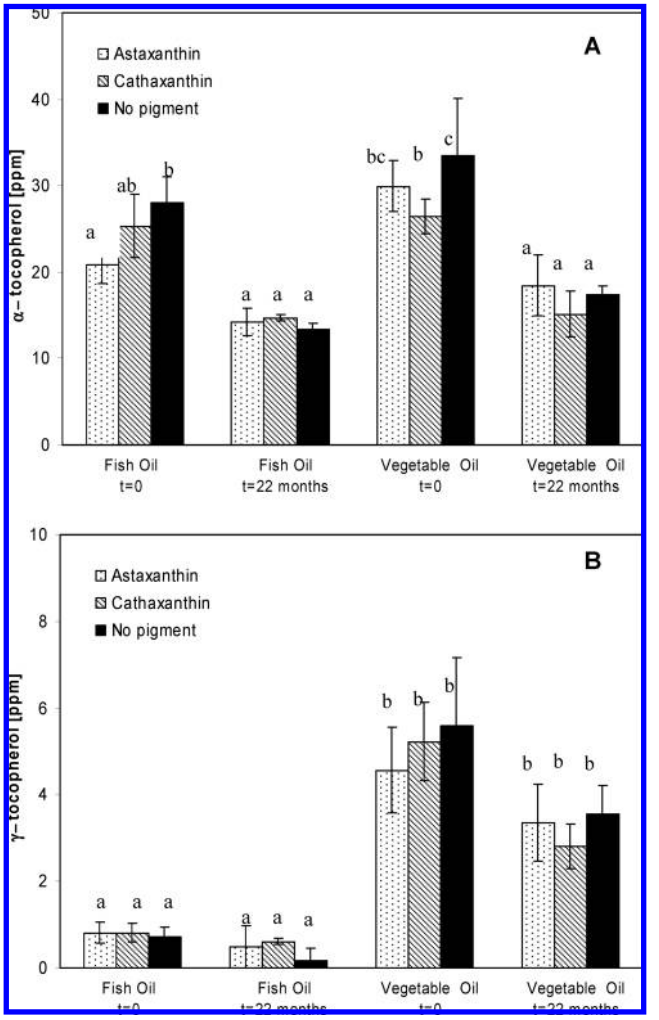

Figure 1. α-Tocopherol (**A**) and γ-tocopherol (**B**) contents expressed in milligrams per kilogram of fish in rainbow muscle trout fed either fish oil or vegetable oil at the beginning ($t = 0$) of the frozen storage period and after 22 months ($t = 22$) at −20 °C, $n = 3$. Different letters at a given time are significantly different with at least $P < 0.05$.

astaxanthin and canthaxanthin, respectively) might be due to loss or degradation during feed preparation as the pigments are sensitive to light, heat, and air. However, these initial differences in carotenoid content in the feed did not result in different contents of pigment in the fish muscle. The carotenoid contents in the muscle at $t = 0$ were not significantly different and around 10 mg/kg for both astaxanthin and canthaxanthin as seen in **Table 5**.

Principal Component Analysis. To obtain an overview of the results, a PCA was performed on both chemical and sensory data using all samples at all time points (0, 4, 8, 13, 18, and 22 months), thereby correlating feeding regime (including oil type, pigments, and tocopherols), chemical changes, and sensory analysis. A PCA models the data by computing so-called latent variables, which are linear functions of the original variables, estimated to contain, in decreasing order, the main structured information in the data. The *X*-axis depicts the first principal component (PC), which is the latent variable that explains most of the variation in the data set. The *Y*-axis depicts the second principal component, which is the latent variable that explains the second most variation in the data set. The PCA indicated that three PCs were necessary to explain the model. Together these three PCs explained approximately 53% of the variation in the data. The score plot and the loading plot for

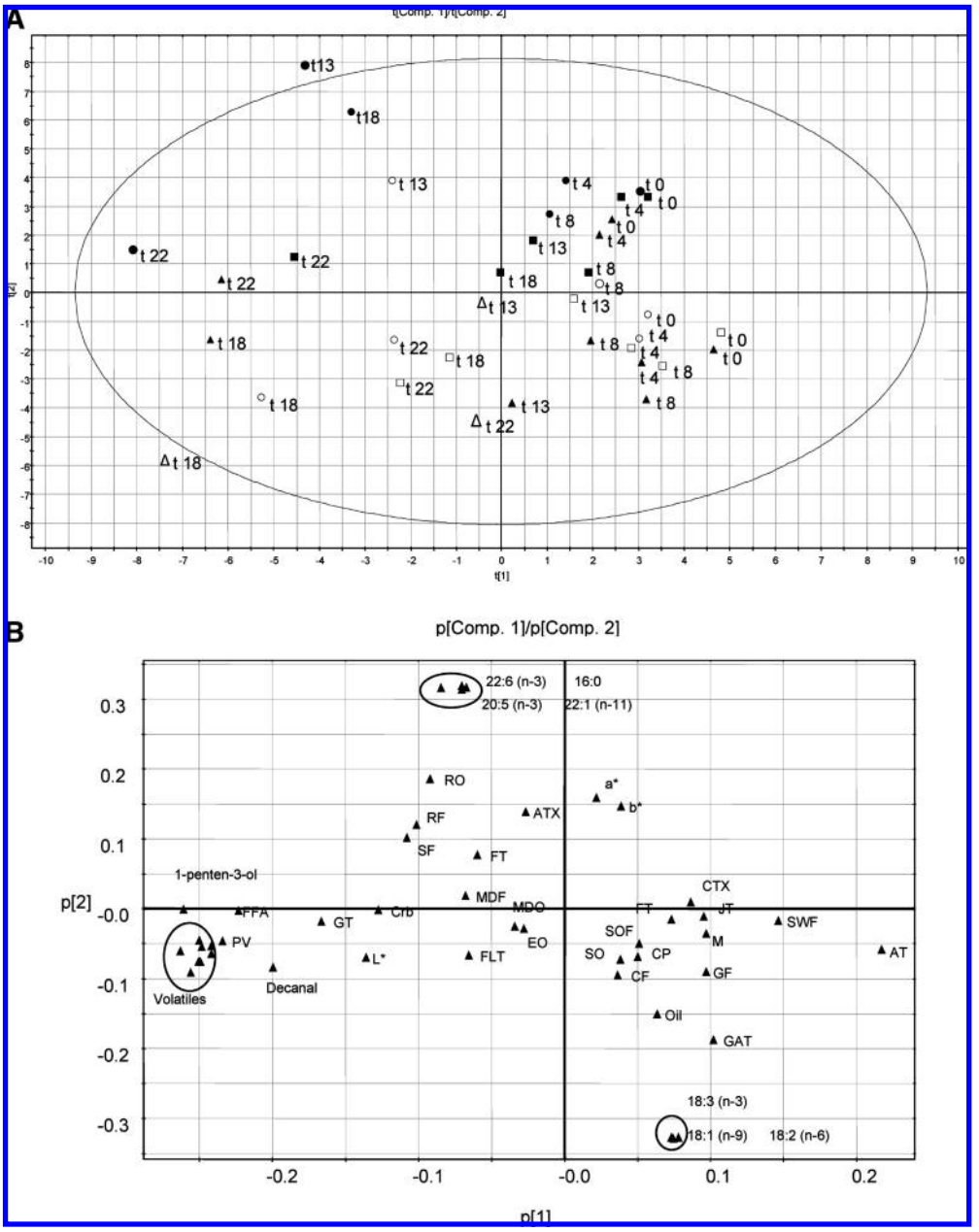

Figure 2. PCA of the chemical and sensory analysis for the entire storage period with (**A**) score plot and (**B**) loading plot for all data sets: (▲) fish oil/no pigment; (■) fish oil/canthaxanthin; (●) fish oil/astaxanthin; (△) vegetable oil/no pigment; (□) vegetable oil/canthaxanthin; (○) vegetable oil/astaxanthin. Chemical analysis abbreviations: *a**, *b**, *L*, redness, yellowness, and lightness; ATX, astaxanthin; CTX, canthaxanthin; GAT, γ-tocopherol; AT, α-tocopherol; Crb, carbonyls; FFA, free fatty acid; PV, peroxide value; oil, oil content. Volatiles: nonanal, 1 penten-3-ol, hexanal, *t*,*t*-2,4-heptadienal, 1-octen-3-ol, *t*-2-octenal, *t*,*c*-2,6-nonadienal. Sensory analysis abbreviations: see **Table 3**.

components 1 and 2 are given in panels **A** and **B**, respectively, of **Figure 2**.

Samples in the initial part of the storage period (t0 to t13) were located to the right in the score plot with time samples moved to the left (**Figure 2A**). Hence, PC1 mainly explained the differences between $t = 0$ and stored fish. Generally, fish fed fish oil were located in the upper part of the score plot, whereas fish fed vegetable oil were located in the bottom (**Figure 2A**). PC2 thus mainly explained differences between the fish oil and the vegetable oil fed fish. When fish oil fed fish were compared at the same time point, astaxanthin fed fish were almost always located farthest to the left followed by the no-pigment fish and the canthaxanthin fish to the right. This trend was not as clear for the vegetable oil fed fish. In the loading plot all of the volatiles, peroxides (PV), and free fatty acids (FFA) were located to the left in the diagram, and in combination with findings from the score plot this showed that their formation increased with time (**Figure 2A**). This location of the variables also indicated that astaxanthin fed fish oxidized more rapidly than the fish without pigment, whereas the canthaxanthin fed fish oxidized more slowly. Rancid odor and flavor were located slightly to the left together with the grainy texture, indicating that the intensity of the attributes increased during storage and most in astaxanthin fed fish. Both α- and γ-tocopherol were negatively correlated with rancidity. Moreover, particularly, the rancid odors and flavors were also located in the upper part of the diagram, although not in the very top of the plot, suggesting that fish oil fed fish developed more rancid odors than vegetable oil fed fish. Sensory attribute relating freshness such as green and juicy correlated strongly with canthaxanthin content,

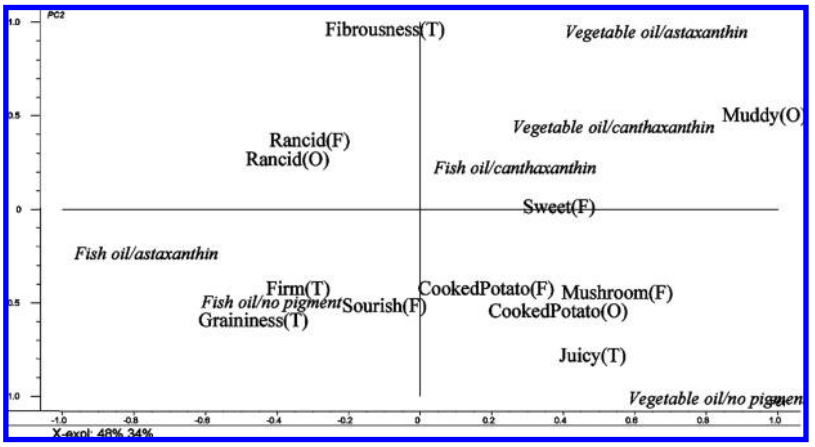

Figure 3. PCA of the sensory attribute for rainbow trout muscle fed either fish oil or vegetable oil after 22 months at −20 °C: (O) odor; (F) flavor; (T) texture. Scores are in plain and loadings in italic text.

whereas astaxanthin content with its location close to the PC2 axis was less well explained by the model. Most of the other sensory attributes as well as volatiles, PV, and FFA were located close to the PC1 axis, indicating that only small differences between fish oil and vegetable oil fed samples could be observed. In the loading plot EPA and DHA were located in the upper part of the plot, and in contrast the fatty acids characteristic for vegetable oil were located at the bottom of the plot. This is in agreement with the location of the corresponding samples in the score plot (**Figure 2B**).

To illustrate the results obtained from the sensory evaluation during storage, the sensory data are presented for all diets after 22 months of frozen storage as biplot (score and loading plots on the same graph) from a PCA (**Figure 3**). PC1 and PC2 explained 82% of the variation in sensory data. After 22 months at freezing temperature, fish fed fish oil had a grainier, more fibrous, and firmer texture compared to fish fed vegetable oil. In contrast, fish fed vegetable oil were juicier. In general, they also had less rancid odor and flavor compared to the fish fed fish oil. Fish fed vegetable oil had cooked potato, sweet, and mushroom flavors, whereas fish fed fish oil had more sourish flavors. For the fish fed fish oil the highest rancid odor and flavor were observed when astaxanthin was present in the feed. In contrast, the lower score for rancidity was observed when canthaxanthin was present in the feed. The picture was more complex and difficult to describe for fish fed vegetable oil, but fish receiving the no-pigment feed had a lower score for both rancid odor and flavor than both samples fed carotenoids. These results were also true when the entire storage period was considered with all sampling points and all diets.

Feed Type and Fish Muscle Oxidation. Irrespective of the feeding regimen, PVs were low for up to 8 months of storage and no significant differences were observed between samples before 13 months of storage (**Figure 4**). Similarly, the volatiles did not develop before 12 months of storage (**Figures 5** and **6**). As also observed from the PCA, the oil type did not seem to affect the oxidation dramatically, and no differences were observed between fish oil and vegetable oil in samples without any pigments. The volatiles 1-penten-3-ol and hexanal were chosen as markers for omega-3 and omega-6 oxidation products, respectively. Other volatile compounds were detected, but their development was similar to those of 1-penten-3-ol and hexanal. At 18 months of storage the statistical analysis of the data obtained from the volatiles indicated that the samples were significantly different. The fish oil fed fish reached a higher value for 1-penten-3-ol and the vegetable oil fed samples a higher value for hexanal.

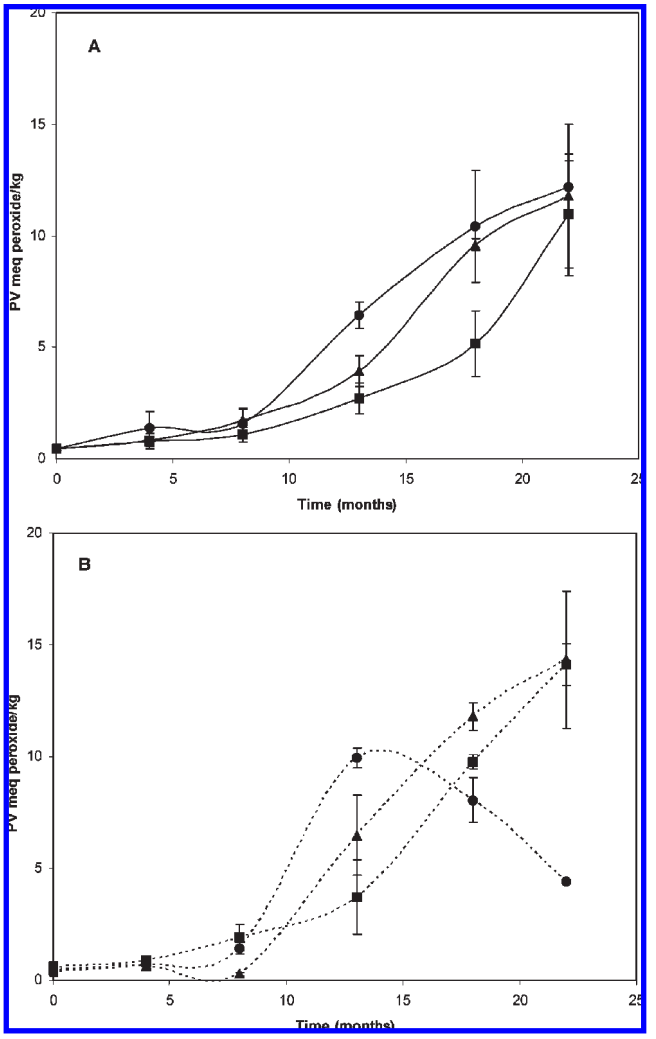

Figure 4. Development of lipid hydroperoxide expressed in milliequivalents of peroxide per kilogram of oil, in rainbow trout muscle fed fish oil (**A**) or vegetable oil (**B**) and stored at −20 °C for up to 22 months, $n = 3$: (▲) no pigment; (■) canthaxanthin; (●) astaxanthin.

Table 4 shows the results from carbonyls, which suggest a lower level of protein oxidation for fish fed fish oil compared to fish fed vegetable oil. Results from both α- and γ-tocopherol contents in the muscle at the start and at the end of the storage period are presented in **Figure 1**. Muscle from fish fed vegetable oil had in general a more severe loss of α- and γ-tocopherol

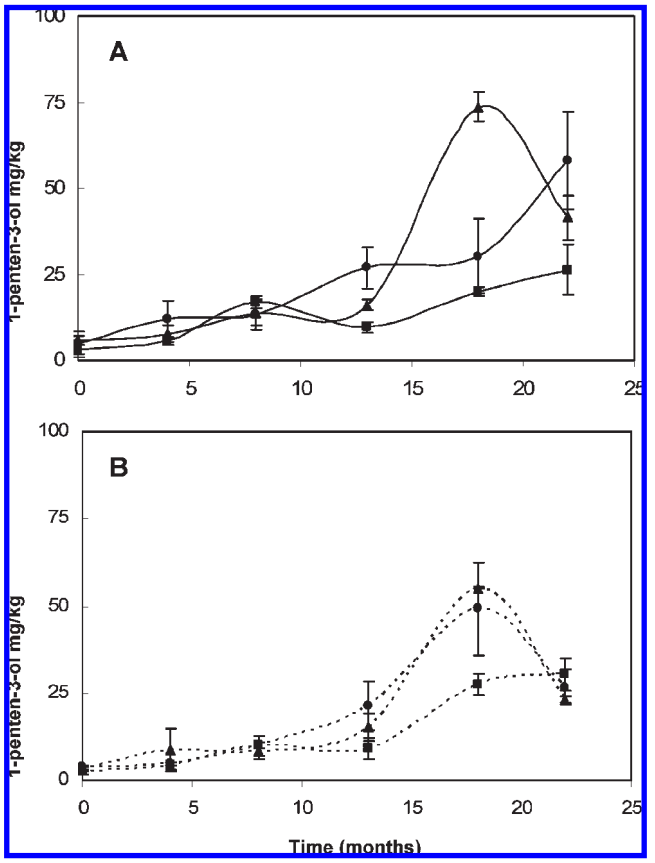

Figure 5. Development of 1-penten-3-ol in rainbow trout muscle fed either fish oil (**A**) or vegetable oil (**B**) and stored at −20 °C for up to 22 months expressed in milligrams per kilogram of fish, $n = 3$: (▲) no pigment; (■) canthaxanthin; (●) astaxanthin.

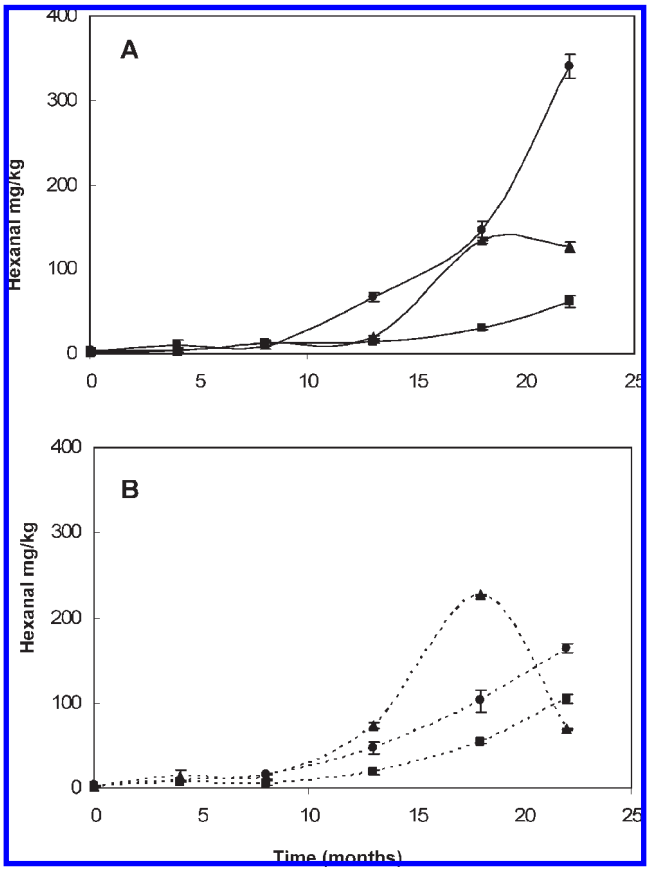

Figure 6. Development of hexanal in rainbow trout muscle fed either fish oil (**A**) or vegetable oil (**B**) and stored at −20 °C for up to 22 months expressed in milligrams per kilogram of fish, $n = 3$: (▲) no pigment; (■) canthaxanthin; (●) astaxanthin.

compared to fish fed fish oil. However, irrespective of the oil type there was no difference between the samples after 22 months of storage.

A significant difference between fish fed different pigments was observed at 13 months as previously mentioned. Irrespective of the difference in initial content of carotenoids in the feed, the fish muscles show similar levels of pigment (10 ppm). Canthaxanthin showed a small antioxidative effect in the muscle tissue for both oil types (**Figure 2**). This was confirmed by a very slow development of peroxides (**Figure 4**) and volatiles (1-penten-3-ol and hexanal, respectively; **Figures 5** and **6**). The carbonyl values obtained after 22 months also indicated that canthaxanthin was able to prevent protein oxidation independent of the oil type (**Table 4**).

However, in contrast to canthaxanthin, the effect of astaxanthin content seemed to some extent to depend on the oil type and on the oxidation parameter measured. Overall, no evident and systematic effect of astaxanthin could, however, be observed (**Figures 4**−**6**). Likewise, the carbonyl values in **Table 4** indicated no effect of astaxanthin on the development of protein oxidation as observed by a significant increase in protein carbonyls content with time.

The carotenoid data presented in **Table 5** showed that canthaxanthin was consumed as a consequence of oxidation in the muscle with a loss of this compound of approximately 30−40% after 22 months of storage irrespective of the oil type. In addition, the data for the a^* value indicated a significant loss in redness for samples with canthaxanthin over time (**Table 5**). The muscle from fish fed canthaxanthin showed the same loss in α-tocopherol content irrespective of the oil type (**Figure 1**). In contrast, samples with astaxanthin consumed more α-tocopherol during storage of muscle from animals fed vegetable oil compared with muscle from animals fed fish oil. Results from color measurement were more difficult to explain as no significant loss in redness was observed in the samples after 22 months irrespective of the oil type. However, astaxanthin content in the tissue after 22 months was lower for fish fed fish oil, but not in fish fed vegetable oil (**Table 5**).

DISCUSSION

Feed Type and Fish Muscle Composition. The oil type did not affect the total fat content of the muscle tissues that reached approximately 8%, irrespective of the oil type (not shown). This is in agreement with the findings of Schlechtriem et al. (*15*), who investigated the replacement of fish oil by vegetable oil in salmon and reported no impact on the oil type on the total fat content of the fish muscle. In contrast, the present data show that the fatty acid profile of the oil and the antioxidant used in the feed were reflected in the muscle of the trout as also reported by others for salmonoids (*12*, *16*, *17*). As expected, fish fed fish oil contained a higher level of EPA (C20:5 n-3) and DHA (C22:6 n-3), whereas fish fed vegetable oil contained a higher level of linoleic acid (C18:2 n-6) and linolenic acid (C18:3 n-3). The carotenoid content of the muscle also reflected the level of carotenoids present in the feed with little difference between astaxanthin and canthaxanthin deposition in the muscle. No impact of the oil type on the carotenoid content in the flesh was observed. The level of dietary fat has been shown to affect the deposition of astaxanthin in the muscle (*26*). However, only a few studies have investigated the effect of the oil type on the

Table 4. Protein Carbonyl Groups of Protein in Rainbow Trout Fed Different Diets during Frozen Storage at −20 °C for up to 22 Months, $n = 3$[a]

	fish diet					
	fish oil			vegetable oil		
	astaxanthin	canthaxanthin	none	astaxanthin	canthaxanthin	none
carbonyl (nmol/mg of protein), $t = 0$	1.83 a (±0.40)	1.66 a (±0.30)	2.16 a (±0.14)	1.83 a (±0.21)	2.13 a (±0.46)	2.21 a (±0.48)
carbonyl (nmol/mg of protein), $t = 22$	5.26 b (±0.64)	1.87 a (±0.16)	4.24 b (±1.38)	8.54 c (±1.70)	1.76 a (±0.35)	7.37c (±1.84)

[a] Numbers with different letters at a given time point are significantly different with at least $P < 0.05$.

Table 5. Carotenoid Content (Astaxanthin or Canthaxanthin) Expressed in Milligrams per Kilogram of Fish and a^* Value of Rainbow Trout Muscle from Fish Fed Different Diets during Frozen Storage at −20 °C for up to 22 Months, $n = 3$[a]

	fish diet			
	fish oil		vegetable oil	
	astaxanthin	canthaxanthin	astaxanthin	canthaxanthin
carotenoids (mg/kg of fish)				
$t = 0$	9.79 a (±2.96)	10.72 a (±4.39)	8.52 a (±2.17)	9.75 a (±3.26)
$t = 22$	5.84 a (±2.11)	7.50 a (±3.06)	9.72 a (±2.19)	6.01a (±2.12)
a^* value				
$t = 0$	14.92 a (±2.56)	11.26 a (±2.40)	16.81 a (±3.34)	13.39 a (±4.35)
$t = 22$	13.99 a (±3.44)	8.48 a (±2.21)	16.1 a (±2.26)	4.98 a (±2.30)

[a] Numbers with different letters at a given time are significantly different with at least $P < 0.05$.

deposition of other carotenoids. Choubert et al. (*27*) examined the impact of the oil type (fish oil vs olive oil) on the astaxanthin level both in tissue and in plasma. They found that fish fed olive oil had a higher level of astaxanthin in their plasma compared to fish fed fish oil but that muscle astaxanthin levels were not different for fish fed olive oil or fish oil. Another study in salmon reported that plasma astaxanthin was unaffected by the level of PUFA (*28*). It was suggested that oils containing different levels of sterols and stanols might interfere with carotenoid deposition in muscle (*29*).

As for the different tocopherols, they seemed to be absorbed differently in the muscle, with α-tocopherol being more easily absorbed than γ-tocopherol. Indeed, the level of γ-tocopherol in the feed with vegetable oil was 70 mg/kg, which is almost 2 or 3 times as much as the level of α-tocopherol, and the highest levels in the flesh were around 6 and 30 mg/kg, respectively. Ackman et al. (*30*) reported that α-tocopherol was stored in phospholipid-rich tissues and γ-tocopherol in the adipocytes, and this might explain the low level of γ-tocopherol found in the muscle despite the high level present in the feed. The presence of carotenoid in the feed seemed to affect the level of tocopherol deposited in the muscle. Thus, fish fed a diet containing no pigment had generally a higher level of α-tocopherol in the muscle and to some extent also a higher level of γ-tocopherol irrespective of the oil type used. Similarly, Jensen et al. (*12*) also observed that the deposition of tocopherol in the tissue was highest for fish fed a lower level of carotenoids.

It seems likely that interaction between carotenoids, tocopherols, fatty acids, and minor components present in the feed such as phytosterols and vitamins affects the composition of the muscle tissue. Additionally, there is also more and more evidence that tocopherol and carotenoids are not only acting as antioxidants but might play a role in regulating signal transduction and gene expression, thereby affecting the entire metabolism (*31*, *32*). A recent paper also suggested that tocopherol could affect the fatty acid metabolism and the biosynthesis of n-3 PUFA in fish (*33*). More investigations dealing with the impact of the feed composition on fish metabolism are needed to gain a better understanding of how the different components present in the feed affect fish physiology and thereby fish muscle quality.

Feed Type and Fish Muscle Oxidation. The effect of feeding vegetable oil versus fish oil on the development of oxidation in frozen fish is not very well documented, but one could expect that more stable fish would be produced if more saturated oils are used in the diet. The impact of feeding fish oil or vegetable oil on the fish oxidative stability and eating quality was rather small in our study. Nevertheless, according to the two PCA plots (**Figures 2** and **3**) fish fed fish oil were perceived by the sensory panel as being more oxidized. Fish oil fed fish had a grainier and tougher texture compared to fish fed vegetable oil after 22 months of storage. The effect of the different oil types on the oxidative stability of the fish was expected to be more pronounced, especially due to the fact that the vegetable oil also contained a much larger amount of both α- and γ-tocopherol compared to the fish oil. However, fish fed vegetable oil seemed more stable, but further studies are necessary to clarify how the oil type alone affects oxidative stability. In addition, in our study the oil type was investigated irrespective of its antioxidants status. It is possible that antioxidants present in situ in the oil may have affected the oxidative stability of the fish. Antioxidant levels present in the different oils were not identical, making the conclusion on antioxidative stability of the fish based on the oil type alone more difficult to justify.

Carotenoids are believed to be able to scavenge free radicals and to be good antioxidants, and astaxanthin has been reported to be a better antioxidant than canthaxanthin (*34*). However, our results indicated that canthaxanthin was better at protecting fish muscle against oxidation compared to astaxanthin, particularly in fish fed a fish oil diet. Ingemansson et al. (*35*) also reported no effect of astaxanthin on lipid oxidation during frozen storage of trout. In our study, not only was canthaxanthin better at protecting the fish from lipid oxidation, but it also showed some ability to limit protein oxidation. Moreover, the sensory data indicated that canthaxanthin in the feed resulted in less rancid trout fillets after frozen storage when the feed contained fish oil. A small investigation on the feed applied in the present study showed that canthaxanthin was able

to prevent protein oxidation (*36*). In addition, it has been reported that β-carotene in combination with tocopherol and ascorbic acid was able to prevent oxidation of human serum albumin (*37*).

The antioxidative activity of carotenoids leads to bleaching as a result of their sacrificial protection against radical process (*38*). Bleaching of the fish muscle was observed in the present study for canthaxanthin-fed fish. It has been reported that canthaxanthin faded more quickly than astaxanthin (*38*). This could not always be correlated to protection against lipid oxidation; however, the present data showed that canthaxanthin was also able to protect the protein from oxidative damage, and this might partly explain why it is consumed more rapidly.

The results with astaxanthin were not so clear, as astaxanthin sometimes behaved either slightly as a pro-oxidant or as an antioxidant, and no systematic effect could be found across the oil types used. These results are in agreement with the contradicting results reported in the literature showing both pro-oxidant and antioxidant or no effect of astaxanthin (*11*, *12*, *35*, *36*). In addition, findings that carotenoids could behave as a pro-oxidant have raised concern with respect to their health benefits (*39*, *40*). It has been demonstrated that the oxygen partial pressure and the concentration of carotenoids seem to be important parameters for their antioxidative activity (*41*). It is therefore possible that the high level of astaxanthin used in the feed was responsible for the nonsystematic antioxidant or pro-oxidant effect observed for the astaxanthin-fed fish. Other studies showing a protective effect of astaxanthin used lower levels in the feed and generally ranged between 30 and 50 mg/kg. At the level used in the present study (200 mg/kg) the limit at which astaxanthin behavior switches from being an antioxidant to a pro-oxidant might have been reached. The results also indicated that the effect of astaxanthin was more affected by the oil type than canthaxanthin.

It is possible that the different concentrations of tocopherols in the oils used also had an impact on the carotenoid antioxidative properties. It is believed that carotenoids can interact with tocopherols, regenerating the tocopheryl radical. However, most studies have focused on the interaction between α-tocopherol and β-carotene, but fewer studies have investigated astaxanthin and canthaxanthin. In addition, little is known about the interaction between carotenoids and γ-tocopherols. Besides, as already mentioned above, minor components present in the oil might have interfered, making the results obtained with astaxanthin more difficult to explain.

Conclusions. The results obtained in the present study suggest that the feed composition, that is, oil type and antioxidant, had an impact on the oxidative stability of the fish muscle during subsequent frozen storage. Fish fed vegetable oil were more stable during frozen storage compared to fish fed fish oil. However, the difference in antioxidant status of the oils used might have contributed to such effect. The data also suggested that canthaxanthin (at the level found in the fish muscle, approximately 10 mg/kg) was able to prevent protein oxidation, and this deserves further investigation. If oxidative stability of salmonoids is to be improved, the feed composition (i.e., oil type, protein source, antioxidants levels) should be carefully evaluated with respect to its impacts on both lipid and protein oxidation in fish during storage.

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