**Damage inhibition during frozen storage of horse mackerel
(*Trachurus trachurus*) fillets by a previous plant extract
treatment**

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ABSTRACT

The present study is aimed to investigate the effect of a commercial plant extract (RP) on the stability of fish during frozen storage. For it, horse mackerel (*Trachurus trachurus*) fillets were soaked in water (Water Control) and two RP concentrations (0.333%, RP-1; 1.333%, RP-2) and compared to untreated fillets (Blank Control). Fluorescence detection and thiobarbituric acid index showed a lower oxidation development for both RP treatments than for both Controls, specially in the case of the highest concentration (RP-2). Decrease in glutathione peroxidase activity was found to be slower in the case of RP-2 treatment. The sensory analysis showed an increasing shelf life time according to the sequence: Blank Control < Water Control < RP-1, RP-2.

Keywords: Horse mackerel, frozen storage, plant extract, lipids, proteins, sensory analyses, shelf life.

INTRODUCTION

Most fish and other marine species give rise to products of great economic importance in many countries. Along the latest decades, fatty fish has captivated a big attention from consumer because of the positive role of marine lipids on human nutrition and health (Kinsella 1987; Illingworth and Ullmann 1990). Accordingly, great efforts are being carried out by fish traders and food technologists in being able to store and commercialize fatty fish products in a safely and high quality state.

Freezing and frozen storage have largely been employed to retain fish sensory and nutritional properties (Pigott and Tucker 1987; Erickson 1997). However, marine species have shown a highly unsaturated lipid composition (Ackman 1989) and an important presence of prooxidant molecules that facilitate the rancidity development (Mohri and others 1992; Harris and Tall 1994; Richards and Hultin 2002). In frozen conditions, lipid oxidation compounds have shown to interact with proteins leading to protein denaturation (Mackie 1993; Sikorski and Kolakowska 1994), nutritional losses (Castrillón and others 1996), modification of electrophoretic profiles of proteins (Saeed and Howell 2002) and lost of endogenous antioxidant systems (Undeland and Lingnert 1999).

To extend the shelf life of marine products, a great attention is being given to the use of natural antioxidants (Frankel 1995; Decker 1998). Recent efforts are focused on the positive role of antioxidant molecules present in plant extracts. Thus, successful applications have widely been carried out on marine oils (Thorisson and others 1992; Hamilton and others 1998), minced fish (Ramanathan and Das 1992; Boyd and others 1993) and fillets (He and Shahidi 1997; Khalil and Mansour 1998).

The present work concerns horse mackerel (*Trachurus trachurus*) and its trading as a frozen product. This species has recently attracted a great attention as being considered an underutilized fish species showing large captures in the latest years (FAO 2002). In the present work, horse mackerel fillets are treated with a commercial plant extract (Rosmol-P) to enlarge its shelf life. Rosmol-P is a permitted ingredient that has already shown a favoring effect on retarding lipid oxidation in meat products (Lugasi and others 2000). Sensory analysis and a wide range of lipid and protein damage indices are checked to assess the commercial suitability of such treatment.

MATERIALS AND METHODS

Plant extract preparation

Plant extract was obtained as Rosmol-P (RP) (FitoChem Kft., Monor, Hungary). For it, 100 g mixture of dry hyssop (*Hysoppus officinalis*), brunella (*Prunella vulgaris*), lemon balm (*Melissa officinalis*) and rosemary (*Rosmarinus officinalis*) was percolated with 30% hydro-ethanolic solution to give 500 ml of brown percolate. The solution was mixed with 2 g of activated coal, refrigerated and filtered. The hydro-ethanolic solution was spray dried to get 30 g antioxidant powder. Maltodextrin was used as carrier. Total polyphenol and rosmarinic acid contents were 6600 mg/100 g and 2100 mg/100 g, respectively (Lugasi and others 2000). Concentrations of RP employed in the present experiment were chosen according to previous research (Lugasi and others 2000).

Raw fish, sampling and processing

Fresh horse mackerel (*Trachurus trachurus*) (n = 87) were captured in November 2001 and kept on ice till arrival to the laboratory. The fish were carefully dressed and filleted by hand and divided into four groups. Each group included 21

fishes. One fillets group was directly packaged in polyethylene bags and immediately frozen at -80°C (Blank Control treatment). The other groups were immersed, respectively, in water (Water Control treatment), in a 0.333% aq. RP solution (RP-1 treatment) and in a 1.333% aq. RP solution (RP-2 treatment) in an isothermal room at 4°C . After 45 min, the fillets were removed, packaged in polyethylene bags and frozen at -80°C . After 48 hours at -80°C , all fillets were placed at -20°C . Sampling was undertaken on the initial material and at months 0, 1, 3, 5, 7, 9 and 12 of frozen storage at -20°C . For each fish group, three different fillet batches were considered and studied separately to achieve the statistical study. Once fillets were subjected to sensory analysis, the white muscle was separated and homogenized for carrying out the biochemical analyses.

Lipid damage measurements

Lipids were extracted by the Folch and others (1957) method. Results were calculated as g total lipids/100 g wet muscle.

Free fatty acids (FFA) content was determined according to Lowry and Tinsley (1976). The method is based on a complex formation between the acid group of FFA and cupric acetate in the presence of pyridine at $\text{pH} = 6.1$.; the resulting chromophore is read at 710 nm. Results are expressed as g FFA/100 g lipids.

The thiobarbituric acid index (TBA-i) (mg malondialdehyde/kg fish tissue) was determined according to Ramanathan and Das (1992). The method is based on reaction between a trichloroacetic acid (TCA) extract of the fish muscle and thiobarbituric acid at high temperature ($95-97^{\circ}\text{C}$); the resulting chromophore is read at 532 nm.

Fluorescence formation (Perkin-Elmer LS 3B) at 327/415 nm and 393/463 nm was studied as described elsewhere (Aubourg and others 1998; Aubourg and Medina

1999). The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission pair, and F_{st} is the fluorescence intensity of a quinine sulfate solution (1 µg/ml in 0.05 M H₂SO₄) at the corresponding wavelength. The fluorescence ratio (FR) was obtained from the lipid extract analysis, according to the following calculation: $FR = RF_{393/463nm} / RF_{327/415nm}$.

Protein change measurements

Digestible protein concentration was measured after enzyme digestion according to Akesson and Stahmann (1964). The method is based on reaction of fish sample with pepsin and then pancreatine; the protein content is finally measured by the Kjeldahl method with Tecator Kjeltac System. Results are expressed as percentage related to initial fish tissue value.

Glutathione peroxidase was extracted by homogenizing 2 g fish and 8 ml of a 1.19 % KCl aq. solution during 30 s. The mixture was centrifuged at 2,500g during 10 min. The supernatant was used for the measurement of the enzyme activity according to the Chin and others (1976) method. In it, the supernatant is reacted with reduced glutathione and finally the color development is read spectrophotometrically (412 nm). Results are expressed as U/g fish tissue, where 1 unit of the enzyme decomposes 1 micromol substrate (reduced glutathione) during 1 min.

Sarcoplasmic protein extracts were prepared in a low-ionic-strength buffer composed of 10 mM Tris-HCl pH 7.2 + 50 mM PMSF (pentamethyl sulfonic acid) (Piñeiro and others 1999). For it, 500 mg of fish muscle were homogenized for 60 s in 4 mL of buffer solution, centrifuged at 12,500g for 15 min in a JA20.1 rotor (J221-M centrifuge, Beckman-Coulter, London, UK) at 4°C, and the supernatants recovered. Solubilization of muscle protein was made in a 2% SDS (sodium dodecyl sulfate) buffer

(2% w/v SDS–0.1 DTT–60 mM Tris-HCl, pH 7.5) following the conditions above mentioned, except that before the centrifugation step (now at 20°C), the SDS extracts were boiled at 100°C for 2 min, homogenized for 30 s and finally maintained at room temperature.

Electrophoretic analysis was carried out by means of horizontal commercial SDS-PAGE gels. Samples were mixed with sample buffer according to the Laemmli (1970) procedure. Because of their higher resolution and reproducibility, precast polyacrylamide 245 x 110 x 1 mm commercial gels (Excel-Gel SDS Homogeneous 15%, Amersham Biosciences) for horizontal electrophoresis were selected. Anode and cathode buffer strips (Amersham Biosciences) were also employed. Electrophoretic studies were performed in a Multiphor II electrophoresis system (Amersham Biosciences) provided with a MultiTemp III refrigerated bath circulator (Amersham Biosciences). Running conditions were 1000 V/40 mA/40 W for 165 min. Once the bromophenol blue had reached the anode, gels were fixed and stained by a standard silver staining protocol (Amersham Biosciences). A low molecular weight protein standard (14-67 kDa) from Amersham Biosciences was employed as reference.

Sensory analyses

Sensory analyses were conducted by a taste panel consisting of five experienced judges, according to the guidelines presented in Table 1 (DOCE 1989). Four categories were ranked: highest quality (E), good quality (A), fair quality (B) and rejectable quality (C). Sensory assessment was carried out on raw whole fillets and included the following parameters: general aspect, odor and color.

Statistical analyses

Data from the different biochemical measurements were subjected to the one-way ANOVA method ($p < 0.05$); comparison of means was performed using a least-squares difference (LSD) method (Statsoft 1994). Linear and non-linear (exponential and logarithmic) correlation analyses and Spearman test for nonparametric correlations were performed (Statsoft 1994).

RESULTS AND DISCUSSION

Lipid damage analysis

Lipid contents ranged between 5.33% and 10.75%, according to individual variations in horse mackerel (Aubourg and Ugliano 2002; Aubourg and others 2002). In the present case, horse mackerel was captured in the period (autumn) of the highest lipid content (Hardy and Keay 1972; Bandarra and others 2001).

A gradual increase in FFA content was observed in all kinds of samples as a result of the frozen storage time (Figure 1); very good linear and nonlinear correlation values were obtained in all cases ($r^2 = 0.91-0.96$; Table 2). A clear effect of the plant extract treatment on the FFA formation was not observed; in most cases, mean values obtained were higher in the case of water treated fillets, although significant differences were only obtained at month 7. Examining the extent of lipid hydrolysis was deemed important to the study because of the high lipid hydrolysis development previously observed in horse mackerel during frozen storage (Simeonidou and others 1997; Aubourg and Ugliano 2002; Aubourg and others 2002) and also because of the great incidence of lipid hydrolysis on lipid oxidation (Miyashita and Takagi 1986; Aubourg 2001) and on protein denaturation (Mackie 1993; Sikorski and Kolakowska 1994).

Formation of thiobarbituric acid reactive substances (TBARS) did not show a continuous tendency along the storage (Figure 2), so that correlation values with time were not satisfactory (Table 2). These variations can be explained as a result of the different phases of peroxides decomposition, formation of carbonyls and interaction compounds with nucleophilic molecules (free amino acids, peptides, proteins, aminated phospholipids) present in the muscle. Comparison among treatments showed the following decreasing pattern for the TBA-i: Blank Control > Water Control > RP-1 > RP-2, where RP-2 values showed to be significantly lower ($p<0.05$) than both Controls in all cases. Compared to RP-1 values, RP-2 ones showed lower mean values, although significant differences ($p<0.05$) were not obtained in all cases. An inhibitory effect of the plant extract on TBARS formation was evident, specially in the case of the highest concentration tested. Water treatment also showed an antioxidant effect (Richards and others 1998), since lower values than in the Blank Control were obtained (months 3-12) ($p<0.05$).

Detection of fluorescent compounds (Figure 3) produced as a result of interaction between oxidized lipids and protein-type compounds showed a FR general increase in all kinds of samples as a result of increasing the frozen storage time, according to previous research on frozen fish (Aubourg and others 1998; Aubourg and Medina 1999; Aubourg and others 2002); satisfactory correlation values were obtained in all cases ($r^2 = 0.75-0.84$, logarithmic fitting; Table 2). Comparison among the different kinds of samples showed higher mean values for both Controls than for both RP treatments. No significant differences were obtained between both Controls; neither between both RP treatments. However, both RP treatments showed a lower ($p<0.05$) fluorescence formation than both Controls.

Protein change analysis

Protein digestibility (Figure 4) showed a gradual decrease till month 5 in all kinds of samples, according to previous research on frozen fish (Castrillón and others 1996). Then, no variations were observed till the end of the experiment. A very good nonlinear fitting was observed for all kinds of samples with the storage time (Table 2). Decrease in digestibility has been attributed to oxidized lipids-proteins interactions that lead to losses in labile amino acids (Gardner 1979; Opstvedt and others 1984; Leake and Karel 1985). Comparison among the different kinds of samples showed that no effect ($p>0.05$) from the RP treatments or the water soaking on protein digestibility could be observed.

Glutathione peroxidase activity (Figure 5) decreased in all kinds of samples during the frozen storage time, according to previous research on fish frozen storage (Undeland and Lingnert 1999; Jia and others 1996); no detectable values were obtained at the end of the experiment in any kind of samples. A very good linear correlation was obtained in all cases with the storage time (Table 2). Comparison among the different treatments showed a higher mean value for the RP-2 treatment, although significant differences ($p<0.05$) were only obtained at months 3, 7 and 9. It is concluded that glutathione peroxidase activity decreased during the frozen storage, but the RP treatment was helpful to prolong the presence of such endogenous antioxidant system.

Analysis of the SDS-PAGE profiles in commercial gels from sarcoplasmic protein and SDS soluble protein revealed no differences in protein patterns as a result of the frozen storage, neither as a result of the antioxidant treatment. Figure 6 shows the results obtained for SDS soluble protein in the case of initial fish sample and after 1, 3, 9 and 12 months of frozen storage. Previous research on a fattier fish species (mackerel, *Scomber scombrus*) showed considerable changes in the myofibrillar protein profile as a

1 result of the frozen storage, that were partially inhibited by the use of natural
2 antioxidants (Saeed and Howell 2002). It is concluded that in the present process
3 (freezing and frozen storage), lipid oxidation was not strong enough to modify such
4 electrophoretic profiles.

5 6 **Sensory analysis**

7 The sensory analysis results (Table 3) showed a short shelf life for the untreated
8 fillets, that were unacceptable at month 3. Both RP treatments enlarged the shelf life, so
9 that fillets were still acceptable at month 5. Sensory results did not show differences
10 between both RP treatments. Water treated fillets showed a longer shelf life than the
11 untreated ones (Blank Control) (Richards and others 1998), as were still acceptable at
12 month 3.

13 The main concern relating to the loss of sensory quality was the dryness and
14 myotomes breakdown produced, which led to unacceptable quality for all kinds of
15 samples at month 7. Further, flesh color showed to be the attribute less indicative of
16 quality loss since it showed to be acceptable in all cases at month 7.

17 Nonparametric correlations were studied among the three sensory attributes and
18 the frozen storage time for each of the four treatments checked. In all cases, good
19 correlation values were obtained (Flesh Color: $r^2 = 0.91-0.95$; Flesh Odor: $r^2 = 0.80-$
20 0.95 ; General Aspect: $r^2 = 0.80-0.95$).

21 22 **Final remarks**

23 According to sensory and biochemical (TBA-i, FR and glutathione peroxidase
24 activity) analyses, the use of RP has turned the fish muscle to be less prone to oxidation
25 than their untreated counterparts, so that a prolonged shelf life was obtained for the

1 frozen storage. Water soaking of fillets also showed some inhibition of oxidation
2 according to sensory and TBA-i results when compared to the Blank Control; this
3 inhibitory effect has been explained (Richards and others 1998; Undeland and others
4 1998) as a result of blood removal from fish. Further studies on the positive role of RP
5 on oxidation inhibition of relatively fat fish species during frozen storage are envisaged.
6 A special stress will be given to fish species commercialization as frozen whole fish,
7 because of the great industrial interest in such kind of product (FAO 2002) and the
8 scarce literature related to antioxidant treatment of whole fish (Wasson and others 1991;
9 Li and others 1998).

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FIGURE LEGENDS

Figure 1: Free fatty acid (g/100g lipids) determination during frozen storage of horse mackerel fillets that were pre-treated under different conditions*

* Treatment abbreviations: Untreated (Blank Control), water treated (Water Control) and Rosmol-P treated (0.333% and 1.333%, RP-1 and RP-2, respectively). Bars denote standard deviation of the mean (n=3).

Figure 2: Thiobarbituric acid index (mg malondialdehyde/kg fish tissue) determination during frozen storage of horse mackerel fillets that were pre-treated under different conditions*

* Treatment abbreviations as specified in Figure 1. Bars denote standard deviation of the mean (n=3).

Figure 3: Fluorescence ratio determination during frozen storage of horse mackerel fillets that were pre-treated under different conditions*

* Treatment abbreviations as specified in Figure 1. Bars denote standard deviation of the mean (n=3).

Figure 4: Protein digestibility determination (% related to initial fish tissue value) during frozen storage of horse mackerel fillets that were pre-treated under different conditions*

* Treatment abbreviations as specified in Figure 1. Bars denote standard deviation of the mean (n=3).

Figure 5: Glutathione peroxidase activity determination (U/g fish tissue) during frozen storage of horse mackerel fillets that were pre-treated under different conditions*

* Treatment abbreviations as specified in Figure 1. Bars denote standard deviation of the mean (n=3).

Figure 6: EXCEL-GEL 15% SDS-PAGE of SDS protein extracts from frozen horse mackerel fillets that were pre-treated under different conditions*

* Abbreviations employed: IN (Initial fish tissue), ST (low molecular weight protein standard: 14-67 kDa). Other abbreviations as specified in Figure 1.

TABLE 1

Scale employed for evaluating quality of frozen horse mackerel fillets

Attribute	E (Highest quality)	A (Good quality)	B (Fair quality)	C (Rejectable quality)
General Aspect (dryness, myotomes breakdown, white spots)	Strongly hydrated; totally adhered myotomes; absence of white spots	Still hydrated; myotomes adhered; absence of white spots	Slightly dry; myotomes adhered in groups; small white spots	Dry; myotomes totally separated; big white spots
Flesh Odor	Shellfish	Weakly Shellfish	Slightly sour and incipient rancidity	Sharply sour and rancid
Flesh Color	Strongly pinky	Still pinky	Slightly pale	Yellowish

TABLE 2

Linear correlations* between frozen storage time and different biochemical determinations in horse mackerel fillets that were pre-treated under different conditions**

Index	Blank Control	Water Control	RP-1	RP-2
Free Fatty Acids	0.94 (0.95)	0.92 (0.96)	0.96	0.91 (0.92)
Thiobarbituric Acid Index	0.60 (0.75)	0.28 (0.45)	0.43 (0.57)	0.63 (0.75)
Fluorescence Ratio	0.68 (0.79)	0.61 (0.75)	0.78 (0.84)	0.80 (0.82)
Protein Digestibility	– 0.86 (– 0.96)	– 0.83 (– 0.94)	– 0.83 (– 0.95)	– 0.85 (– 0.95)
Glutathione Peroxidase	– 0.92 (– 0.96)	– 0.92	– 0.95	– 0.98

* Non-linear fittings (logarithmic) are expressed in brackets when equal to or superior to the linear ones.

** Treatment abbreviations as specified in Figure 1.