Research Article

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Effect of the antioxidants composition in diet on the sensory and physical properties of frozen farmed Coho salmon (*Oncorhynchus kisutch*)

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Abstract

BACKGROUND: Great attention has been paid to the antioxidants present in farmed fish feeds, with the replacement of synthetic antioxidants by natural ones being a main objective. In the present study, Coho salmon (*Oncorhynchus kisutch*) was fed a conventional diet that was enriched with different kinds of antioxidants: synthetic antioxidants (butylated-hydroxy toluene and ethoxyquin; diet I), a tocopherols-rich mixture (diet II) and a tocopherols–rosemary extract mixture (diet III). A comparative study of the sensory and physical changes observed in the corresponding frozen products was undertaken.

RESULTS: After 18 months at −18 °C, fish previously fed on diet I showed higher putrid and rancid odours and rancid taste scores, while lower mean typical odour and taste values were attained. Dripping and expressible moisture values obtained for diet II-fish were lower when compared with their counterparts belonging to the diet I; additionally, microstructure analysis revealed that Z-lines integration was better preserved in fish corresponding to diets II and III.

CONCLUSION: Diet II has been recognised as being the most profitable to be employed to maintain the sensory and physical properties of the frozen product when long-term storage is considered. Further research is to be continued to optimise the natural antioxidants profile.

Keywords: *Oncorhynchus kisutch*; diet antioxidants; frozen storage; sensory acceptance; physical properties; quality

INTRODUCTION

Freezing and frozen storage have largely been employed to retain fish sensory and nutritional properties before consumption or further technological process. However, both processes may cause lysis of mitochondria and lysosomes and alter the distribution of enzymes and factors affecting the rate of enzyme reactions in tissues, so that deteriorative damage in frozen fish could be accelerated.[1–3] Additionally, the presence of both a highly unsaturated lipid composition and relevant pro-oxidant compounds can facilitate an important enzymatic and non-enzymatic rancidity development.[4,5] As a result, important detrimental effects on fish muscle properties such as colour, odour, water leaching and texture take place.

To extend the shelf life of frozen fish species, many efforts have been based on the employment of antioxidants. Since synthetic antioxidants have been reported to behave as carcinogenic and mutagenic agents, more attention has been given to the use of natural antioxidants.[6] Accordingly, recent research has focused on the employment of endogenous-type antioxidants (namely, tocopherols and organic acids)[7] or antioxidants present in plant extracts[8] to enhance quality of frozen fish products.

Great attention has also been addressed to the presence of antioxidants in farmed fish feeds. Fish farmers have included a wide range of permitted synthetic[9] and natural[10] antioxidants in order to retain the quality of the corresponding processed food. In this sense, an increasing interest is being accorded to the employment of natural antioxidants during the growing process of fish species;[11,12] thus, in addition to food quality enhancement, the presence of such natural antioxidants in the diet would increase the oxidative stability of the living fish, as well as increase the functional properties in the corresponding food product.

Among farmed fish, Coho salmon (*Oncorhynchus kisutch*), also called silver salmon, has received great attention because of its increasing production in countries like Chile, Japan and Canada in parallel to important capture production in countries such as USA, Russian Federation, Canada and Japan.[13,14] The present work

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focuses on the loss of quality of this fatty species during frozen storage. For this reason, Coho salmon (*Oncorhynchus kisutch*) was fed a conventional diet that was enriched with different types of antioxidant: synthetic antioxidants (butylated hydroxy toluene and ethoxyquin; diet I), a tocopherols-rich mixture (diet II) and a tocopherols–rosemary extract mixture (diet III). The basic aim of the study was to investigate the effect of the antioxidants profile in diet on the sensory and physical properties of the corresponding frozen product.

MATERIALS AND METHODS

Fish and diets

Coho salmon fish used in this study were farmed in three different tanks by EWOS Innovation Research (Colaco, Puerto Montt, Chile), according to the experimental conditions reported in a parallel study.[15] Thus, sandbed-filtered seawater (salinity range, 31.3–33.1 g kg^{-1}) was supplied to each tank over a temperature range of 11.2–12.8 °C. Feeding to satiety was carried out during the lighted period (photoperiod, 5.8–7.8 h) by supplying a diet with the following general composition (g kg^{-1}): 430 (protein), 290 (fat), 70 (moisture), 65 (ash), 13 (crude fibre) and 132 (carbohydrates). The distribution of fat composition into saturated, monounsaturated and polyunsaturated fatty acid groups was 325.4, 269.6 and 401.6 g kg^{-1}, respectively.

According to the objectives of the work, each of the three tanks was fed on a different antioxidant composition.[15] Thus, the first tank was fed on an antioxidant mixture including 19.3, 3.0 and 22.4 mg kg^{-1} of EQ, BHT and total tocopherols, respectively (diet I); the antioxidant mixture supplied to the second tank included 2.9, 0.0 and 101 mg kg^{-1} of EQ, BHT and total tocopherols, respectively (diet II); and the third tank was fed on an antioxidant mixture including 2.3, 0.0, 45, 18 and 13 mg kg^{-1} of EQ, BHT, total tocopherols, carnosic acid and carnosol, respectively (diet III).

Fish processing and sampling

Once Coho salmon individuals attained ca. 2500 g weight, 30 fish per tank were withdrawn, sacrificed by a sharp blow to the head, the gills cut and bled in a water–ice mixture, beheaded, gutted and kept in ice for 24 h until they arrived at our laboratory. The fish were then frozen at −40 °C in individual polyethylene bags, this including vacuum sealing at 400 mbar. After 3 days, the fish were stored in a freezing room at −18 °C. Frozen fish were taken for analysis on months 0, 3, 6, 9, 12 and 18 of storage at −18 °C. From each tank under study, five different individuals were analysed independently ($n = 5$) at each sampling time after fish thawing; thawing was carried out by overnight storage (12 h) in a cool room (4 °C).

In order to achieve the sensory and physical analyses, sampling was carried out according to the extraction methodology previously proposed[16] and adapted to the actual species. Thus, the different muscle zones employed for each kind of quality analysis are indicated in Fig. 1. All chemicals used were reagent grade (Merck, Darmstadt, Germany).

Sensory analysis

The quantitative descriptive analysis (QDA) method was applied in order to assess changes in sensory properties of Coho salmon.[14,16] Ten panellists (five females and five males) with experience in sensory testing were selected and trained according to international standards.[17,18] During the training sessions, the sensory descriptors for thawed and thawed–cooked salmon were discussed and analysed by the panellists on samples of varying quality conditions.

At each sampling time of the present study, 4 cm × 4 cm × 2 cm steaks ($N = 20$) were removed from each individual salmon according to Fig. 1 (zone 4). Individual steaks were then placed in polyethylene bags, coded with three-digit random numbers and employed for thawed ($N = 10$) and thawed–cooked ($N = 10$) fish analysis; each panellist was given a single thawed steak and a single thawed–cooked steak of each individual fish. Cooking was accomplished by suspending the bags in a circulating water bath heated to 100 °C ± 2 °C for 15 min to an internal temperature of 67 °C ± 2 °C and then the samples were submitted independently to the panel.

At each sampling time, sensory evaluation began by the analysis of the salmon muscle in the raw state and was followed by the muscle analysis in the cooked state. Both types of sample were analysed at a single sitting, according to the previous training received by the panellists. At each sitting, samples were submitted independently and in a randomised order to the panellists; once each score sheet was filled in, the following sample to be analysed was received. Diluted tea without sugar was served to panellists to clean the mouth.

The different sensory descriptors (typical, rancid and putrid odour and taste; muscle dehydration; elasticity) were evaluated on a non-structured linear scale with numerical scores from 0 to 10. A score of 0 corresponds to an undetected value for the descriptor, while a score of 10 corresponds to the highest detectable value

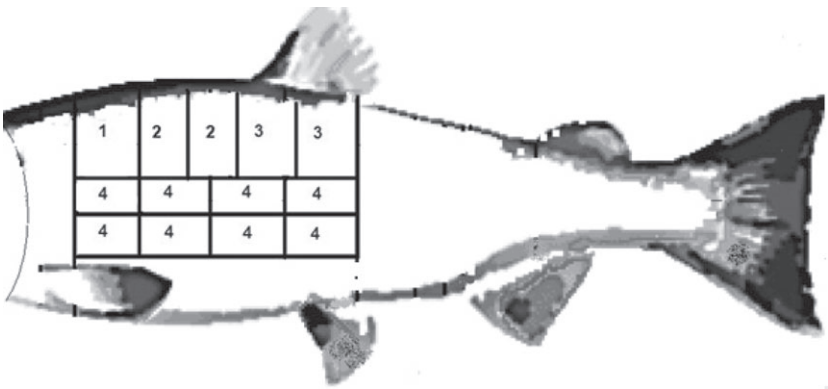

Figure 1. Location of the different fish muscle zones employed for the different sensory and physical analyses: (1) microstructure, (2) water loss (expressible moisture); (3) texture (shear test); (4) sensory analysis (odour, taste, elasticity, colour and muscle dehydration). Dripping loss was obtained in whole fish.

for the descriptor. Odour, muscle dehydration and elasticity were analysed on thawed fish; taste was analysed on thawed–cooked fish. Scores among panellists were averaged. For odour and taste descriptors, a score of 5.0 was considered the borderline of acceptability; scores from the remaining descriptors were comparatively discussed without considering a borderline of acceptability.

In addition, the red colour appearance was evaluated by the Roche SalmoFan™ Lineal card. For it, panellists matched the salmon muscle colour with a 20–34-score card system previously established for salmonids pigmented with astaxanthin.[18] In this card, 20-score and 34-score represent the lightest and the most intense salmon colours, respectively.

Physical analyses

Assessment of water loss

The expressible moisture content was measured as the amount of fluid extracted from fillets subjected to compression.[19] A Universal Testing Machine (LR-5 K; Lloyd Instruments, Hampshire, UK) was used with a load cell of 100 N connected to a computer and a Dapmat 40-0465 software for data analysis (version 3.05; Lloyd Instruments). Cylindrical samples (diameter = 20 mm, length = 20 mm) were extracted according to Fig. 1 (zone 2) and tempered at 4 °C for 4 h prior to analysis. Samples were compressed into 14 mm at a constant speed of 12.7 mm min^{-1}. The fluid exudate was received in a Whatman No. 4 filter paper. The expressible moisture was calculated as the weight difference of the filter paper before and after compression. Results were expressed as g exudate kg^{-1} sample. Each analysis was conducted in quadruplicate for each fish sample.

Dripping loss was measured according to Rørå and Einen.[20] For it, whole fish were packed in sealed bags with an absorbent pad placed between the skin and the vacuum pack. The absorbent pad absorbed all liquid leakage from salmon during freezing, frozen storage and thawing. At each sampling time, vacuum packs were opened and the wet absorbent pad was weighted. Water loss (dripping) was calculated as g kg^{-1} fish.

Texture analysis

Shear test was used to evaluate the firmness and deformation in thawed salmon muscle.[19,21] The force–deformation curve was obtained from a Universal Testing Machine (LR-5 K; Lloyd Instruments) with a load cell of 100 N connected to a computer, this including a Dapmat 40-0465 software data analysis (version 3.05, Lloyd Instruments). Samples of 4 cm × 4 cm × 2 cm from salmon muscle (zone 3; Fig. 1) were extracted in quadruplicate and kept at 4 °C ± 1 °C for 4 h prior to analysis. The samples were sheared using a Warner–Bratzler steel blade (knife-edge 60°; 1.2 mm thick, 155 mm high, and 150 mm width) at a constant speed of 60 mm min^{-1}. In a force versus deformation curve, the peak of maximum force (N) required for shearing the sample was recorded as the shear force and represents the maximum resistance of the sample to shear (N). Deformation (mm) was measured during the downward movement of the blade and was calculated as the deformation (mm) at maximum peak force. Each analysis was conducted in quadruplicate for each salmon individual.

Microstructure analysis

Small pieces (0.5 mm thickness) (zone 1; Fig. 1) of Coho salmon muscle were fixed for 18 h in a 30 g L^{-1} solution of glutaraldehyde in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.2) at room temperature and subsequently rinsed (3 × 20 min) in 0.1 mol L^{-1} sodium cacodylate buffer. Samples were then post-fixed on ice for 90 min in an aqueous solution of 10 g L^{-1} osmium tetroxide in 0.2 mol L^{-1} sodium cacodylate, and rinsed for 30 min in distilled water. The samples were then stained for 60 min in an aqueous solution of 10 g L^{-1} uranyl acetate, dehydrated for 20 min in increasing concentrations of acetone (500, 700, 950 and 1000 g L^{-1}) and embedded in 1:1 epon–acetone overnight. The muscle tissues were then immersed in epoxy resin, being the polymerisation of the resin carried out at 60 °C for 24 h. Samples were cut to 1 µm in a Sorvall MT-IIB ultramicrotome (Dupont Company, Newtown, CT, USA) and were stained with toluidine blue. Ultra-thin sections obtained from the selected areas were stained with 40 g L^{-1} uranyl acetate in methanol and then with lead citrate according to Reynolds.[22] Finally, the sections were analysed by Transmission electron microscopy (Tecnai 12; Philips, Eindhoven, the Netherlands) to 80 kV. Analyses were carried out on fish samples corresponding to months 0, 9 and 18 of frozen storage.

Statistical analyses

Three variance checking methods (Cochran's C test, Bartlett's test and Hartley's test) were performed to verify that each of the quality parameters applied was consistent with a normal distribution. In negative cases, the non-parametric Kruskal–Wallis test was employed ($P < 0.05$); otherwise, data were subjected to variance analysis (ANOVA) to explore differences by two different ways ($P < 0.05$) in order to assess the effect of diet antioxidants supplied and the effect of the frozen storage time. In the event of any significant difference, a multiple range test (HSD, Tukey method) was applied. Correlation analyses between sensory and physical quality parameters and frozen storage time were studied according to the Pearson method; correlations of sensory and physical parameters with chemical quality indices corresponding to parallel research[15] were also analysed. For this, Statgraphics Plus® version 5.1 software (Manugistics 2001; Statistical Graphics Corporation, Rockville, MA, USA) was employed, with a confidence interval at the 95% level being considered in all cases ($P < 0.05$).

RESULTS AND DISCUSSION

Odour and taste assessment

Progressive decreasing values with the frozen storage time could be observed for both typical odour ($r = 0.94–0.76$, linear fitting, $P < 0.05$; Fig. 2) and taste ($r = 0.90–0.80$, logarithmic fitting, $P < 0.05$; Fig. 3) in all kinds of sample; this decrease agrees with previous work on the same species stored under similar frozen conditions (−18 °C for up to 15 months).[13] Meantime, a general low rancid odour ($r = 0.97–0.87$; quadratic fitting, $P < 0.05$; Fig. 2) and taste ($r = 0.98–0.92$; quadratic fitting, $P < 0.05$; Fig. 3) development with storage time could be observed in all cases; for both descriptors, a negligible development in the 0–9 month period was followed by an increase in the 12–18 month interval, with all scores being under 2.7. Finally, a very low putrid odour and taste development could be observed; thus, all values were included in the 0–1 score range (Table 1).

Concerning the comparative study of diets, fish belonging to diet I showed a higher quality decrease at the end of the experiment when compared with their counterparts from diets enriched with natural antioxidants; at that time, higher ($P < 0.05$) putrid and rancid odour and rancid taste scores were obtained in diet-I fish, while lower mean typical odour and taste values were attained in such samples. Between diets enriched with natural antioxidants, a lower ($P < 0.05$) putrid and rancid development in odour and taste

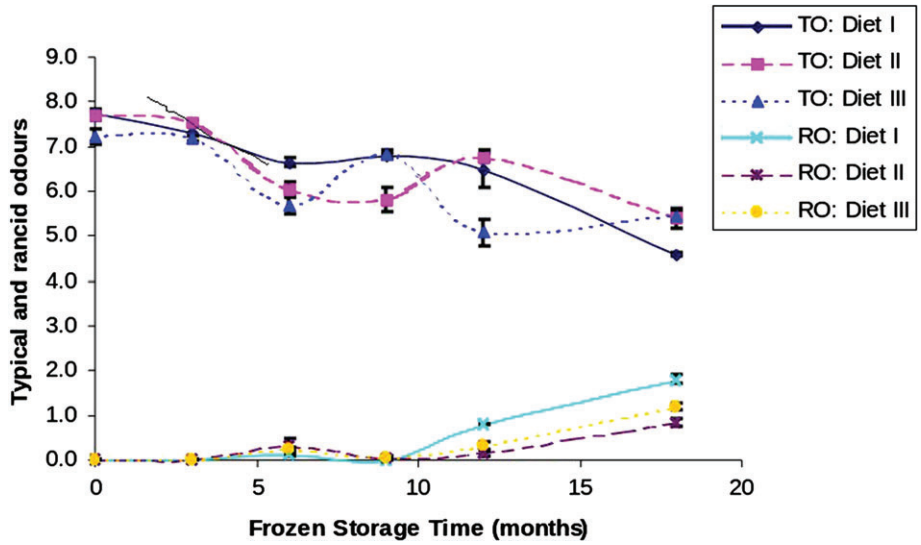

Figure 2. Typical (TO) and rancid (RO) odour development* in frozen Coho salmon muscle which was previously fed on different diets**. *Mean values of five ($n = 5$) replicates. Standard deviations are denoted by bars. **Diets I, II and III include an antioxidant mixture enriched in ethoxyquin/butylated hydroxytoluene, tocopherols and tocopherols/polyphenols (carnosic acid and carnosol), respectively, as described in the Material and Methods section.

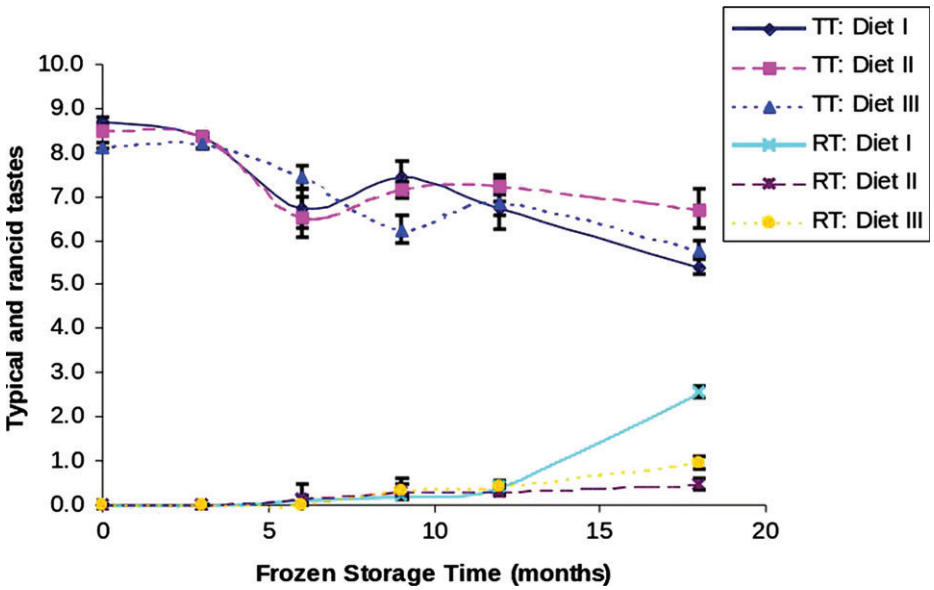

Figure 3. Typical (TT) and rancid (RT) taste development in frozen Coho salmon muscle which was previously fed on different diets*. *Mean values of five ($n = 5$) replicates. Standard deviations are denoted by bars. Diets employed as expressed in Fig. 2.

could be detected at the end of the experiment in salmon fed on diet including high levels of tocopherols (diet II).

Development of rancidity has been shown to be a limiting factor of acceptability for most fatty fish species under frozen storage conditions.[4,7] Lipid oxidation is recognised as a complex process where different kinds of molecules are produced, most of them unstable and thus breaking down into molecules with lower molecular weights or reacting with other compounds.[23,24] Among them, secondary lipid oxidation compounds (namely, carbonyls) are known to be the most closely related to the rancid odour and taste development.[25,26]

In a related study,[15] individuals fed on diet II showed a greater rancidity stability (conjugated diene content, peroxide and anisidine values, thiobarbituric acid index, fluorescence and browning development) and polyunsaturated fatty acid retention (polyene index assessment) when compared with their counterparts from the diet I. Comparison of chemical and sensory indices related to lipid oxidation development showed that rancid odour and taste obtained their best correlation values when compared with the thiobarbituric acid index ($r = 0.87–0.75$ and $0.95–0.71$, respectively, $P < 0.05$).

The antioxidant content in salmon muscle was also analysed in a parallel study.[27] In it, BHT and EQ contents in fish muscle were found in all cases below 0.01 and 0.03 mg kg^{-1} concentration, respectively. Additionally, feeding on diet II led to frozen samples showing higher contents of γ- and δ-tocopherol when compared with their counterparts previously fed on diet I; however, no effect on α-tocopherol presence was implied. Further, a marked loss of α-tocopherol content was observed with frozen time for all kinds of samples, in agreement with the general rancidity development

Table 1. Detection* of putrid odour, putrid taste and colour in frozen Coho salmon that was previously fed on different diets**

Frozen storage time (months)	Putrid odour			Putrid taste			Colour (Roche card)		
	Diet I	Diet II	Diet III	Diet I	Diet II	Diet III	Diet I	Diet II	Diet III
0	0.0^{a} (0.0)	0.0^{a} (0.0)	0.0^{a} (0.0)	0.0^{a} (0.0)	0.0^{a} (0.0)	0.0^{a} (0.0)	26.2^{bc} (1.2)	26.5^{d} (0.7)	27.1^{cd} (1.0)
3	0.0^{a} (0.0)	0.0^{a} (0.0)	0.0^{a} (0.0)	0.0^{a} (0.0)	0.0^{a} (0.0)	0.0^{a} (0.0)	26.8^{c} (0.7)	26.3^{d} (0.5)	27.7^{d} (0.7)
6	0.0^{a} (0.0)	0.1^{ab} (0.1)	0.1^{ab} (0.1)	0.1^{ab} (0.1)	0.0^{a} (0.0)	0.0^{a} (0.0)	26.6^{c} (0.8)	25.9^{cd} (0.7)	25.9^{bc} (0.7)
9	$^{z}0.0^{a}$ (0.0)	$^{y}0.2^{b}$ (0.1)	$^{z}0.0^{a}$ (0.0)	$^{y}0.2^{bc}$ (0.1)	$^{y}0.3^{b}$ (0.2)	$^{z}0.0^{a}$ (0.0)	24.4^{a} (0.5)	24.0^{a} (0.6)	24.8^{ab} (0.8)
12	$^{zy}0.1^{a}$ (0.1)	$^{z}0.1^{b}$ (0.0)	$^{y}0.2^{b}$ (0.1)	$^{z}0.0^{a}$ (0.0)	$^{zy}0.1^{ab}$ (0.1)	$^{y}0.3^{b}$ (0.1)	25.2^{ab} (0.4)	25.2^{bc} (0.4)	24.2^{ab} (1.2)
18	$^{x}1.0^{b}$ (0.1)	$^{z}0.2^{b}$ (0.1)	$^{y}0.4^{c}$ (0.1)	$^{y}0.3^{c}$ (0.1)	$^{z}0.1^{ab}$ (0.1)	$^{y}0.4^{b}$ (0.1)	24.3^{a} (0.5)	24.3^{ab} (0.8)	24.0^{a} (0.7)

*Mean values of five replicates ($n = 5$); standard deviations are included in brackets. For each descriptor and for each diet, mean values followed by different letters (a–d) denote significant differences ($P < 0.05$) as a result of the frozen storage time. For each descriptor and for each frozen storage time, mean values preceded by different letters (z, y, x) indicate significant differences ($P < 0.05$) among diets. No letters are expressed in cases where no significant differences ($P > 0.05$) were found.
**Diets supplied as expressed in Fig. 2.

observed throughout the frozen storage in sensory and chemical parameters.

Determination of colour changes

The Roche card employment (Table 1) showed a decreasing tendency for pink colour intensity with storage time in all kinds of individuals ($r = 0.91–0.80$; linear fitting, $P < 0.05$); this result agrees with previous research carried out on Atlantic salmon (*Salmo salar*) frozen at −10 and −20 °C for a 34-week period.[26] However, no differences could be assessed in the present study among individuals from the different diets, so that an effect of the antioxidant composition in diet on the pink colour intensity in thawed fish could not be proved.

Colour plays an important role in the appearance presentation and acceptability of seafood, especially in those related to salmonid species. Astaxanthin (AX) is well known as the main pigment responsible for the pink colour of salmonid fish species and could deteriorate either due to non-enzymatic or to enzymatic pathways.[28] Thus, previous studies have shown that AX content decreases during the frozen storage of Atlantic salmon (*Salmo salar*)[26] and rainbow trout (*Oncorhynchus mykiss*).[29] However, and in agreement with the present results, related research concerning colour analysis (CIE 1976 L^*, a^*, b^*; lightness, greenish/reddish and bluish/yellowish values, respectively) in Coho salmon[27] showed no effect on AX content and a^* and b^* colour parameters during the frozen storage of Coho salmon corresponding to the three diets under study, while a general L^* value increase was implied. Additionally, no effect of the antioxidant profile could be depicted on AX content and colour (L^*, a^* and b^*) parameters.[27] This AX content retention and accordingly, colour properties retention, may be explained on the basis of the protection exerted by the natural and synthetic antioxidants present in the different diets.[15]

Texture parameters assessment

Firmness assessment (Table 2) has shown a general increase with storage time throughout the 0–9 month period, followed by a score decrease at month 12 and a firmness increase at the end of the experiment. Concerning comparison among the different kinds of samples, some significant differences could be observed; however, a definite pattern related to the effect of the antioxidants profile in diet on firmness value could not be assumed.

Cohesivity analysis (Table 2) showed in most cases the highest mean values in month-9 samples. After that time, a general cohesivity decrease could be observed. Throughout the frozen storage, some differences were obtained among individuals from the different diets; thus, a lower mean value was obtained at the end of the experiment for samples previously fed on the diet enriched with synthetic antioxidants (diet I). However, a definite trend concerning the effect on the cohesivity score of the antioxidants profile included in the diet could not be proved.

Elasticity was evaluated by sensory procedure (Table 2), according to the muscle recovery after exerting a short pressure. A marked elasticity loss could be observed in all kinds of samples with frozen storage time ($r = 0.90–0.79$; quadratic fitting, $P < 0.05$), this leading to values included in the 4–5 range for individuals fed on diet III and diet I at month 9 and at months 12 and 18, respectively. Comparison among diets did not lead to a definite effect on elasticity values during the frozen storage period.

Texture is considered to be one of the most important quality attributes of seafood, which often determines consumer acceptance and hence, the marketability of such products. Protein denaturation has widely been described to be produced during the frozen storage of fish.[1,2] Different hypotheses have been proposed to explain the denaturation development that finally leads to a drier and firmer muscle texture. The connective tissue holding the cells together is reported to be degraded as muscle damage increases and blocks of cells become readily separated from each other, so that lower cohesivity and water holding capacity properties would be observed in muscle; additionally, elasticity decrease has been explained as a result of myofibrillar protein denaturation and aggregation.[3,30]

In the case of fatty fish, the role of fat in the texture changes has been found to be important due to cross-linking of peptide chains by reaction with lipid oxidation compounds produced during processing and storage[1,24] and leading to an increased firmness and decreased water holding capacity.[31] In agreement with present rancid odour and taste values, a parallel study[15] showed that frozen Coho salmon muscle acquired a higher rancidity stability if previously fed on diet II when compared with its counterpart fed on diet I. Present results have shown that, in spite of this lipid stability advantage, no definite effect on texture properties could be outlined in fish corresponding to diet II.

Determination of water loss

In all three quality parameters tested (Table 3), increasing water loss values with frozen storage time could be observed for all

Table 2. Comparative analysis of texture (firmness and cohesivity) and elasticity values* measured in frozen Coho salmon that was previously fed on different diets**

Frozen storage time (months)	Firmness (N)			Cohesivity (mm)			Elasticity		
	Diet I	Diet II	Diet III	Diet I	Diet II	Diet III	Diet I	Diet II	Diet III
0	$^{z}17.7^{a}$ (3.3)	$^{z}15.2^{a}$ (1.4)	$^{y}27.3^{ab}$ (4.2)	$^{y}55.3^{c}$ (0.1)	$^{y}55.8^{c}$ (0.6)	$^{z}53.6^{b}$ (0.1)	7.5^{c} (0.3)	7.6^{c} (0.3)	7.7^{e} (0.2)
3	$^{z}23.3^{bc}$ (2.3)	$^{y}32.0^{cd}$ (6.0)	$^{z}19.6^{a}$ (5.6)	$^{z}51.9^{b}$ (0.9)	$^{zy}53.0^{b}$ (2.0)	$^{y}54.8^{c}$ (0.2)	$^{y}7.5^{c}$ (0.2)	$^{y}7.5^{c}$ (0.1)	$^{z}7.0^{d}$ (0.2)
6	$^{z}25.8^{c}$ (6.2)	$^{y}30.8^{c}$ (2.3)	$^{y}33.5^{c}$ (0.9)	55.2^{c} (0.1)	55.2^{c} (0.2)	54.1^{bc} (2.1)	$^{zy}6.3^{b}$ (0.5)	$^{z}6.0^{ab}$ (0.2)	$^{y}6.9^{d}$ (0.2)
9	45.9^{d} (5.6)	43.3^{de} (6.0)	38.2^{c} (5.4)	$^{y}56.6^{d}$ (0.1)	$^{z}55.4^{c}$ (0.0)	$^{zy}55.8^{c}$ (1.2)	$^{y}6.0^{b}$ (0.3)	$^{y}6.4^{b}$ (0.3)	$^{z}4.5^{a}$ (0.3)
12	24.1^{c} (6.5)	25.5^{bc} (4.9)	26.5^{ab} (4.1)	53.8^{bc} (1.5)	54.3^{bc} (0.7)	54.8^{c} (0.2)	$^{z}4.6^{a}$ (0.4)	$^{y}5.8^{ab}$ (0.5)	$^{y}6.3^{c}$ (0.3)
18	41.4^{d} (6.2)	46.6^{e} (4.7)	39.5^{c} (9.4)	$^{z}44.4^{a}$ (2.1)	$^{y}48.0^{a}$ (0.0)	$^{zy}47.2^{a}$ (1.5)	4.8^{a} (0.2)	5.4^{a} (0.5)	5.1^{ab} (0.3)

*Mean values of five replicates ($n = 5$); standard deviations are included in brackets.
For each parameter and for each diet, mean values followed by different letters (a–e) denote significant differences ($P < 0.05$) as a result of the frozen storage time. For each parameter and for each frozen storage time, mean values preceded by different letters (z, y) indicate significant differences ($P < 0.05$) among diets employed. No letters are expressed in cases where no significant differences ($P > 0.05$) were found.
**Diets supplied as expressed in Fig. 2.

Table 3. Sensory (fish muscle dehydration) and physical (whole fish dripping and expressible moisture of fish muscle) assessment* of water loss in frozen Coho salmon that was previously fed on different diets**

Frozen storage time (months)	Fish muscle dehydration			Whole fish dripping (g kg^{-1} fish)			Expressible moisture of fish muscle (g kg^{-1} muscle)		
	Diet I	Diet II	Diet III	Diet I	Diet II	Diet III	Diet I	Diet II	Diet III
0	$^{x}1.5^{b}$ (0.1)	$^{y}1.2^{a}$ (0.1)	$^{z}0.9^{a}$ (0.1)	0.41^{a} (0.26)	0.53^{a} (0.26)	0.64^{a} (0.08)	$^{zy}2.23^{bc}$ (1.06)	$^{y}3.82^{ab}$ (1.61)	$^{z}1.03^{a}$ (0.25)
3	$^{z}1.1^{a}$ (0.1)	$^{y}1.4^{b}$ (0.1)	$^{y}1.4^{b}$ (0.1)	$^{z}0.48^{a}$ (0.06)	$^{x}1.18^{b}$ (0.12)	$^{y}0.68^{a}$ (0.10)	0.98^{a} (0.40)	2.56^{a} (1.15)	2.71^{b} (1.18)
6	1.5^{b} (0.1)	1.6^{b} (0.2)	1.5^{ab} (0.7)	$^{y}1.74^{b}$ (0.07)	$^{x}2.35^{c}$ (0.31)	$^{z}1.47^{b}$ (0.13)	3.94^{cd} (1.42)	5.03^{ab} (1.79)	5.27^{c} (1.18)
9	$^{z}1.8^{c}$ (0.2)	$^{y}3.2^{c}$ (0.6)	$^{y}3.9^{d}$ (0.7)	$^{z}1.96^{b}$ (0.37)	$^{z}1.98^{c}$ (0.27)	$^{y}2.97^{d}$ (0.25)	$^{y}4.54^{de}$ (0.91)	$^{y}5.16^{b}$ (0.53)	$^{z}2.71^{b}$ (0.69)
12	$^{z}2.7^{d}$ (0.2)	$^{y}3.2^{c}$ (0.1)	$^{yz}3.4^{cd}$ (0.7)	$^{y}3.60^{c}$ (0.38)	$^{y}3.29^{d}$ (0.28)	$^{z}2.43^{cd}$ (0.35)	3.59^{cd} (0.97)	4.54^{ab} (0.87)	5.74^{c} (1.47)
18	3.0^{d} (0.3)	3.1^{c} (0.8)	3.0^{c} (0.1)	$^{y}4.80^{d}$ (0.06)	$^{z}1.98^{c}$ (0.53)	$^{z}1.84^{bc}$ (0.27)	$^{y}7.00^{e}$ (1.56)	$^{z}4.69^{b}$ (0.69)	$^{zy}5.28^{c}$ (0.82)

*Mean values of five replicates ($n = 5$); standard deviations are included in brackets. For each quality index and for each diet, mean values followed by different letters (a–e) denote significant differences ($P < 0.05$) as a result of the frozen storage time. For each quality index and for each frozen storage time, mean values preceded by different letters (z, y, x) indicate significant differences ($P < 0.05$) among diets. No letters are expressed in cases where no significant differences ($P > 0.05$) were found.
**Diets supplied as expressed in Fig. 2.

kinds of samples. This general behaviour can be explained on the basis of an increasing protein denaturation, being this explained as a result of different factors such as partial protein dehydration, interaction with oxidised lipids, free fatty acids and formaldehyde and alteration of the protein micro-environment.[1,2]

Measurement of muscle dehydration showed some lower ($P < 0.05$) scores for individuals previously fed on the diet enriched with synthetic antioxidants (months 3 and 9); however, no differences ($P > 0.05$) could be detected at the end of the experiment. Concerning dripping and expressible moisture assessments, a definite trend about the effect of the antioxidant composition in the diet could not be concluded; however, lower mean values ($P < 0.05$) for both parameters were observed at month 18 in fish previously fed on diet II when compared with samples corresponding to diet I.

Microstructure analysis

Concerning frozen samples at time 0 (Fig. 4), striated muscle of diet I-fish consisted of an orderly structure of actin and myosin filaments. Thus, light bands (I-bands and dark A-bands) can be observed alternately arranged, while a thin dark line (Z-line or membrane of Krause) is found to bisect each I-band. A bright area (H-area or Hansen membrane) is located in the centre of the A-band, while a narrow dark line (M-line) runs through the centre of the H-band. Finally, the endomysium is observed to remain intact around each of the different fibres.

At that frozen time (month 0), the addition of a tocopherols-rich mixture in the diet of salmon (diet II) led to slight changes in the myofibrils morphology. Thus, sarcomeres showed well-marked areas for the I bands, A bands and Z line, while the H area showed a diffuse line of the Hansen membrane. The muscle fibres showed a slight shift and the Z line was observed somewhat misaligned. Concerning fish corresponding to diet III, no loss of alignment in the myofibrils morphology could be observed; thus, the sarcomeres network was composed of well-aligned and defined Z lines.

After month 9 of frozen storage (Fig. 4), fish belonging to diets I and III showed some displacement and misalignment of myofibrils, this including the Z-line, M-line and I-band; however, no loss of the sarcomeres basic unit in the striated muscle could be depicted. Misaligned sarcomeres were observed especially for fish corresponding to diet II; thus, discontinuities in the Z-line, I-band and A-band were produced as a result of myofibrils displacement, although no loss of the basic unit of the striated muscle was revealed.

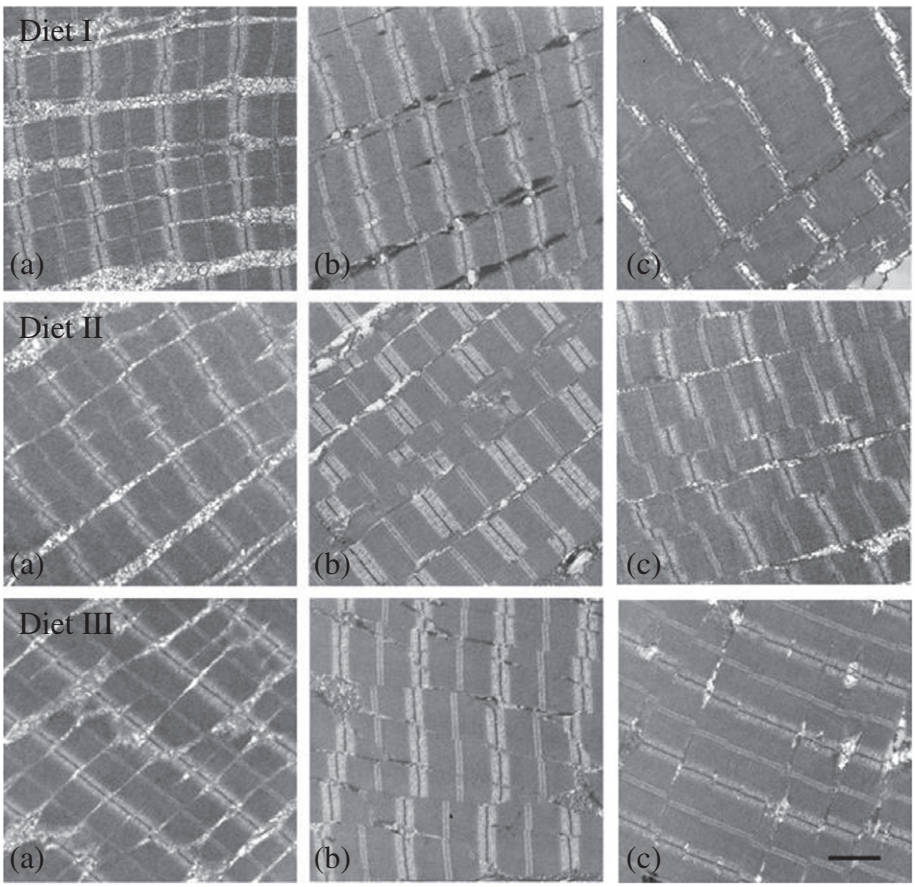

Figure 4. Evolution of the muscle structure of frozen (a: 0 months; b: 9 months; c: 18 months) Coho salmon muscle which was previously fed on different diets*. *Diets employed as expressed in Fig. 2. The bar indicates a 1 μm length.

At the end of the experiment (Fig. 4), marked changes related to the microstructure of the sarcomeres were observed in diet I-fish samples. Thus, the basic unit of the striated muscle did not present well-demarcated areas of the Z-line, A-band, and H-area. Moreover, I-band was observed misaligned. The disintegration of the Z-line has been consistently reported as a post-mortem phenomenon, being this fragmentation generally associated with calpain (Ca^{2+}-dependent protease) activity.[32] Meantime, the microstructure of the sarcomeres remained unchanged for Coho salmon muscle previously fed on diets II and III, this indicating that the addition of natural antioxidants does not affect this parameter until month 18 of storage. It is concluded that the typical characteristics of striated muscle and the sarcomeres morphology were influenced throughout the frozen storage by the antioxidants profile included in the previous diet so that a better preservation of frozen samples previously fed on diets II and III was proved at the end of the frozen storage.

CONCLUSIONS

After 18 months at −18 °C, fish previously fed on a conventional diet enriched with natural antioxidants (diets II and III) showed lower putrid and rancid odours and rancid taste scores, while higher mean typical odour and taste values were attained. Concerning physical parameters, dripping and expressible moisture values obtained at month 18 for diet II-fish were lower when compared with their counterparts belonging to the diet enriched with synthetic antioxidants (diet I). Additionally, microstructure analysis showed that the Z-lines integration was better preserved in fish corresponding to diets II and III after frozen storage for 18 months when compared with their corresponding samples belonging to diet I. Between diets enriched with natural antioxidants, a lower putrid and rancid development in odour and taste could be detected at the end of the experiment in salmon fed on diet including an excess of tocopherols (diet II).

According to the present results, the employment of diet II has been recognised as being the most profitable to be employed to maintain the sensory and physical properties of the frozen product when a long-term storage is considered. In agreement with the wide range of benefits attributed to natural antioxidants, further research is currently under development to optimise the natural antioxidants profile to be employed on each farmed species.

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