Lipid and Protein Changes Related to Quality Loss in Frozen Sardine (Sardina pilchardus) Previously Processed Under High-Pressure Conditions

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Abstract This research focuses on biochemical changes related to quality loss in frozen (-18 °C for 9 months) sardine (Sardina pilchardus) previously subjected to high-pressure (HP) processing (125-200 MPa). The inhibition ($p < 0.05$) of lipid hydrolysis development (lower free fatty acid formation and lipase activity), observed in frozen sardine as a result of the previous HP treatment, increased with the pressure level applied. Several parameters including peroxide value, thiobarbituric acid index, fluorescent compounds, and polyenes showed that the applied HP conditions prior to sardine freezing did not increase lipid oxidation. Also, HP did not induce a substantial modification of acid phosphatase and cathepsins B and D activities, and the electrophoretic patterns of sarcoplasmic and myofibrillar protein fractions did not change. However, HP processing led to a decrease in myofibrillar protein content in frozen pressure-treated fish, an effect that was higher in 175- and 200-MPa treated samples. In conclusion, this research showed that pressure treatments in the 125-200-MPa range with holding time of 0 min cause only minor modifications in biochemical indicators of deterioration through- out the subsequent frozen storage of samples for up to 9 months. This study shows the need to optimize HP conditions, particularly in the case of applications combining HP treatments, frozen storage, and thawing to obtain products with high quality and commercial viability.

Keywords Sardina pilchardus · High-pressure processing · Frozen storage · Lipids · Proteins · Enzymes

Introduction

High-hydrostatic-pressure technology has been shown to retain the sensory and nutritional properties of foods while inhibiting microbial activity and leading to shelf-life extension and safety assurance (Norton and Sun 2008; Mújica-Paz et al. 2011). Concerning high-pressure (HP) application to seafood, previous research accounts for a wide range of studies showing practical and beneficial effects when employed prior to a subsequent processing or storage step. This is the case of products subjected to further refrigerated (Erkan et al. 2010) and chilled (Ortea et al. 2010) storage or processed by cold smoking (Lakshmanan et al. 2005). In the case of freezing, frozen storage, and thawing treatments, pressure-shift technology shows advantages in terms of protein denaturation, water- holding capacity, and toughening (Chevalier et al. 2000; Tironi et al. 2010). When products stored frozen are considered, an enhanced sensory acceptance was observed in samples of Atlantic mackerel (Scomber scombrus; Aubourg et al. 2013) and horse mackerel (Trachurus trachurus; Torres et al. 2014) subjected to a previous HP treatment.

Along with advantages, HP processing of seafood has shown negative effects reflecting in the content of sensitive lipids (i.e., high unsaturation degree) and proteins (i.e., high content of reactive amino acids) of marine species (Ashie et al. 1996; Campus 2010). Relatively severe HP conditions have been reported to induce lipid oxidation (i.e., formation of per- oxides and carbonyls compounds), accompanied by off odors, browning, and modifications of the quaternary, tertiary, and secondary protein structures (Hendrickx et al. 1998; Tabilo-Munizaga et al. 2016). Consequently, the optimization of the HP conditions to be applied to fish species ought to achieve microbial stabilization while retaining the original sensory and nutritional properties. This optimization should take into ac- count the changes associated with further processing and storage.

Sardine is a small pelagic fish species captured and consumed in large amounts in South Europe and North Africa. With the exception of negligible amounts sold in the retail market, this species is mainly used as fresh or raw material destined for further processing. One of the factors limiting its commercial use is the difficulty of its preservation under frozen conditions. Thus, previous research has shown an important endogenous pro-oxidant activity and significant quality loss during frozen storage (Verma et al. 1995; Aubourg et al. 1998). Concerning HP treatment of sardine, the gel-forming conditions of sardine muscle by HP processing (150-300 MPa for 10-30 min at 0-7 °C) have been analyzed and optimized (Pérez-Mateos and Montero 1997). Hernández-Andrés et al. (2008) studied the effect of pressure (300 MPa, 20 min, 7 °C) on the electrophoretic profiles of myofibril proteins. Microbial activity and lipid oxidation development were inhibited when an HP treatment (300 MPa, 10 min, 7 °C) was combined with functional films during refrigerated (7 °C) storage of sardine muscle (Núnez-Flores et al. 2013). An HP treatment
(300 MPa, 15 min, 20 °C) combined with refrigerated (5 °C) storage of cold-smoked sardine was also effective (Gómez- Estaca et al. 2007).

This research focuses on the quality retention of sardine during frozen storage under commercial conditions (9 months at -18 °C). The effect of a previous HP treatment prior to freezing was followed by determining changes in lipids (hydrolysis and oxidation), myofibril and sarcoplasmic protein fractions, and in

the activity of key enzymes (lipase, acid phosphatase, cathepsin B, and cathepsin D). Previous research on HP treatments at 150, 300, and 450 MPa for 0, 2.5, and 5 min of Atlantic mackerel (Aubourg et al. 2013) and horse mackerel (Torres et al. 2014), followed by frozen storage under accelerated conditions (-10 °C), showed that 150 MPa for 0-min holding time was optimum to retain the sensory and physical properties of these two pelagic species. Consequently, pressure conditions chosen for the present sardine study were 150 MPa and a lower (125 MPa) and two higher (175 and 200 MPa) values, all with zero value as pressure holding time.

Materials and Methods

Raw Fish, HP Processing, Frozen Storage, and Sampling

Sardine (156 individuals) caught near the Bask coast in north- ern Spain was obtained at the Ondarroa harbor (Bizkaia, Spain) and transported under ice to the AZTI (Derio, Spain) pilot plant for HP treatment within 6 h after catch. Whole- sardine individuals were placed in flexible polyethylene bags (three individuals per bag) and vacuum sealed at 400 mbar. The length and weight of the specimens ranged 17-23 cm and 145-175 g, respectively.

HP treatments were performed in a 55-L high-pressure unit (WAVE 6000/55HT; Hiperbaric, Burgos, Spain). Pressure values chosen were 125, 150, 175, and 200 MPa, all applied with a pressure holding time of 0 min (i.e., effect of pressure come up and depressurizing time). Water used as the pressurizing medium increased pressure at a 3-MPa s^{-1} rate, thus yielding 41.7, 50, 58.3, and 66.7 s as the corresponding come up times for the different treatments. In all cases, the decompression time was less than 3 s. The high-pressure equipment employed cold pressurizing water to maintain isothermal conditions (20 °C) during HP treatment.

HP-treated sardines were analyzed after 48 h (month 0) and after 1, 3, 6, and 9 months of storage at -18 °C. Fish without HP treatment and subjected to the same freezing and frozen storage conditions (30 individuals) were used as control (0.1- MPa pressure condition). Similarly, six initial individuals were distributed into three batches (two individuals per batch) and analyzed as initial fish material. Three batches or replicates (n = 3) for each processing condition or control were analyzed independently. In each case, the corresponding ex- tract from the fish white muscle pooled from two individual fish was employed.

Lipid Extraction and Quality Index Determination

Lipids were extracted by the Bligh and Dyer (1959)method employing a chloroform-methanol (1:1) mixture for the single-phase solubilization of fish muscle lipids. Quantification results were expressed as g lipid kg^{-1} muscle. The free fatty acid (FFA) content in this lipid extract was determined by the Lowry and Tinsley (1 976) spectrophotometric method (715 nm; Beckman Coulter DU 640, London, UK) based on the complex formation with cupric acetate pyridine. Results were calculated both as g FFA kg^{-1} muscle and as g FFA kg^{-1} lipids.

The peroxide value (PV) in the lipid extract was deter- mined by peroxide reduction with ferric thiocyanate, according to the Chapman and McKay (1949) method. Results were expressed as meq active oxygen kg^{-1} lipids.

The thiobarbituric acid index (TBA-i) was determined as described by Vyncke (1970) and based on the reaction between a trichloroacetic acid (TCA) extract of the fish muscle and TBA. Content of thiobarbituric acid reactive substances (TBARSs) was spectrophotometrically measured at 532 nm, and results were expressed as mg malondialdehyde kg^{-1} muscle.

The formation of fluorescent compounds was determined in the aqueous phase obtained during the lipid extraction by measurements at 393/463 and 327/415 nm (Aubourg et al. 1998). Relative fluorescence (RF) was defined as the F/F_{st} ratio, where F is the fluorescence measured at each excitation and emission maximum and F_{st} is the fluorescence intensity of a quinine sulfate solution (1 (ig mL^{-1} in 0.05 M H2SO4) at the corresponding wavelength. To quantify the formation of fluorescent compounds, fluorescence ratio (FR) was calculated as the ratio between the two RF values, $^{FR = RF}$393/463 nm$^{/RF}$327/415 nm.

Lipid extracts were converted into fatty acid methyl esters (FAMEs) by reaction with acetyl chloride and then analyzed using a Perkin-Elmer 8700 gas chromatograph (Waltham, MA, USA) equipped with a fused silica capillary column SP-2330 (0.25 mm i.d. x 30 m, 0.20-^m film; Supelco Inc., Bellefonte, PA, USA) and using nitrogen at 10 psi as carrier gas (linear flow rate of1.0 mL min^{-1}) and a flame ionization detector (FID) at 250 °C (Aubourg et al. 1997). Peaks corresponding to fatty acids were identified by comparison of the retention times of two standard mixtures (Qualmix Fish, Larodan, Malmo, Sweden; FAME Mix, Supelco Inc., Bellefonte, PA, USA). Peak areas were automatically integrated (TotalChrom v. 6.3.2 Perkin-Elmer), and content of each fatty acid was calculated as g 100 g^{-1} total fatty acids by employment of C 19:0 fatty acid as internal standard. The polyene index (PI) was calculated as the following fatty acid ratio: (C 20:5^3 + C 22:6^3),C 16:0.

Protein Damage Assessment

Sarcoplasmic (low-salt-soluble) and myofibril (high-salt-soluble) protein extraction from the sardine muscle

was per- formed by modification of a previously published protocol (Pazos et al. 2011). Briefly, 0.5 g was homogenized using an Ultra-Turrax high-performance disperser in 5 mL of Tris buffer (10 mM Tris-HCl, pH 7.2), containing 5-mM protease inhibitor (phenylmethylsulfonyl fluoride (PMFS)). The sarcoplasmic protein fraction was obtained from the supernatant after centrifugation of the homogenate at 40,000xg (4 °C, 20 min). The pellet was then homogenized with a saline solution (0.6 M NaCl, 10 mM Tris buffer, 5 mM PMFS, pH 7.2), and myofibril proteins were isolated from the supernatant after centrifugation at 4,500xg (4 °C, 20 min). Sarcoplasmic and myofibril proteins were stored at -80 °C until use. Protein concentrations were determined in both fractions by bicinchoninic acid assay (Smith et al. 1985). Contents were calculated as g kg^{-1} muscle.

One-dimensional (1-D) laboratory-made 10 % polyacrylamide gels (v/v; acrylamide N,N'-ethylene-bis acrylamide, 200:1) with upper stacking gel (4 % polyacrylamide) were loaded with 20 or 30 mg of protein per lane. The 1-D gels were run in a Mini-PROTEAN 3 cell (Bio-Rad) with an aqueous running buffer composed by1.44 % (w/v) glycine, 0.67% Tris-base, and 0.1 % SDS (Laemmli 1970). Gels were finally stained overnight with the Coomassie dye PhastGel Blue R- 350 (GE Healthcare).

Protein breakdown was complementarily measured by trimethylamine (TMA) formation. TMA-N values were obtained by means of the picrate method, as previously de- scribed (Tozawa et al. 1971). This involves the preparation of a 5 % (w/v) TCA extract of the sardine muscle. The results were expressed as mg TMA-N kg^{-1} muscle.

Enzymatic Activity Analysis

The enzymatic extract was prepared by homogenization of fish muscle (10 g) with 50 mL of ice-cold distilled water for 2 min, using an IKA Ultra-Turrax T25 homogenizer for 2 min and at 8000 rpm (IKA®-Werke GmbH & Co., Staufen, Germany), and then kept in ice with occasional stirring during 30 min. After this time period, it was centrifuged at 14,600xg for 20 min at 4 °C. The supernatant was filtered through a Whatman no. 1 filter and stored at -20 °C prior to enzymatic activity quantifications.

Lipase activity was determined following the titrimetric enzymatic assay described by Sigma-Aldrich (1999), and results were expressed as pmol FFA min^{-1} g^{-1} muscle. Acid phosphatase activity assessment was carried out by the methodology described by Ohmori et al. (1992), using p- nitrophenylphosphate (p-NPP) as substrate, with only minor modifications. Enzymatic extracts (0.250 mL) were mixed with substrate solution (0.225 mL of 4 mM p-NPP in 0.1 mM acetate buffer and 1 mM ethylenediamine- tetraacetic acid (EDTA), pH 5.5). After incubation at 37 °C for 15 min, the reaction was stopped by adding 1 mL of 100 mM KOH and the p-nitrophenol (p-NP) released was measured at 400 nm (Lambda 35 UV/Vis spectrophotometer, Perkin-Elmer Instruments, Waltham, MA, USA). Acid phosphatase activity was expressed as nmol p-NP min^{-1} g^{-1} muscle.

Cathepsin B activity was evaluated by the methodology described by Lakshmanan et al. (2005), being its activity expressed as fluorescence unit (FU) min^{-1} g^{-1} muscle. Cathepsin D activity was analyzed following the protocol de- scribed by Buckow et al. (2010), with few modifications. Enzyme extracts (0.2 mL) were mixed with substrate solution (0.6 mLof2 % denatured hemoglobin (w/v) in 200 mM citrate buffer, pH 3.7). After incubation for 3 h at 37 °C, the reaction was stopped by the addition of 0.6 mL of 10 % TCA (w/v), and after vigorous stirring, the precipitate was removed by centrifugation for 15 min, at 18,000xg (Elmi Micro Centrifuge CM-50, Porvoo, Finland). Soluble peptides were spectrophotometrically measured at 280 nm (Lambda 35 UV/ Vis spectrometer, Perkin-Elmer Instruments) and converted to tyrosine equivalents using a previously built calibration curve. Cathepsin D activity was expressed as pg tyrosine min g muscle.

Statistical Analysis

Fish samples corresponding to all the conditions tested were analyzed after 0, 1, 3, 6, and 9 months offrozen storage time. Data (n = 3) obtained from the different biochemical parameters were subjected to one-way ANOVA method ($p < 0.05$)to explore differences due to two different variables, i.e., the pressure level and the frozen storage time. Comparison of means was performed using a least squares difference (LSD) method (Statsoft, Statistica, version 6.0, Tulsa, OK, USA). Correlation analysis among parameters (frozen storage time and biochemical indices) was also carried out. Results are referred to linear fittings unless indicated.

Results and Discussion

Assessment of Lipid Hydrolysis Development

FFA formation via lipid hydrolysis is described in Fig. 1a (g kg^{-1} muscle). Values were calculated on the basis of the lipid content in sardine muscle (5.3-9.5-g lipids kg^{-1} muscle range; data not shown) throughout the 0-9-month period. An inverse relationship between FFA content and the pressure applied was observed, being the best correlation values for the 3-6-month period ($r^2 = 0.71$-0.77). The initial fresh fish value (0.07 ± 0.01 g FFA kg^{-1} muscle) led to a marked FFA increase in all kinds of samples after the freezing step (month- 0 frozen samples); however, this increase was found to be lower ($p < 0.05$) in samples corresponding to the two highest

pressure levels applied (175 and 200 MPa). Afterward (3-9- month period), the FFA formation showed a progressive in- crease for all samples throughout the frozen storage (r^2 = 0.90-0.93, linear fitting). Compared to control fish, the 175-MPa treated fish showed a lower ($p < 0.05$) lipid hydro- lysis for the 0-6-month period; in the case of samples corresponding to the highest pressure, a lower ($p < 0.05$) FFA content was obtained at months 0, 3, and 6, with mean values lower than those for the control fish throughout the storage period. Consequently, a pressure treatment prior to freezing can be concluded to have an inhibitory effect on the FFA formation. Similar conclusions were obtained when FFA con- tent was expressed on a lipid content basis (g kg^{-1} lipids).

Accumulation of FFA via lipid hydrolysis in fish muscle has no nutritional significance, but it has undesirable

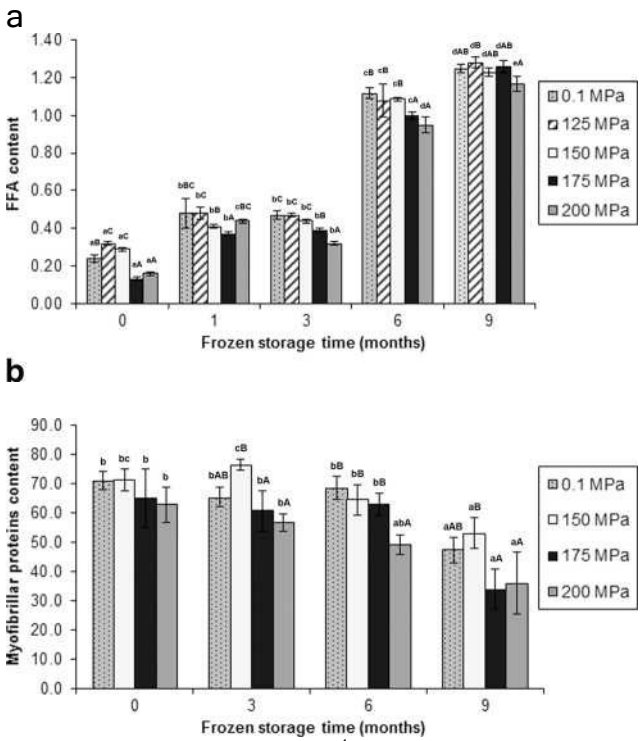

Fig. 1 Free fatty acid (FFA; g kg^{-1} muscle) (a) and myofibrillar protein content (g kg^{-1} muscle) (b) assessment in frozen sardine previously processed under different high-pressure conditions*. *Mean values of three replicates (n = 3); standard deviations are indicated by bars. For each frozen storage time, values accompanied by different letters (A-C) denote significant differences ($p < 0.05$) as a result of the pressure treatment. For each pressure treatment condition, means followed by different lowercase letters (a-e) indicate significant differences ($p < 0.05$) as a result of frozen storage time. No letters are included when significant differences were not found

secondary effects including muscle texture changes and lipid oxidation acceleration (Sikorski and Kolakowska 1994) and off-odor and off-taste development (Refsgaard et al. 2000). Research concerning the effect on FFA formation after HP treatment followed by a frozen storage is, to our knowledge, not available in the case of sardine, while this information can be considered scarce when other fish species are concerned. Thus, two close pelagic fish species, Atlantic mackerel (S. scombrus; Vázquez et al. 2013) and horse mackerel (Trachurus trachurus; Torres et al. 2013), were subjected to HP treatment (150, 300, and 450 MPa for 0, 2.5, and 5 min) prior to freezing (-20 °C) and frozen storage (-10 °C for 3 months). The results revealed a markedly reduced FFA con- tent for all kinds of frozen samples that were previously HP treated; this FFA content reduction was higher by increasing the previous pressure level applied as well as by increasing the previous pressure holding time. By checking different frozen storage conditions to the present ones, Ohshima et al. (1992) found that enzymatic degradation of phospholipids (i.e., phospholipase action) in cod (Gadus morhua) muscle was success- fully inhibited during storage at -2 °C for 6 days when previously treated at pressures above 400 MPa for 15 and 30 min; however, no effect was observed in samples frozen under the same conditions if previously submitted to a 200-MPa pressure. On the other hand, freezing by pressure release (i.e., pressure shift freezing; 140 MPa) was compared with air- blast freezing (-20 °C) in turbot (Scophthalmus maximus) filets (Chevalier et al. 2000); as a result, FFA formation during subsequent storage at -20 °C for 75 days did not show differences between both freezing conditions.

Assessment of Lipid Oxidation Development

The formation of primary lipid oxidation compounds (PV; Table 1) showed a marked increase ($p < 0.05$) in all fish samples after the freezing step (month-0 samples); this increase was found higher ($p < 0.05$) in 175- and 200-MPa samples when compared to control one. At month 0, a direct relation- ship (r^2 = 0.80) between peroxide content and pressure applied was detected; however, a definite trend between both parameters could

not be observed later on (1-9-month period). The highest PVs were observed at month 3 in all conditions; after this time, a general content decrease was observed till the end of the experiment. Comparison among the different samples showed some significant differences, although a general trend could not be foreseen concerning the previous HP treatment effect. Thus, at time 0, the lowest mean PVs were observed for samples corresponding to the control and 125-MPa conditions, while 200-MPa samples showed the lowest mean values for the 3-6-month period.

Concerning the formation of secondary lipid oxidation compounds (TBARS; Table 2), a general increase with storage time was detected after freezing (month-0 samples). At this time, a lower value was obtained by previous pressure in- crease, so that an inverse relationship between TBA-i and pressure applied was obtained (r^2 = 0.85); however, a definite relationship between both parameters could not be implied for the 1-9-month period. Throughout the whole storage period, a general increase of TBARS content was detected in all samples (r^2 = 0.75-0.93, quadratic fitting), showing a marked
increase at the end of the storage time. This increase is in agreement with the marked decrease found at the same time for the peroxide content (Table 1). Comparison among samples showed lower mean TBARS values for the 0-1-month period in samples corresponding to the 200-MPa treatment. After this period, scarce differences could be observed, so that a general trend could not be implied.

The formation of tertiary lipid oxidation compounds (namely, fluorescent interaction molecules) is presented in Table 3. As for peroxides and TBARS, a general content increase was observed after the freezing step (month-0 samples), with significant lower values in 175-MPa samples when compared with their counterparts from control and 125-MPa conditions. At month 0, a poor inverse correlation value could be observed between the FR value and the pressure applied (r^2 = 0.59), while a definite trend could not be observed for the 1-9-month period between both parameters. Throughout storage, fluorescent compound formation can be considered as very low or even negligible. For the 3-9-month period, lower mean values were obtained in the 125-MPa treated fish samples.

Also related to lipid damage, PI values were also assessed (data not shown) and ranged 2.64-2.85 (initial fish value 2.80 ± 0.07), showing no effect ($p < 0.05$) of the pressure treatment and the frozen storage time. This result can be considered in agreement with the above-mentioned scarce differences found among samples during the different steps of the lipid oxidation development.

Lipid oxidation is a complex process producing many different compounds; most of them are unstable and thus breaking down into smaller-molecular-weight ones or reacting with other compounds, mostly nucleophilic-type, present in fish muscle. In the present case, peroxides content reached a maximum value at month 3 in all kinds of samples that was followed by a general decrease. This decrease led to a general TBARS formation increase but hardly modified the tertiary lipid oxidation compound formation. It can be concluded that formation of secondary lipid oxidation compounds was the main event related to lipid oxidation development in the present study.

Table 1 Peroxide value (PV; meq active oxygen kg^{-1} lipids) assessment in frozen sardine muscle previously processed under different high-pressure conditions

Frozen storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
Initial fish	0.68a (0.11)	0.68a (0.11)	0.68a (0.11)	0.68a (0.11)	0.68a (0.11)
0	1.89bA (0.80)	2.31cA (0.37)	2.48bAB (1.09)	3.63cAB (1.00)	3.55cB (0.17)
1	5.85cB (0.31)	4.49dB (1.21)	6.37cB (1.50)	1.64bA (0.18)	4.29cB (1.94)
3	10.61dB (2.27)	11.62eB (1.24)	13.48dB (2.02)	6.28dA (1.23)	4.73cA (1.15)
6	5.84cAB (2.13)	4.21dAB (1.37)	7.17cB (1.83)	4.39cdAB (2.11)	3.26bcA (1.08)
9	1.83abAB (1.01)	1.30bA (0.08)	3.79bB (1.17)	3.28cB (0.51)	1.54bA (0.53)

Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each frozen storage time, mean values followed by different capital letters (A and B) indicate significant differences ($p < 0.05$) as a result of the pressure treatment. For each pressure treatment condition, means followed by different lowercase letters (a–e) indicate significant differences ($p < 0.05$) as a result of frozen storage time

Table 2 Measurement of the thiobarbituric acid index (TBA-i; mg malondialdehyde kg^{-1} muscle) in frozen sardine muscle previously processed under different high-pressure conditions

Frozen storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
Initial fish	0.18a (0.08)	0.18a (0.08)	0.18a (0.08)	0.18a (0.08)	0.18a (0.08)
0	0.50bC (0.07)	0.41bcBC (0.10)	0.41bcABC (0.13)	0.32bB (0.02)	0.23aA (0.04)
1	0.53bB (0.11)	0.38bAB (0.06)	0.51bcB (0.09)	0.44cAB (0.05)	0.32aA (0.05)
3	0.49b (0.02)	0.53c (0.06)	0.52bc (0.09)	0.50c (0.05)	0.50b (0.08)
6	0.63B (0.03)	0.58cAB (0.10)	0.46bA (0.01)	0.54cdAB (0.12)	0.52bAB (0.11)
9	0.76c (0.03)	0.77d (0.05)	0.66c (0.11)	0.74d (0.11)	0.80c (0.07)

Mean values of three replicates ($n = 3$); standard deviations are indicated in brackets. For each frozen storage time, mean values followed by different capital letters (A–C) indicate significant differences ($p < 0.05$) as a result of the pressure treatment. For each pressure treatment condition, means followed by different lowercase letters (a–d) indicate significant differences ($p < 0.05$) as a result of frozen storage time. No letters are included when no significant differences are found

Previous research accounts for different studies concerning the effect of HP treatment on different kinds of sardine products, although studies concerning the frozen sardine products are, to our knowledge, not included. Thus, an inhibitory effect was observed on TBARS formation at 300 MPa/10 min/7 °C combined with functional gelatin-lignosulfonate film to preserve sardine filets during chilled storage (Núnez-Flores et al. 2013). Peroxide and TBARS contents were also lowered in cold-smoked sardine (Sardina pilchardus) by combining a 300-MPa/15-min/20 °C treatment and gelatin-based functional edible films (Gómez-Estaca et al. 2007).

The lipid changes observed in this study can be explained by two opposite mechanisms. First, it is possible that the inhibitory effect of pressure on the endogenous enzyme activity should reduce the lipid oxidation development during the frozen storage period in agreement with previous research (Torres et al. 2013; Vázquez et al. 2013). However, iron-bound protein denaturation during HP treatment has been reported to facilitate the increase of free metal ion content, which would lead to a lipid oxidation increase in fish meat after HP treatment (Campus 2010). Consequently, previous related research accounts for contradictory results when the effect of HP treatment on lipid oxidation development in frozen fish is evaluated. Closely related to this study, a marked inhibition of tertiary lipid oxidation compound formation in frozen (-10 °C up to 3 months) mackerel (Vázquez et al. 2013) and horse mackerel (Torres et al. 2013) by previous HP treatment (150, 300, and 450 MPa for 0.0, 2.5, and 5.0 min) was observed. This effect was increased by increasing the pressure level or the pressure holding time. However, in a 6-day frozen storage (-2 °C) study, Ohshima et al. (1992) reported an increased TBARS formation in frozen cod (G. morhua) and mackerel (S. scombrus) muscle previously subjected to HP treatments (616, 408, and 204 MPa for 15 and 30 min). Additionally, a comparison of pressure shift freezing at 140 MPa with air-blast freezing (-20 °C) did not provide differences on TBARS formation during subsequent storage at -20 °C for 75 days of turbot (S. maximus) filets (Chevalier et al. 2000).

Analysis of Protein Changes

No HP treatment or frozen storage time effects were observed in the analysis of sarcoplasmic protein content

Table 3 Fluorescence ratio assessment in frozen sardine muscle previously processed under different high-pressure conditions

Frozen storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
Initial fish	0.30a (0.07)	0.30a (0.07)	0.30a (0.07)	0.30a (0.07)	0.30 (0.07)
0	0.45bB (0.03)	0.43bB (0.01)	0.41bAB (0.06)	0.35aA (0.04)	0.42AB (0.05)
1	0.38ab (0.08)	0.31a (0.05)	0.33a (0.07)	0.40ab (0.05)	0.38 (0.01)
3	0.33aAB (0.06)	0.30aA (0.02)	0.34aAB (0.05)	0.36aAB (0.03)	0.49B (0.10)
6	0.36abAB (0.09)	0.34aA (0.04)	0.48bB (0.03)	0.43abAB (0.07)	0.43AB (0.05)
9	0.54cB (0.03)	0.38abA (0.06)	0.42abAB (0.11)	0.45bA (0.03)	0.45AB (0.07)

Mean values of three replicates ($n = 3$); standard deviations are indicated in brackets. For each frozen storage time, mean values followed by different capital letters (A and B) indicate significant differences ($p < 0.05$) as a result of the pressure treatment. For each pressure treatment condition, means followed by different lowercase letters (a–c) indicate significant differences ($p < 0.05$) as a result of frozen storage time. No letters are included when no significant differences are found

(21.9-27.4-g kg^{-1} muscle range; data not shown).

However, a marked effect of pressure applied and frozen storage time was observed on myofibrillar protein content (Fig. 1b). Thus, an inverse ratio between myofibrillar protein content and pressure applied (r^2 =0.67)was implied at month 0, being this inverse relationship maintained throughout the whole study. Meantime, a myofibrillar protein content decrease with storage time (r^2 = 0.86-0.94) resulted in a significant loss at the end of storage time for all samples and also after 6 months for the 200-MPa treated samples. Since the content of these proteins de- creased by increasing the pressure level applied, lower mean values were observed throughout the whole experiment in fish subjected to the highest HP treatment. Electrophoretic analysis of the sarcoplasmic and myofibrillar protein fractions (data not shown) showed that the pressure treatment and frozen storage time did not modify the 1-D electrophoretic pattern.

This higher sensitivity of myofibrillar protein fraction in comparison to the sarcoplasmic one contradicts most previous re- search (Campus 2010; Tabilo-Munizaga et al. 2016). Thus, and concerning the combination of HP processing and frozen stor- age, opposite results to those observed in this study were previ- ously reported for mackerel (Pazos et al. 2015); in it, HP processing (150-200 MPa, 0 min) and frozen storage (-18 °C for 9 months) did not change the electrophoretic pattern of myofibrillar proteins, but a 28.7-kDa band loss was observed in the sarcoplasmic fraction of frozen fish samples previously treated at 175 and 200 MPa and identified as phosphoglycerate mutase. Also differing from the present study, a marked decrease in sarcoplasmic protein content was evident in chilled HP-treated (170 and 200 MPa, 30 s) Coho salmon (Oncorhynchus kisutch); as in the study on mackerel, a partial loss of phosphoglycerate mutase was detected and identified by mass spectrometry (Ortea et al. 2010). However, and in agreement with the actual research, Hernández-Andrés et al. (2008) reported a marked degradation of myofibrillar proteins (i.e., actin and myosin) in sardine muscle after a 300-MPa/20-min/7 °C treatment.

After freezing (month-0 comparison), the TMA-N content (Table 4) increased strongly ($p < 0.05$) in all kinds of samples; at this time, an inhibitory effect on TMA formation for samples previously treated at the two highest pressure levels was obtained, showing an inverse ratio between the TMA-N con- tent and the pressure applied (r^2 =0.67). After this time, scarce differences were attained among samples so that a definite trend could not be concluded, concerning the effect of the pressure applied. However, all kinds of samples showed progressive increases ($p < 0.05$) with frozen storage time (r^2 =0.90-0.95), with a sharp increase at the end of the experiment. In agreement with the close relationship between TMA formation and protein breakdown, a good inverse correlation value was obtained between the TMA-N value and myofibrillar protein content (r^2 =0.79-0.91).

TMA formation can be interpreted as the conversion of trimethylamine oxide (TMAO) into TMA by non-enzymatic processes or by endogenous enzymes or produced by micro- organisms before the HP or the freezing process. An alternative is protein breakdown caused by the HP treatment and by further damage during frozen storage. Results similar to those obtained in this study were observed during the frozen storage (-18 °C) of control and 125-200-MPa treated mackerel (Pazos et al. 2015), showing a progressive TMA content in- crease throughout the 9-month frozen storage. However, when applying higher pressure levels (200, 300, and 400 MPa) and longer pressure holding times (5 and 15 min), Senturk and Alpas (2013) observed a higher TMA formation as a result of the HP treatment when compared to control fish.

Enzyme Activity Analysis

A marked inverse ratio was observed between lipase activity (Fig. 2a) and pressure applied throughout the whole experiment (r^2 =0.73-0.92). Lipase activity showed negligible changes throughout the 0-6-month frozen

storage of all samples, while an increase ($p < 0.05$) was generally observed after 9 months. Control samples showed the highest mean values throughout the whole storage period. Differences were significant ($p < 0.05$) for all pressurized samples during months 1-6 of frozen storage. At month 9, lower values ($p < 0.05$) were observed in 175- and 200-MPa samples when compared to control fish. Lipase activity inhibition and FFA formation showed a good agreement ($r^2 = 0.67$-0.82). Lipolysis has been associated to quality deterioration during the frozen storage of fish muscle (Sikorski and Kolakowska 1994; Verma et al. 1995). In this context, an important role has been accorded to lipase enzymes catalyzing the hydrolysis of tri- glycerides to FFA, glycerol, and partially hydrolyzed glycerides (Hendrickx et al. 1998; Aubourg et al. 1998). In agreement with this study, Fidalgo et al. (2014) observed a reduction in the lipase activity in frozen mackerel previously treated under stronger HP conditions (150, 300, and 450 MPa for 0, 2.5, and 5 min); in such study, the inhibitory effect observed increased with the pressure level and the pressure holding time of the HP treatment.

The barely detectable differences in acid phosphatase activity observed in all fish samples during the 0-3-month frozen storage period (Fig. 2b) were followed by a general in- crease at months 6 and 9, yielding a good correlation with storage time ($r^2 = 0.94$-0.99). Thus, a definite trend could not be concluded between phosphatase activity and pressure applied for the 0-3-month period and month 9, while an in- verse ratio ($r^2 = 0.77$) was obtained at month 6. Comparisons among HP treatments showed some significant differences but with no defined trend. Good linear correlation values were obtained for the acid phosphatase activity when compared to

Table 4 Trimethylamine (TMA; mg TMA-N kg^{-1} flesh) assessment in frozen sardine muscle previously processed under different high-pressure conditions

Frozen storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
Initial fish	0.23a (0.04)	0.23a (0.04)	0.23a (0.04)	0.23a (0.04)	0.23a (0.04)
0	2.48bB (0.24)	3.31bcB (0.83)	2.51bB (0.26)	1.58bA (0.16)	1.52bA (0.42)
1	3.70c (0.18)	3.60b (0.26)	3.64c (0.60)	3.79c (0.40)	3.30c (0.08)
3	3.55cA (0.12)	4.41cB (0.33)	4.39cB (0.28)	4.35cB (0.17)	4.52cB (0.16)
6	5.88dAB (0.48)	5.87dAB (0.49)	6.57dB (0.18)	5.56dA (0.37)	6.08dAB (1.04)
9	8.20eAB (0.57)	9.36eB (0.41)	8.64eAB (1.07)	8.64eAB (0.07)	8.09eA (0.05)

Mean values of three replicates ($n = 3$); standard deviations are indicated in brackets. For each frozen storage time, mean values followed by different capital letters (A and B) indicate significant differences ($p < 0.05$) as a result of the pressure treatment. For each pressure treatment condition, means followed by different lowercase letters (a–e) indicate significant differences ($p < 0.05$) as a result of frozen storage time. No letters are included when no differences are found

chemical parameters such as TMA ($r^2 = 0.86$-0.95) and FFA (0.91-0.94) contents.

The analysis of cathepsin B activity showed a general de- crease at month 1, followed by a marked increase at month 3 andthenatmonth9(Fig.3a). Although the comparison among samples showed some significant differences, no definite HP treatment effect could be concluded; additionally, a definite relationship between cathepsin B activity and pressure applied could not be implied. However, good correlation values were obtained with chemical indices related to quality loss such as the TBA-i ($r^2 = 0.77$-0.89) and the TMA-N content ($r^2 = 0.78$-0.86). Cathepsin D activity assessment showed a progressive increase with storage time in control

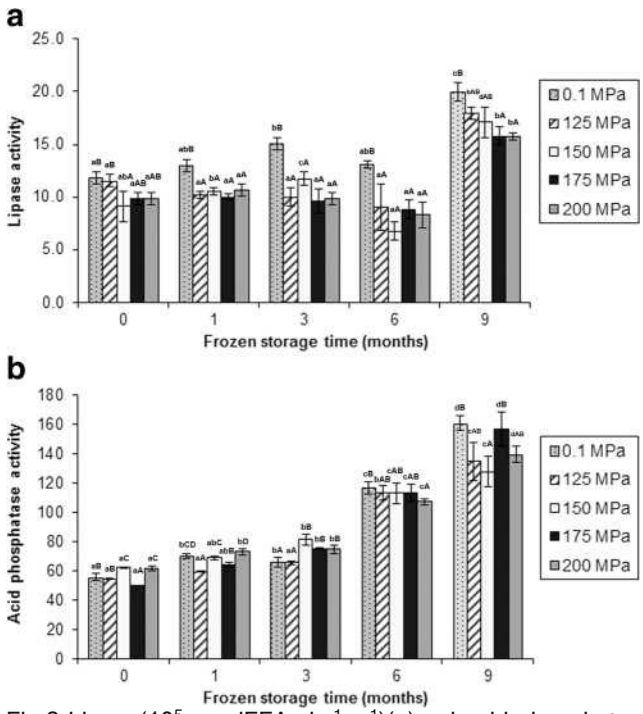

Fig.2 Lipase(10^5 pmolFFAmin^{-1} g^{-1})(a)and acid phosphatase(mmol p-NP min^{-1} g^{-1}) (b) activities in frozen sardine muscle previously processed under different high-pressure conditions*. *Mean values of three replicates (n = 3); standard deviations are indicated by bars. For each frozen storage time, values accompanied by different letters (A-D) denote significant differences ($p < 0.05$) as a result of the previous treatment. For each pressure treatment condition, means followed by different lowercase letters (a-d) indicate significant differences ($p < 0.05$) as a result of frozen storage time

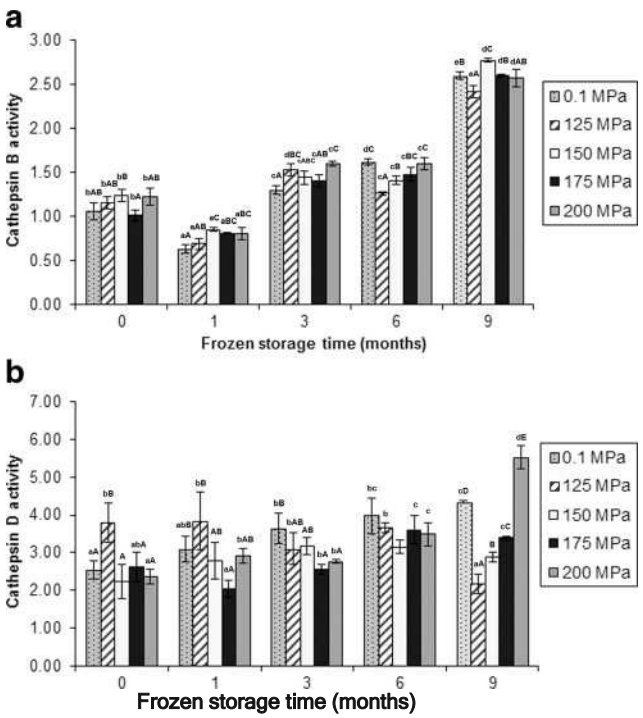

Fig. 3 Evolution of cathepsin B (10^5 FU min^{-1} g^{-1}) (a) and cathepsin D (pg tyrosine min^{-1} g^{-1}) (b) activities in frozen sardine muscle previously processed under different high-pressure conditions*. *Mean values of three replicates (n = 3); standard deviations are indicated by bars. For each frozen storage time, mean values accompanied by different capital letters (A-E) indicate significant differences ($p < 0.05$) as a result of the pressure treatment. For each pressure treatment condition, means accompanied by different lowercase letters (a-e) indicate significant differences ($p < 0.05$) as a result of frozen storage time. No letters are included when significant differences were not found

and 200-MPa samples, while no effect was observed in other samples (Fig. 3b). Although the effect of the previous pressure treatment showed no general trend by comparison of the different kinds of samples, lower values ($p < 0.05$) were observed after 9 months of frozen storage in 125-, 150-, and 175-MPa samples when compared to control fish. Thus, a definite trend could not be concluded between cathepsin D activity and pressure applied for the 0-1- and 6-9-month periods; however, an inverse ratio was observed at month 3 ($r^2 = 0.86$).

Previous research on the contribution of proteolytic systems to muscle degradation in HP-treated fish has focused on two groups of endogenous enzymes, i.e., calpains and cathepsins. Their effects on fish muscle depend on several factors that may cause activation or inactivation with reversible or irreversible effects and all showing a great dependence on the applied HP conditions. Activity level of enzymes can in- crease due to

the low-temperature disruption of lysosomes and by activity recovery caused by enzyme renaturation that may occur during frozen storage. Consequently, the enzyme activity observed in this study may be the result of two opposite effects. HP treatment conditions may lead to a marked activity inhibition by disrupting bonds determining their secondary, tertiary, and quaternary conformations (Ashie et al. 1996; Campus 2010). On the other side, HP treatment may disrupt lysosomal membranes and cause release of proteases into the fish muscle, provoking a hydrolytic activity increase (Ohmori et al. 1992; Chéret et al. 2005).

In agreement with the present research, the activity in frozen mackerel of acid phosphatase and cathepsins B and D was not modified by pressure (125-200 MPa, 0 min) treatments prior to freezing (Pazos et al. 2015). However, the application of higher pressure and holding times (150-450 MPa, 0-5min) showed an inhibitory effect on the cathepsin B activity, which increased with the pressure level and holding time applied on the same fish species (Fidalgo et al. 2014). Additionally, previous research accounts for different experiments applying higher pressures than in the present research. Thus, Hernández-Andrés et al. (2008) found an inhibitory effect on proteolytic activity after HP treatment (300 MPa, 10 min, 7 °C). Chéret et al. (2005) observed that after a 500-MPa/5- min treatment, cathepsin B activity in sea bass (Dicentrarchus labrax) muscle increased almost fivefold; however, Teixeira et al. (2013) observed for the same fish that 100-450-MPa treatments did not affect the activity of cathepsin B. Further, Ohmori et al. (1992) showed that 200-300-MPa treatment increased the acid phosphatase activity in the cytosolic fraction but decreased it in the lysosomal fraction.

Conclusions

Changes in biochemical indicators related to quality loss were analyzed during frozen storage (-18 °C, 9 months) of sardine previously treated under different HP conditions. Among the different damage pathways considered, lipid hydrolysis development (FFA content), formation of secondary lipid oxidation compounds (TBA-i), and protein denaturation or breakdown (myofibrillar protein content) showed to be the most important deteriorative events of quality loss in the present study. Inhibition ($p < 0.05$) of lipid hydrolysis development (lower FFA formation and lipase activity) could be observed as a result of the HP treatment, this effect being stronger when the pressure level applied was increased. Several lipid oxidation indices (PV, TBARS, fluorescence ratio, and PI) showed that the HP treatment did not increase the development of lipid oxidation. Additionally, the HP treatment did not lead to a substantial modification of the activity of acid phosphatase and cathepsins B and D, as well as in the electrophoretic pattern of sarcoplasmic and myofibrillar protein fractions. Furthermore, the contents of sarcoplasmic proteins and TMA were not affected by treatments in the pressure range used in this study. However, HP processing led to a myofibrillar protein content decrease, this effect being higher in samples corresponding to the 175- and 200-MPa treatments. In conclusion, this research showed that pressure treatments with no holding time in the 125-200-MPa range cause only minor modifications in biochemical indicators throughout the subsequent frozen storage of samples for up to 9 months. This study shows the need to optimize HP conditions, particularly in the case of applications combining HP treatments, freezing, frozen storage, and thawing to obtain products with high quality and commercial viability.

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