



Quality loss during the frozen storage of sea salmon (*Pseudoperca semifasciata*). Effect of rosemary (*Rosmarinus officinalis* L.) extract

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ABSTRACT

Lipid and protein alterations during the frozen storage ($-11\text{ }^{\circ}\text{C}$) were analyzed in minced sea salmon muscles to evaluate the effect of the application of rosemary extract (200 and 500 mg/kg). Lipid oxidation reached maximum TBA values between 3 and 4 months of storage in untreated muscles. The main polyunsaturated fatty acids affected were 22:6- ω 3, 22:5- ω 3 and 20:4- ω 6 acids. Phospholipid hydrolysis was also detected. Rosemary extract reduced lipid oxidation for 6 months (500 mg/kg, muscle with 10.8 g/kg lipids) or 3 months (200 mg/kg, muscle with 5.3 g/kg lipids). Myofibrillar proteins showed a decrease of extractability (80%) after 2 months of storage. Myosin denaturation was evident by DSC at 3 months, while myosin and actin peaks disappeared at 6 months. A diminution of extractable polypeptides of high molecular weight was recorded by SDS-PAGE after 3 months. The available lysine content suffered a reduction starting at 3 months of storage, suggesting some interaction involving the free amino groups of lysine. Fluorescent compounds' determination did not show changes due to the interaction of lipid oxidation products and proteins, while protein alterations could not be reduced by the rosemary extract. Furthermore, the antioxidant reduced the loss of red color in the muscle.

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1. Introduction

Freezing and frozen storage is a very important method of fish preservation. However, some deteriorative changes occur during the freezing, frozen storage and thawing of fish muscle, including modifications of flavor, odor, texture and color (Matsumoto, 1979). These alterations are dependent on the processing as well as on the chemical composition of the muscle. Protein denaturation – myofibrillar proteins specially – is an important factor of quality loss of frozen fish that can be produced by diverse factors (Badii & Howell, 2002; Shenouda, 1980). This process has been associated with texture changes such as “hardening” and with functional alterations such as liquid loss and changes in gelation properties (Awad, Powrie, & Fennema, 1969). Lipid hydrolysis and oxidation occur during the frozen storage of fish, even in those with low lipid content due to their high proportion of polyunsaturated fatty acids (PUFAs), and have an important influence on product acceptability (Aubourg, Rey-Mansilla, & Sotelo, 1999). One of the consequences of this process is the production of volatile compounds, inducing

flavor deterioration. In addition, primary – free radicals and hydroperoxides – and secondary – aldehydes, dialdehydes, epoxides and others – oxidation products can react with other cellular components such as proteins, peptides, free amino acids, phospholipids and nucleic acids, forming conjugated Schiff base type products which present high color intensity and characteristic fluorescence properties (Aubourg, 1999a; Frankel, 1998). The interaction of oxidized lipids with proteins would contribute to protein denaturation and can affect the nutritional and organoleptic quality of foods. A decrease in the biological value of proteins by the reaction with the ϵ -amine groups of lysine, the oxidation of methionine and other amino acids' changes, as well as modifications of flavor, color (browning), and texture due to the cross-linking of the polypeptide chains have been observed (Aubourg, 1999a; Pokorny, Rěblová, Kourimská, Pudil, & Kwiatkowska, 1993).

Antioxidants are widely used to prevent the negative consequences of lipid oxidation (Shahidi, 1997, chp 1). Since carcinogenic activity of synthetic antioxidants (BHT, BHA and TBHQ) has been demonstrated (Lindberg Madsen & Bertelsen, 1995; Wu, Lee, Ho, & Chang, 1982), natural antioxidants are being extensively studied, and spices and herbs have been identified as important sources of these compounds. Diverse studies have evaluated the antioxidant properties of the rosemary (*Rosmarinus officinalis* L.) (Lindberg

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Madsen and Bertelsen, 1995). This spice contains certain compounds such as carnosol and carnosic acid, with high antioxidant activity, and others with minor activity (Offord, Guillot, Aeschbach, Loliger, & Pfeifer, 1997, chp 6). These substances would act by a hydrogen atom – donating mechanism (Frankel, 1998). Capabilities of rosemary extracts in retarding oxidation of different fish oils have been informed (Bhale, Xu, Prinyawiwatkul, King, & Godber, 2007; Yoshioka, Yamada, & Wada, 2002). Szczepanik and Stodolnik (2003) demonstrated the ability of aqueous extracts of rosemary to prevent the β -carotene destruction in fresh and frozen muscles from different fish species. Although in the past a major obstacle to the use of natural plant extracts such as rosemary has been the imposition of undesirable flavours and odours, extracts with non-interfering sensory characteristics are produced at the present (McBride, Hogan, & Kerry, 2007). Some works have evaluated the effects of rosemary extracts on sensory acceptability. Addition of rosemary extract at concentrations of 1 and 2.5 g/kg to beef burgers did not affect the perception of warmed over flavor, but it was affected by concentrations of 10 g/kg. In addition, this concentration resulted in lower preference scores (McBride et al., 2007). In other work, Akarpat, Turhan, and Ustun (2008) demonstrated that beef patties treated with rosemary extract presented high scores of overall acceptability (color, taste and odor).

Since sea salmon is an appreciated and well valued fishing source, the analysis of the quality loss during its frozen storage is proposed in this work, focusing in deteriorative aspects related to the lipid and protein alterations. In addition, the effect of the application of rosemary extract as antioxidant was evaluated. An analysis of the possible relationship between protein alterations and lipid oxidation evolution was approached.

2. Materials and methods

2.1. Materials

Sea salmon (*Pseudoperca semifasciata*) was caught by commercial vessels in the southwest Argentinean sea. Bovine albumin, trinitrobenzene sulfonic acid (TNBS) and electrophoretic grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of analytical grade.

2.2. Samples

Fish was kept in ice up to arrival to the laboratory (about 48 h). Fishes were cleaned, skinned and filleted, fillets were washed by water immersion. Minced muscle was prepared using a food processor. Two storage experiments were performed involving two different batches of muscle: *muscle A*: obtained from a fish of 11 kg captured in spring; and *muscle B*: obtained from fishes weighing between 2.2 and 3.5 kg captured in winter. A representative portion of each minced muscle was treated with *Guardian* Rosemary extract 09 (DANISCO, Copenhagen, Denmark), which was applied by spraying (treated sample, T) with a subsequent homogenization step. The antioxidant was applied as an aqueous dispersion (1/10 wt/wt). Two concentrations of rosemary extract in fish muscle were evaluated: 200 mg/kg (T200) in muscle B, and 500 mg/kg (T500) in muscle A (final concentrations in meat). Samples (30–50 g) of minced fillets treated (T200 and T500) or untreated (UT) with rosemary extract were packed in low density polyethylene bags (100 μ m) with high oxygen permeability.

2.3. Freezing process and frozen storage

Packaged samples were placed in a plate freezer (Mini Freezer, White Martin, Brazil) using liquid nitrogen as cryogenic fluid.

Temperature was set at -30°C . When the center of samples reached -18°C , they were removed from the freezer. Samples were stored during 9 months in a freezing room, monitoring its temperature each hour by a button data logger temperature. The average temperature value during the overall period of storage was $-11 \pm 2^{\circ}\text{C}$. Sample thawing at different times of storage was performed in a cold room at 4°C during 12–14 h. Two samples corresponding to each treatment were used for analytical measurements at each sampling time.

2.4. Proximal composition of minced muscles

Protein (Kjeldahl method) and ash content (550°C) were measured by duplicate. Total lipid content (Bligh & Dyer, 1959) and moisture (drying at 105°C – 24 h) were determined by triplicate.

2.5. Lipid fraction composition

Analysis of lipid fraction was performed as was previously described in Tironi, Tomás, and Añón (2007). Briefly, triglyceride (TG) and phospholipid (PL) fractions were separated from the total lipid extract by preparative thin layer chromatography using a hexane:ethyl ether:acetic acid (80:20:2) solvent system. Methyl esters were prepared and purified on silica gel G plates using hexane:ether (95:5); spots were located under UV light after spraying with dichlorofluorescein, and recovered into hexane after thoroughly mixing the silica support with methanol:water:hexane (1:1:1). A Varian 2700 gas chromatograph connected to a Star Chromatography Workstation (version 4.51) was used for fatty acid analysis. The GC instrument was equipped with two (2 mm \times 2 m) glass columns packed with 15% SP 2330 on Chromosorb WAW 100/120 (Supelco Inc., CA, USA) and connected to two flame ionization detectors operated in the dual-differential mode. Injector and detector temperatures were 220°C and 230°C , respectively, and N_2 (30 ml/min) was the carrier gas. The column oven temperature was programmed from 150°C to 230°C at a rate of $5^{\circ}\text{C}/\text{min}$. The fatty acid methyl esters were identified according to the retention times by comparison with commercially available standards (Sigma-Aldrich, MO, USA).

2.6. 2-Thiobarbituric acid assay (TBA number)

TBA determinations were performed on TCA extracts from 2 g of minced muscle according to Tironi et al. (2007). Two independent extracts were obtained for each sample and subjected to the TBA reaction (30 min, 70°C) (Botsoglou et al., 1994) in duplicate. TBA number was calculated as follows:

$$\text{TBA number (mg MDA/kg muscle)} = \frac{\text{Abs} \times \text{M} \times \text{Va} \times \text{Ve} \times 1000}{\varepsilon \times l \times m}$$

where MDA: malonaldehyde; Abs: absorbance at 532 nm; M: molecular weight of MDA (72 g); Va: sample volume (2 ml); Ve: extract volume (16 ml); ε : molar extinction coefficient ($1.56 \times 10^5 \text{ M}^{-1}$); l: optical path (1 cm); m: muscle weight.

2.7. Determination of fluorescent compounds

Fluorescent compounds' determinations were carried out in a Perkin Elmer LS 50 B spectrofluorometer driven by an FLWinLab software, on the organic and aqueous extracts obtained from 5 g of sea salmon muscle. Emission spectra at each excitation maxima (325 and 388 nm) previously determined for sea salmon muscle (Tironi et al., 2007) were recorded as a function of the frozen storage time,

measuring fluorescence intensity F at the emission maximum. Relative fluorescence RF was calculated according to $RF = (F \times V) / (F_{st} \times m)$, where F : sample fluorescence intensity; V : extract volume; F_{st} : standard fluorescence intensity (350 nm/450 nm); and m : sample mass (Aubourg, Pérez-Martín, Medina, & Gallardo, 1992). A quinine sulfate solution (1 µg/ml in 0.05 mol/l H_2SO_4) was used as standard. Wavelength shifts in both phases were calculated as $\delta F = RF_{388/460} / RF_{325/412}$, and the shift between the phases was obtained as $\delta Forg / \delta Faq$. Three independent extractions were carried out from each sample.

2.8. Differential scanning calorimetry (DSC)

Differential scanning calorimetric studies were performed in a Polymer Laboratories calorimeter (Rheometric Scientific Ltd.) with a Plus V 5.41 software. The equipment was calibrated at a heating rate of 10 °C/min using indium as standard. Samples (18–22 mg wet weight) were placed in DSC hermetic pans and scanned from 15 to 100 °C at a $\beta = 10$ °C/min by triplicate. As reference, a capsule with distilled water was used. Denaturation enthalpies (ΔH_{myosin} , ΔH_{actin} and ΔH_{total}) were estimated by measuring the corresponding areas under the DSC transition curve. Specific areas (partial area/total mg of dry sample) were calculated.

2.9. Muscle protein extractability

Minced muscle (2.5 g) was homogenized with 50 ml 0.03 mol/l Tris-HCl, 0.6 mol/l KCl, pH = 7.0 buffer solution. After centrifugation (1500 g, 30 min, 4 °C) the supernatant containing the total extractable proteins (TEP) was separated. Extractable myofibrillar proteins (MEP) fractions were obtained from 10 ml – or 20 ml in the case of stored samples due to their low protein concentration – TEP extract which was mixed with 90 ml of distilled water and stored at 4 °C (16–24 h). Then, the mixture was centrifuged (2100 g, 30 min, 4 °C) and the supernatant was removed. The pellet containing the myofibrillar proteins was suspended with 5 ml – or 2 ml in the case of stored samples – of 0.03 mol/l Tris-HCl, 0.6 mol/l KCl (pH = 7) solution. Protein concentration in the extracts (2 extracts/sample) was determined by duplicate by a modified biuret method (Robson, Goll, & Temple, 1968).

2.10. Electrophoresis of extractable fractions

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble fractions was performed. Samples were treated with a pH 9.0 buffer containing 8 mol/l urea, 3 g/l SDS and 0.5 g/l 2-mercaptoethanol (2-ME), and a similar protein mass (30 µg) was loaded onto each lane. Slab SDS-PAGE was carried out by the Laemmli discontinuous buffer system (Laemmli, 1970) in a Mini Protean II Dual Slab Cell (BIO-RAD). A 30–150 mg/g gradient resolving gel was used. Gels were stained with Coomassie Brilliant Blue R-250 (2 g/l) and analyzed with a Molecular Analyst Software (BIO-RAD).

2.11. Transmission electron microscopy (TEM)

MEP fractions were adjusted to a protein concentration of 0.2 mg/ml. Two hundred mesh grids with a formvar film were placed in a sample drop for 3 min. Then, the grids were placed in a drop of saturated solution of uranyl acetate for 1 min. They were dried and examined with a JEOL 100 CX II transmission electron microscope operated at 80 kV.

2.12. Determination of available lysine

Available lysine was determined on a protein pellet obtained from the muscle, as was previously described (Tironi et al., 2007). Briefly, the minced muscle was treated with absolute ethanol and after centrifugation (1500 g, 10 min, 4 °C), acetone was added to the pellet and separated by centrifugation (3 times). A measured sample of pellet was treated with methanol, and reacted with TNBS in 0.5 mol/l borate, pH = 9.2 buffer, shaking during 1 h at room temperature. Reagent blank tubes RB (without sample) and blank tube B were also prepared. After agitation, saturated solution of glycine was added to all tubes except the B one (replacing by water). After agitation, proteins were precipitated with 100 g/l trichloroacetic acid (TCA) and centrifugated (1500 g, 10 min, 15 °C). Absorbance at 345 nm was determined in the supernatant (dilution 1/20) and available lysine content was calculated as follow:

$$\% \text{ lysine (g/100g pellet)} = \frac{(RB - S) \times m_{TNBS} \times M_{lysine} \times 100}{RB \times M_{TNBS} \times m_p}$$

where RB = reactive blank tube absorbance, S = sample tube absorbance, m_{TNBS} = mass of TNBS (4 mg), M_{lysine} = molecular weight of lysine, M_{TNBS} = molecular weight of TNBS, m_p = mass of pellet. The content of available lysine respect to the total protein content in the pellet (by Kjeldahl) was determined.

2.13. Color determination

Color parameters on the CIELAB system a^* , b^* and L^* were determined using a Minolta CR300 colorimeter (NJ, USA). For each sample, five measures were performed on different locations, taking two samples for each time-rosemary extract concentration condition.

2.14. Statistical analysis

Data were analyzed by means of the analysis of variance (ANOVA) according to the General Linear Model Procedure. When differences were significant ($p < 0.05$) mean values were evaluated by Least Significant Differences (LSD) by the Fisher test using a SYSTAT statistical package (Wilkinson, 1990).

3. Results and discussion

3.1. Proximal composition of the sea salmon muscle

Main components of sea salmon muscles (A and B) were determined. The results showed a protein content of 18.85 ± 0.21 g/100 g for muscle A and 17.45 ± 0.15 g/100 g for muscle B. Moisture was 79.75 ± 0.04 g/100 g and 80.55 ± 0.42 g/100 g, while ash content was 1.28 ± 0.08 g/100 g and 1.21 ± 0.05 g/100 g, for muscles A and B, respectively. Lipid content presented very different values among the muscles: 1.08 ± 0.15 g/100 g for muscle A and 0.53 ± 0.08 g/100 g for muscle B.

3.2. Lipid oxidation analysis

The progression of lipid oxidation secondary products – malonaldehyde and others – during the frozen storage was evaluated by the TBA number determination.

3.2.1. UT muscles

Fig. 1 shows the results obtained for muscles of different lipid content: muscle A (Fig. 1a) and muscle B (Fig. 1b). During the first

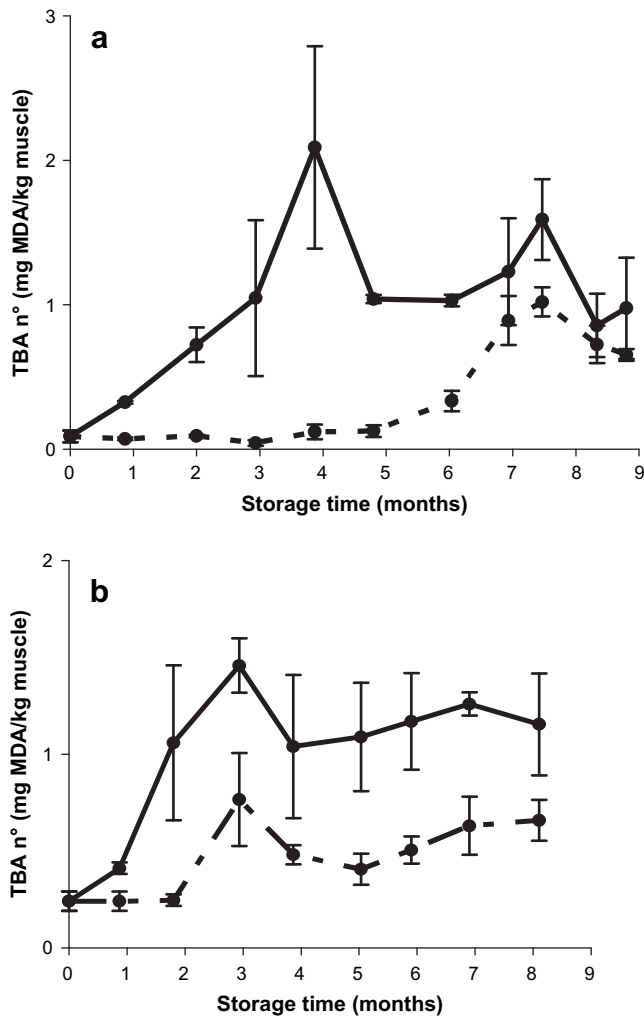


Fig. 1. TBA number as a function of the frozen storage time of sea salmon muscles with different lipid content, in the absence (UT) or presence (T) of rosemary extract: a) 10.8 g/kg of lipids, – UT, – T500; b) 5.3 g/kg of lipids, – UT, – T200. Measures were performed on two independent extracts by duplicate each one.

months of storage, both muscles presented a progressive increase of the TBA number until a maximum was reached around 4 months for the muscle with the highest lipid content (TBA number = 2.1 ± 0.7), and at 3 months for muscle with the lowest lipid content (TBA number = 1.5 ± 0.1). As can be observed in the figures, TBA values presented an important dispersion at each storage time, suggesting that the lipid oxidation process occurred in an inhomogeneous fashion in the samples. These results are in line with those reported for other non fatty fish species. Determinations performed in different regions of hake (*Merluccius merluccius*) muscle (0.5–1% of lipids) showed a maximum TBA number at 6 months of storage at -11°C . Although measurements were carried out on separated zones of muscle, values presented important dispersions as in the present work (Aubourg et al., 1999). In the case of blue whiting (*Micromesistius poutassou*) muscle (lipid content: 0.43–0.60%) stored at -10°C , the TBA number presented a maximum at 5 months (Aubourg, 1999b). A similar behavior was observed in cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) (lipid contents: 0.45–0.65%) (Aubourg & Medina, 1999). The highest TBA number reached during the frozen storage of sea salmon muscle was comparable to those recorded during the chilled storage (Tironi et al., 2007).

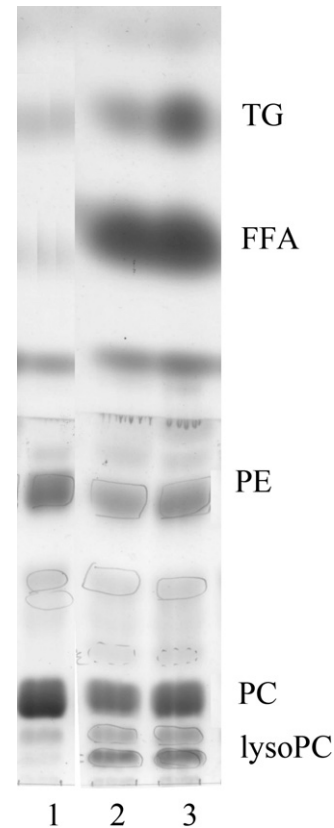


Fig. 2. TLC of the total lipid fraction from minced sea salmon muscle (5.3 g/kg lipids) as a function of the frozen storage time and the addition of rosemary extract: 1) initial time, 2) UT, 4 months, and 3) T200, 4 months.

3.2.2. T muscles

As shown in Fig. 1a the application of 500 mg/kg of rosemary extract to muscle A (T500) produced a very important reduction in the development of lipid oxidation secondary products. In T500 samples the TBA number did not change along the first 6 months of storage. A significant increase ($p < 0.05$) was recorded at 7 months, and the highest value (about 1) was reached at 7.5 months. This value is about a 50% of the maximal value reached by the UT sample (at 4 months). The addition of 200 mg/kg of rosemary extract to muscle B (T200) prevented lipid oxidation during the first 2 months, but a significant increase was observed at 3 months ($p < 0.05$) (Fig. 1b). No additional changes in the TBA number were recorded after this time of storage. As in the case of T500, the maximal TBA level reached by T200 was almost a half of that recorded in the untreated sample. However, this level was reached by both samples – UT and T200 – at the same time.

Results showed that rosemary extract in a concentration of 200 mg/kg (T200) was able to reduce by a half the production of oxidized compounds. However, a concentration of 500 mg/kg can delay the development of lipid oxidation for some months in addition to a 50% of reduction of the maximal amount of lipid oxidation products detected.

3.3. Changes in the lipid fraction

Modifications in the lipid fractions of UT and T200 muscles were analyzed after 4 months of storage, when both samples had reached the maximum level of lipid oxidation. Different lipid fractions – polar and neutral lipids – were separated by TLC.

Table 1

Fatty acids' content of sea salmon muscle as a function of the frozen (-11°C) storage time and the addition of rosemary extract (UT and T200 muscles).

Fatty acid (g/100 g of total fatty acids)		Storage time (months)		
		0	4	
			UT	T200
Saturated		25.8 ^a	30.7 ^b	27.8 ^a
Monounsaturated		17.4 ^a	20.1 ^b	17.4 ^a
ω 3-PUFAs		44.3 ^a	38.3 ^b	43.5 ^a
	Total			
	18:3	1.0 ^a	2.3 ^c	1.5 ^b
	20:5	7.5 ^a	3.5 ^c	4.1 ^b
	22:5	1.8 ^a	1.3 ^b	1.6 ^a
	22:6	33.9 ^a	26.1 ^c	36.2 ^b
ω 6-PUFAs		11.3 ^a	9.6 ^b	9.9 ^b
	Total			
	18:2	1.2 ^a	1.3 ^a	1.4 ^a
	20:4	5.8 ^a	3.8 ^b	3.8 ^b
	22:5	4.3 ^a	4.1 ^a	4.7 ^a

Each value is represented as the mean of two determinations. Different letters (a,b) in the same line indicate significant differences ($p < 0.05$).

3.3.1. UT muscles

After 4 months of storage at -11°C , an important increase in the amount of free fatty acids (FFA), a decrease of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), as well as the appearance of a lysophosphatidylcholine (lysoPC) band were observed (Fig. 2). These results indicate a marked hydrolysis of phospholipids during the frozen storage. Lipolysis has been largely detected in frozen muscles, affecting the phospholipid fraction in some cases and phospholipids and neutral lipids in others (Aubourg, 1999b; Aubourg et al., 1999). The presence of free fatty acids in the frozen muscle has been associated with a reduced acceptability because of both its interaction with proteins causing texture modifications (Shenouda, 1980; Aubourg, 1999b), and its relationship with lipid oxidation (Hand & Liston, 1988). Changes in fatty acids composition

were evaluated by gas chromatography. After 4 months of frozen storage, a decrease of the ω 3-polyunsaturated fatty acids (PUFAs) was recorded (about 6% respect to the total fatty acids) (Table 1). This fact could be related to the oxidation of these compounds. This limited decrease of the ω 3-PUFAs suggested that the oxidation affects mainly the TG fraction, similar to that found during the chilled storage of sea salmon in a previous work (Tironi et al., 2007). Saturated and monounsaturated acids presented a relative increase due to the decrease of the ω 3 acids previously mentioned. However, this trend was not observed for the ω 6-PUFAs, suggesting that the oxidation process also affected this type of fatty acids. Changes in the composition of the ω 3 and ω 6 fractions were also analyzed. In UT samples, 22:6 ω 3-acid (DHA) content showed an important diminution (about 8%) (Table 1). The 20:5 ω 3 and 22:5 ω 3 acids were also reduced (4 and 0.5%, respectively), suggesting that all these fatty acids could be involved in the oxidation process. In the case of the ω 6-fraction, the 20:4 (arachidonic) acid content decreased about 2% (Table 1). Since the arachidonic acid is mainly present in the phospholipid fraction, its diminution may be related to the hydrolysis process, which would release this acid and make it susceptible to oxidation. During the chilled storage of sea salmon no changes in this fatty acid were detected due to the less extension of the hydrolysis (Tironi et al., 2007).

3.3.2. T muscles

Similarly to UT samples, T200 presented phospholipid hydrolysis (Fig. 2). The most important TLC difference between UT and T200 samples at 4 months of storage was the intensity of the TG band, which was diminished in the UT samples (Fig. 2). This fact could be attributed to the occurrence of lipid oxidation on the TG fraction, decreasing its content. T200 samples presented a smaller reduction of the ω 3 fraction (only about 1%) than the UT sample (about a 6%). The DHA content did not change after 4 months of storage, although the 20:5 ω 3 acid showed a decreased amount (Table 1). Regarding the ω 6-fraction, the modifications observed in

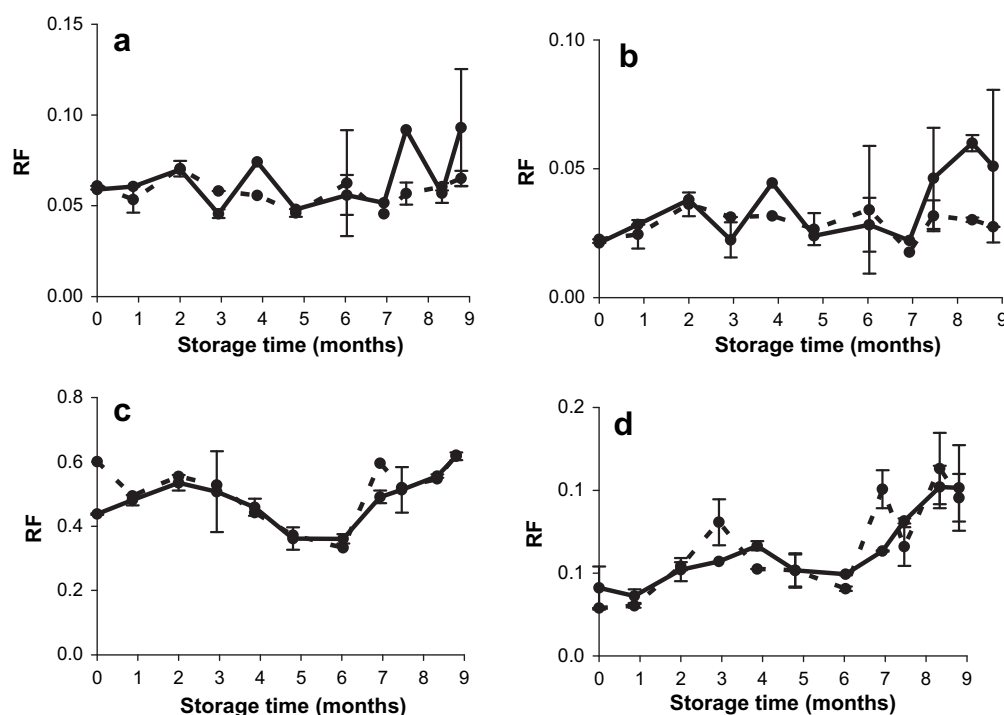


Fig. 3. Relative fluorescence (RF) as a function of the frozen (-11°C) storage time in sea salmon muscle (10.8 g/kg lipids): UT —, and T500 ----. a) Organic phase, 325/412 nm; b) organic phase, 388/460 nm; c) aqueous phase, 325/412 nm; d) aqueous phase, 388/460 nm. Measures were performed on three independent extracts.

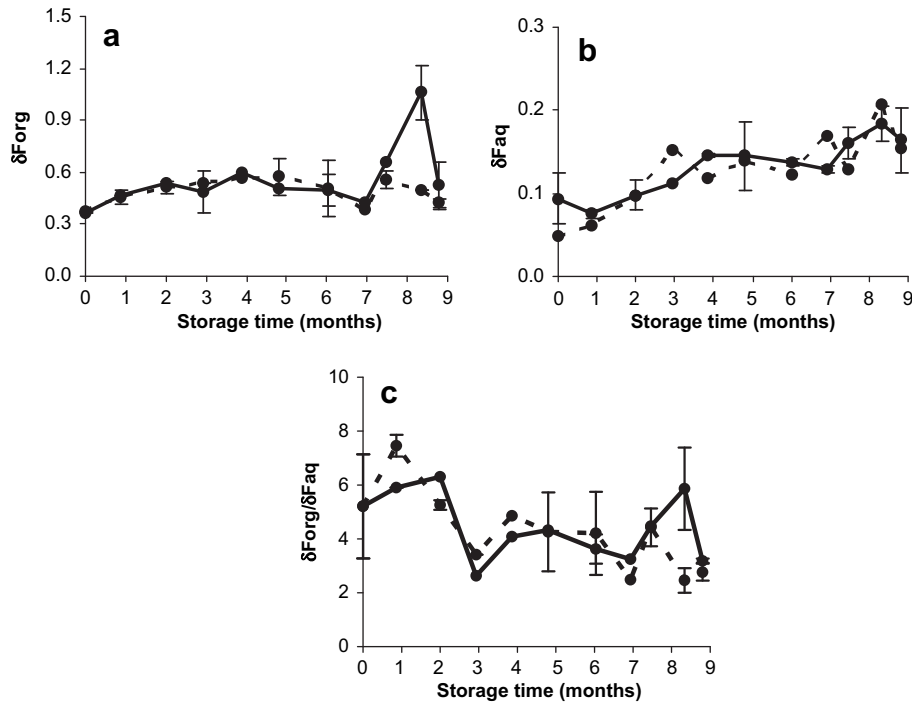


Fig. 4. Wavelength shift $\delta F = RF_{388/460}/RF_{325/412}$: a) in the organic phase δF_{org} , and b) in the aqueous phase δF_{aq} ; and phase shift $\delta F_{org}/\delta F_{aq}$ (c), as a function of the frozen (-11°C) storage time of sea salmon muscle (10.8 g/kg lipids): UT —, and T500 ----. Measures were performed on three independent extracts.

T200 samples were similar to those found in the UT system (decreasing 20:4 acid content).

Results showed that rosemary extract (200 mg/kg – T200) was able to inhibit the oxidation of the 22:6 ω 3-PUFA, which is the main acid involved in this process. However, this type of antioxidant could not protect other fatty acids (22:5 ω 3 and 20:4 ω 6). Thus, their oxidation could be responsible of the increasing TBA number in T200 samples after 4 months of storage.

3.4. Fluorescent compounds

3.4.1. UT muscles

The organic phase showed significant increments ($p < 0.05$) in the fluorescence at 325/412 at 7.5 and 9 months for the muscle with the highest lipid content (Fig. 3a). In the aqueous phase, fluorescence at 325/412 showed a diminution at 5 months in both cases (muscles A and B), increasing again at 7.5 months in the muscle with 1.08% of lipids (Fig. 3c); while fluorescence at 388/460 increased significantly ($p < 0.05$) in this muscle at the end of the storage (Fig. 3d). The wavelength shift of the fluorescence compounds – represented by δF_{org} and δF_{aq} – as well as the phase shift – $\delta F_{org}/\delta F_{aq}$ – have demonstrated a good correlation with the progression of the oxidative damage (Aubourg, 1999a). δF_{org} presented a progressive increase as a function of the frozen storage time, being statistically significant at 2 months ($p < 0.05$) for the muscle with the lower lipid content (data not shown) and at 7.5 and 8.5 months in the other case (Fig. 4a). δF_{aq} showed also a trend to increase during the frozen storage, with significant differences ($p < 0.05$) at 4 months for the muscle with 1.08% of lipids (Fig. 4b), and at 7 months for the muscle with 0.53% of lipids (data not shown). $\delta F_{org}/\delta F_{aq}$ values did not present a definite trend as a function of the frozen storage time in any case (Fig. 4c). Results are in agreement with those informed by other authors. During the frozen storage (-10°C) of blue whiting (*M. poutassou*) increments in both δF_{org} – beginning at 5 months, being maximal

at 7 months – and δF_{aq} – beginning at 7 months, being maximal at 9 months – were detected (Aubourg, 1999b). In the same way, on haddock muscle (*M. aeglefinus*), δF_{org} increased at 7 months, being maximal at 9 months, and δF_{aq} increased at 9 months; while in the case of cod (*G. morhua*), δF_{org} raised at 7 months, decreasing thereafter, and δF_{aq} began to increase at 7 months (Aubourg et al., 1999). Conversely, in fatty species such as *Sardina pilchardus*, δF_{org} and δF_{aq} presented a significant increase at very short storage times (10 and 60 days, respectively), in

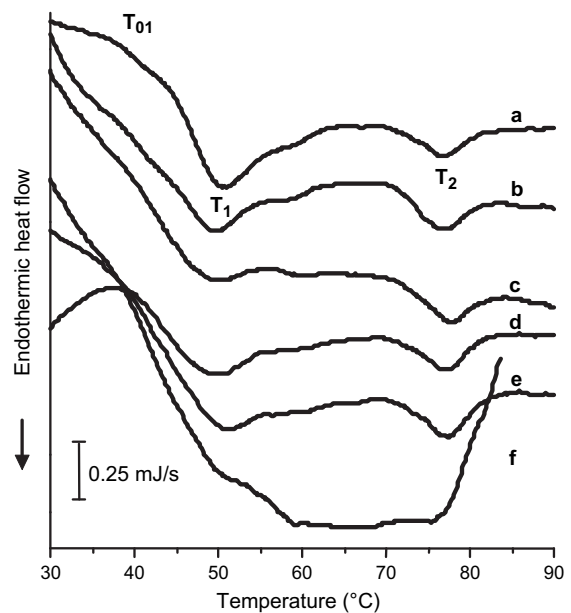


Fig. 5. DSC thermograms of UT sea salmon muscle as a function of the frozen (-11°C) storage time: a) fresh muscle; b) 1 month; c) 2 months; d) 3 months; e) 4 months; f) 6 months.

Table 2

DSC parameters as a function of the frozen ($-11\text{ }^{\circ}\text{C}$) storage time of UT sea salmon muscle.

Storage time (months)	Myosin peak			Actin peak	
	T_{01} ($^{\circ}\text{C}$)	T_1 ($^{\circ}\text{C}$)	ΔH_1 (J/g)	T_2 ($^{\circ}\text{C}$)	ΔH_2 (J/g)
0 (fresh)	42.8 ± 0.4^a	50.7 ± 0.04^a	9.22 ± 0.07^a	76.8 ± 0.2^a	0.87 ± 0.35^a
1	37.8 ± 0.9^b	49.7 ± 0.5^a	9.38 ± 0.84^a	77.5 ± 0.9^a	1.24 ± 0.40^a
2	38.2 ± 1.5^b	49.7 ± 0.3^a	9.16 ± 2.14^a	76.9 ± 0.9^a	1.13 ± 0.25^a
3	38.0 ± 2.1^b	49.6 ± 1.2^a	6.29 ± 1.20^b	77.9 ± 0.4^a	0.80 ± 0.35^a
4	38.3 ± 1.2^b	50.3 ± 0.7^a	6.62 ± 1.37^b	77.2 ± 0.3^a	1.31 ± 0.12^a

Each value is represented as the mean \pm SD of three determinations. Different letters (a,b) in the same column indicate significant differences ($p < 0.05$).

agreement with a very rapid increment on the TBA number which reached its maximum value at 10 days of frozen storage at $-10\text{ }^{\circ}\text{C}$ (Aubourg, Medina, & Gallardo, 1998).

3.4.2. T muscles

For T200 samples, neither the organic nor the aqueous phase presented significant differences ($p < 0.05$) compared to UT samples (data not shown). When 500 mg/kg of rosemary were added (T500), significant differences ($p < 0.05$) in the RF at 325/412 and 388/460 in organic phase between 7.5 and 9 months were recorded, RF being larger for UT samples (Fig. 3a and b). On the other hand, δForg and δFac presented larger values for T500 ($p < 0.05$) at 8.5 months (Fig. 4a and 4c). The aqueous phase did not present significant differences between UT and T500 samples.

The present results suggest that fluorescence compounds' determination was not useful to evaluate the progression of lipid oxidation in the frozen sea salmon muscle. Changes of fluorescence in aqueous phase were detected in both UT and T (200 and 500 mg/kg) samples – where lipid oxidation was inhibited or reduced. So, it was not possible to relate these changes with any possible interaction between lipid oxidation products and proteins. In organic phase, there were some differences between UT and T500 samples at longer storage time (after 8 months) suggesting the formation of organic soluble interaction compounds. Such compounds have been related to membrane phospholipids since they could act as amine donor groups (Aubourg, 1999a; Tappel, 1980).

3.5. Protein alterations

3.5.1. UT muscles

Fig. 5 shows the thermograms obtained by DSC of sea salmon muscle corresponding to different frozen storage times, in comparison with the fresh muscle (trace a). After 1 month of storage, a lower T_{01} (onset temperature or temperature to which

the protein unfolding begins) of the myosin denaturation peak could be observed (Table 2). However, the corresponding peak temperature T_1 (denaturation temperature) was constant along 4 months. In addition, a “shoulder” around $60\text{ }^{\circ}\text{C}$ became evident as a function of the storage time (Fig. 5c–e). Actin did not present changes in its denaturation temperature (Table 2). After 6 months of frozen storage, the thermogram did not present the characteristic peaks of myosin and actin (Fig. 5f). Myosin denaturation enthalpy was constant during the first 2 months, decreasing significantly ($p < 0.05$) at 3 months of storage (Table 2). The results suggest that myosin would experience a denaturation process at this time, affecting around a 30% of the structure according to the corresponding enthalpy value in comparison to that of fresh muscle. Actin denaturation enthalpy did not present significant changes during the first 4 months of storage (Table 2). Since samples did not present defined peaks at 6 months of storage, enthalpy values could not be obtained; however, the presence of some non-denatured muscle proteins cannot be excluded. Studies performed in other fish species (cod and tilapia) stored at $-20\text{ }^{\circ}\text{C}$ showed a decrease in the T_p and denaturation enthalpy (30–40%) of myosin, with no changes in the actin peak (Poulter, Ledward, Godber, Hall, & Rowlands, 1985). In cod stored at $-10\text{ }^{\circ}\text{C}$, myosin peak was broader, presenting a new transition at lower temperature after 2 weeks of storage (Hastings, Rodger, Park, Matthews, & Anderson, 1985). Sea salmon alterations are in agreement with the general behavior of frozen muscle proteins. The wide myosin peak – with a lower T_0 – would be related to the alteration of one region of the molecule inducing a high thermal sensitivity. At a long storage time, a partial loss of the native structure of this protein could be evidenced. However, previous studies have not informed such a drastic modification on the thermal profile as the one detected in sea salmon after 6 months of storage. A possible cause of this difference could be that in the present case muscle was stored in the minced state and probably the previous processing induced a greater alteration of protein structures.

The influence of the freezing process on the protein extractability was evaluated by comparison between the frozen/quick thawed (not stored) muscle and the fresh one. Results did not show significant differences ($p < 0.05$) either for TEP or MEP, in agreement with other works in the literature involving diverse types of muscles and freezing/thawing conditions (Anderson & Ravesi, 1970; Wagner & Añón, 1985). Extractable protein values of fresh muscle differed between the analyzed muscles. For muscle B TEP values were: 7.4 ± 0.2 g/100 g muscle or 42.1 ± 0.9 g/100 g total proteins; MEP: 1.6 ± 0.1 g/100 g muscle or 9.5 ± 0.2 g/100 g total proteins; with a rate MEP/TEP of $22.4 \pm 0.1\%$. In the case of muscle A the corresponding values were: TEP: 14.4 ± 0.5 g/100 g muscle or 77.2 ± 2.9 g/100 g total proteins; MEP: 6.7 ± 0.2 g/100 g muscle or

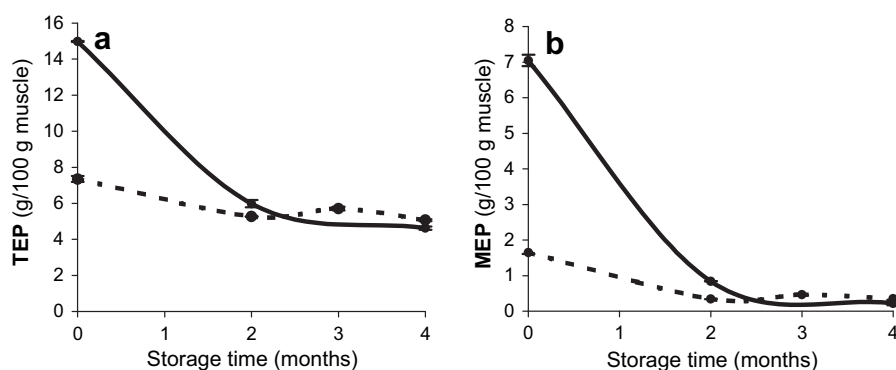


Fig. 6. Total extractable proteins (TEP) (a), and myofibrillar extractable proteins (MEP) (b), as a function of the frozen ($-11\text{ }^{\circ}\text{C}$) storage time of UT sea salmon muscles: — muscle A, --- muscle B. Measures were performed on two independent extracts by duplicate each one.

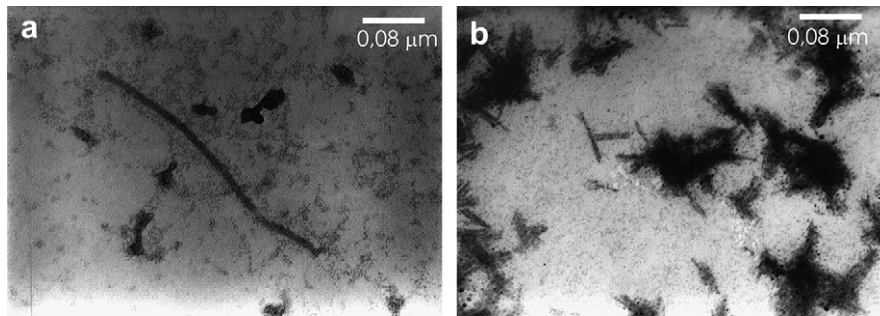


Fig. 7. TEM micrograph of the MEP fraction of UT sea salmon muscle: a) fresh; b) frozen (-11°C) stored during 2 months.

36.0 ± 1.3 g/100 g total proteins; with a rate MEP/TEP of $46.7 \pm 1.3\%$. These results indicated a high extractability of the myofibrillar proteins in the second case, suggesting differences in the conformational state of these proteins in the UT muscles. Several factors can affect the muscle protein extractability, such as the fish age, the lipid content and the protein–lipid interactions, the processing, the pre- or post-spawning and post-migration states, the nutritional and stress state before the capture, and the *rigor mortis* state (Leinot & Cheftel, 1990). Protein extractability was evaluated as a function of the frozen storage (-11°C) time. Fig. 6 shows the progression of the TEP (a) and MEP (b) for both UT muscles during the first 4 months of storage. As can be seen, TEP and MET decreased until reaching similar values for both muscles at 2 months of storage, without additional changes thereafter. Thus, an extensive aggregation and insolubilization process would occur during the first 2 months, involving a large proportion of the myofibrillar proteins. Diverse studies have demonstrated that the myofibrillar proteins – and not the sarcoplasmic proteins – were insolubilized during the frozen storage of fish (Awad et al., 1969), this process being dependent on the storage temperature (Careche, Del Mazo, Torrejón, & Tejada, 1998). Taking into account the information obtained from DSC and extractability analysis, it is possible to observe that

the aggregation process – causing the reduction of the MEP at 2 months of storage – would occur previous to the denaturation process of the myosin which was detectable after 3 months. Notwithstanding, changes in thermal stability were evidenced at shorter times. Studies in bovine muscle showed that protein denaturation occurred before the aggregates' formation (Wagner & Añón, 1986). In addition, it is remarkable that protein deterioration was very rapid in sea salmon in comparison with other muscles, which showed a progressive decrease of protein solubility. As previously mentioned, a factor potentially involved in the deterioration rate would be muscle mincing; although species-related characteristics cannot be ruled out.

The aggregation process of the myofibrillar proteins during the frozen storage can be evidenced by transmission electron microscopy (TEM) (Jarenbäck & Liljemark, 1975). Proteins from fresh muscle presented a characteristic filament structure (Fig. 7a), which would be related to actomyosin filaments. After 2 months of storage, different structures were observed, which can be characterized as bounded filaments forming “packs” (Fig. 7b). These new structures may have arisen from interactions between actomyosin filaments. It is important to note that the micrographs were obtained from the extractable myofibrillar proteins' fraction, thus,

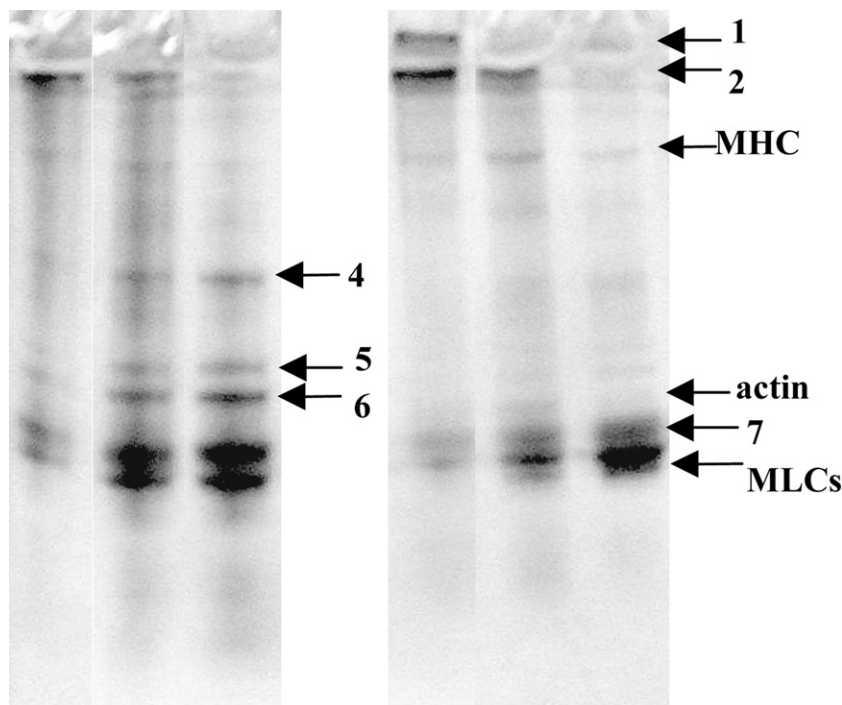


Fig. 8. SDS-PAGE (with 2-ME) of the extractable fractions of UT sea salmon muscle as a function of the frozen (-11°C) storage time. Lanes 1, 2 and 3: TEP extract: 0, 3 and 6 months, respectively; lanes 4, 5 and 6: MEP extract: 0, 3 and 6 months, respectively. 30 μg of protein was loaded in each lane.

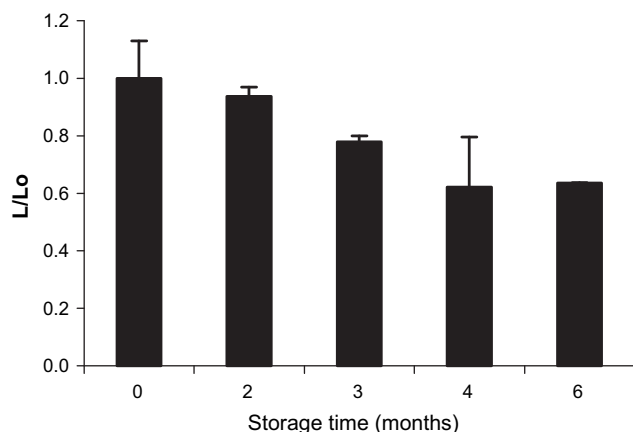


Fig. 9. Available lysine (L) expressed as a fraction of the corresponding value of fresh muscle (L_0), as a function of the frozen ($-11\text{ }^{\circ}\text{C}$) storage time of UT sea salmon muscle. Determinations were performed by duplicate.

myofibrillar proteins remaining in the extractable fraction would evidence some structural alteration. However, the most important modifications are expected to occur in the residual material (non-extractable fraction), which was not analyzed here.

The electrophoretic patterns of the TEP extract of UT muscle stored frozen for 3 months exhibited a decrease in a band of high molecular weight (*band 2*), which virtually disappeared after 6 months (Fig. 8 – lanes 1, 2, and 3). The MEP fraction presented similar results (Fig. 8 – lanes 4, 5, and 6) since this band is probably constituted by myofibrillar proteins forming some protein species of high molecular weight. In addition, the MEP fraction suffered the disappearance of the *band 1* at 3 months of storage, and a decrease of the myosin heavy chain (MHC) band after 6 months. Some low molecular weight proteins (bands 5 and 6 in the TEP extract, band 7 and myosin light chains, MLC, in both extracts) presented a high intensity, while the intensity of other bands decreased. The SDS-PAGE analysis suggested that the main protein species involved in the formation of aggregates are the very high molecular weight polypeptides, which were constituted by myofibrillar proteins, probably corresponding to the filaments observed by TEM.

The possible loss of available lysine during the frozen storage of sea salmon because of its reaction with other compounds such as the lipid oxidation products was evaluated. Frozen but not stored ($t=0$) muscle did not present differences in its available lysine content compared to the fresh muscle. After 2 months of storage, there were not significant changes ($p > 0.05$), suggesting that the very important extractability loss experienced by the myofibrillar proteins at this time was not related to a cross-linking process involving free amine groups of lysine. However, a significant decrease ($p < 0.05$) in the available lysine content was recorded after 3 months of storage (about 20%) and after 6 months (about 40%) (Fig. 9). It is remarkable that the decrease in the available lysine content coincided with the beginning of myosin denaturation – as measured by DSC – and that an additional decrease was paralleled by a total loss of myosin structure (6 months).

3.5.2. T muscles

All the protein analysis performed (DSC, extractability, TEM, SDS-PAGE and available lysine) did not reveal significant changes as a result of the application of rosemary extract (T200 and T500 samples) (data not shown). So, in all cases, results were similar to those shown before for the UT sample. According to this, protein alterations recorded during the frozen storage could not be prevented by the antioxidant. Although other studies have postulated a correlation between the increase in the TBA number and the

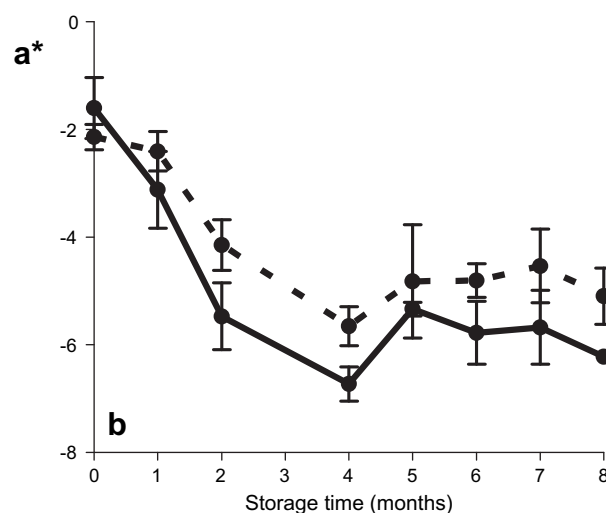
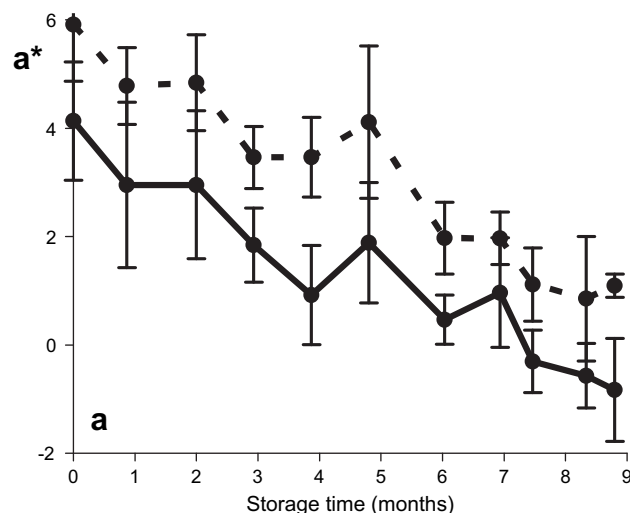


Fig. 10. Color parameter a^* as a function of the frozen ($-11\text{ }^{\circ}\text{C}$) storage time of sea salmon muscle: a) 10.8 g/kg lipids; UT —, T500 ----; b) 5.3 g/kg lipids; UT —, and T200 ----. Determinations were performed on two samples, measuring in five different locations.

decrease in the available lysine content in herring (*Clupea harengus*) stored at $-8\text{ }^{\circ}\text{C}$ (Kuusi, Nikkilä, & Savolainen, 1975), the results of the present study could not relate protein modifications to the appearance of lipid oxidation products.

3.6. Color

Parameters a^* , b^* and L^* were determined during the frozen storage of UT, T200 and T500 samples. It is important to remark that muscles A and B presented different initial parameters (Fig. 10a and b). Muscle A could be characterized as a “more red” muscle while muscle B was “more white”. T200 samples showed no significant differences ($p > 0.05$) in L^* and b^* compared to UT samples (data not shown). In contrast, a^* presented a progressive diminution as a function of the storage time for both samples (Fig. 10b), the decrease being significantly more important ($p < 0.05$) for UT samples at all storage times (except at 5 months). When 500 mg/kg of rosemary were used, L^* , b^* and a^* results were comparable to those previously described, being more evident the preservation of the red color – the difference between UT and T500 samples for parameter a^* was more important – in this case than in T200 samples (Fig. 10a). Results showed a relationship between the oxidation process and the loss of red color. However,

this loss could not be totally inhibited, suggesting that other factors – different from oxidation – are involved in this alteration. Similar results have been obtained in other fish species (Wetterskog & Undeland, 2004).

4. Conclusions

During frozen storage ($-11\text{ }^{\circ}\text{C}$), sea salmon muscle experienced diverse alterations in the lipid and protein fractions. The most rapid and evident protein alteration was an extractability loss, which occurred before that other modifications, such as protein denaturation and changes in the electrophoretic profiles of extractable fractions, were evident. Results suggest that after the aggregation and insolubilization process – along the first 2 months of storage – other protein changes were produced, inducing myosin denaturation. Changes in the nature of the aggregates and the type of interaction involved in their formation may be postulated. Although this hypothesis cannot be completely confirmed from the experimental data, results of available lysine determination are in this sense. The available lysine content decreased at 3 months of storage, suggesting some type of covalent interaction involving the free amino groups of lysine. A relationship between the protein alterations and the lipid oxidation could not be demonstrated. The application of rosemary extract as antioxidant was effective to reduce and delay the appearance of lipid oxidation as well as the loss of red color. Although both concentrations of rosemary extract were not assayed in the same batch of muscle, a dependence of the effectiveness on extract concentration can be proposed since each one is analyzed in relation to its corresponding untreated system, showing T200 a less evident effect than T500. Also, this is supported by the fact that the lower antioxidant concentration (200 mg/ml) was applied in the muscle that presented the lower lipid content; so, a larger antioxidation level could be expected due to the larger antioxidant/lipid ratio. Results showed the rosemary extract as a promissory natural antioxidant to be used in minced frozen sea salmon products.

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