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4 Lipid and protein changes related to quality loss in frozen
5 sardine (*Sardina pilchardus*) previously processed under
6 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 values, thiobarbituric acid index, fluorescent compounds and polyenes, showed that the applied HP conditions prior to sardine freezing did not increase lipid oxidation ($p>0.05$). Also, HP did not induce ($p>0.05$) 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 with no holding time in the 125-200 MPa range with holding time of 0 min cause only minor modifications in biochemical indicators of deterioration 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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Running Title: Biochemical changes in frozen sardine previously pressurized.

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 inactivating inhibiting microbial populations activity and leading to shelf-life extension and safety enhancement 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 peroxides and carbonyls compounds), accompanied by off-odors, browning and modifications of the quaternary (dissociation of oligomers), tertiary (modification of hydrophobic and ionic interactions), and secondary (modification of hydrogen bonds) 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

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nutritional properties. This optimization should take into account 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 treatments of sardine, the gel-formingification 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 (Núñez-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 (125, 150, 175 and 200 MPa) 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.

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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.

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MATERIALS AND METHODS

Raw fish, HP processing, frozen storage and sampling

Sardine (156 individuals) caught near the Bask coast in Northern 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 hours 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.

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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). ver paper previo o su explicac en carta. 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. Cold The high-pressure equipment employed cold pressurizing water was used to maintain temperature isothermal conditions (20°C) during HP treatment. at room temperature (20°C).

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HP-treated sardines (120 individuals) were analyzed after 48 hours 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 extract from the fish white muscle pooled from two individual fish was employed.

~~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.~~ In this study focusing on commercial frozen storage conditions (-18°C), the pressure levels chosen for sardine, a close pelagic fish species, were 150 MPa, and a lower (125 MPa) and two higher (175 and 200 MPa) values, all with zero holding time.

Lipid extraction and quality indices dDetermination of ILipid deterioration amage analysindices

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 (1976) spectrophotometric method (715 nm, Beckman Coulter DU 640, London, UK) based on

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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 determined 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 (TBARS) 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 nm 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 µg 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:

$$FR = RF_{393/463 \text{ nm}} / RF_{327/415 \text{ nm}}.$$

Lipid extracts were converted into fatty acid methyl esters (FAME) 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 µm film, Supelco Inc., Bellefonte, PA, USA) and using nitrogen at 10 psi as carrier gas (linear flow rate of 1.0 mL min⁻¹) and, a flame ionization detector (FID) at 250 °C, and C 19:0 fatty acid as internal standard for quantitative analysis (Aubourg et al. 1997). Peaks corresponding to fatty acids were identified by comparison

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of the retention times of two standards 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 20:5ω3 + C 22:6ω3) / C 16:0.

Protein damage assessment

Sarcoplasmic (low-salt-soluble) and myofibril (high-salt-soluble) proteins extraction from the sardine muscle was performed by modification of a previously published protocol (Pazos et al. 2011). Briefly, 0.5 g were homogenized using an Ultra-Turrax high-performance disperser in ten 5 mL volumes of Tris buffer (10 mM Tris-HCl, pH 7.2), containing 5 mM protease inhibitor (phenylmethylsulphonyl 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. 1-D gels were run in a Mini-PROTEAN 3 cell (Bio-Rad) with an aqueous running buffer composed by 1.44

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% (w/v) glycine, 0.67 % Tris-base, and 0.1 % SDS (Laemmly 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 described (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 8,000 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 14,600×g for 20 min at 4 °C. The supernatant was filtered through a Whatman n° 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 μmol 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.

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Cathepsin B activity was evaluated by the methodology described by Lakshmanan et al. (2005), being its activity expressed as fluorescence units (FU) $\text{min}^{-1} \text{g}^{-1}$ muscle. Cathepsin D activity was analyzed following the protocol described by Buckow et al. (2010), with few modifications. Enzyme extracts (0.2 mL) were mixed with substrate solution (0.6 mL of 2 % 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 18,000×g (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 μg tyrosine $\text{min}^{-1} \text{g}^{-1}$ muscle.

Statistical analysis

Fish samples corresponding to all the conditions tested were analyzed after 0, 1, 3, 6 and 9 months of frozen storage time. Data ($n = 3$) obtained from the different biochemical parameters were subjected to one-way ANOVA method ($p < 0.05$) to explore differences as a result of 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

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257 FFA formation via lipid hydrolysis is described in Figure 1 (Panel A; g kg⁻¹ muscle).
 258 Values were calculated on the basis of the lipid content in sardine muscle (5.3-9.5 g
 259 lipids kg⁻¹ muscle range; data not shown) obtained throughout the 0-9-month period. A
 260 an inverse relationship between FFA content and the pressure applied was observed,
 261 being the best correlation value; this correlation attained the best values for the 3-6-
 262 month period ($r^2 = 0.71-0.77$). The initial fresh fish value (0.07±0.01 g FFA kg⁻¹
 263 muscle) led to a marked FFA increase in all kinds of samples after the freezing step
 264 (month-0 frozen samples); After freezing (month-0 frozen samples), a marked FFA
 265 content increase ($p<0.05$) was observed in all cases (initial fresh fish value: 0.7±0.1 g
 266 kg⁻¹ muscle); however, this increase was found to be lower ($p<0.05$) in samples
 267 corresponding to the two highest pressure levels applied (175 and 200 MPa).
 268 Afterwards (3-9-month period) the FFA formation showed a progressive increase for all
 269 samples throughout the frozen storage ($r^2 = 0.90-0.93$, linear fitting). Compared to
 270 control fish, the 175-MPa-treated fish showed a lower ($p<0.05$) lipid hydrolysis for the
 271 0-6-month period; in the case of samples corresponding to the highest pressure, a lower
 272 ($p<0.05$) FFA content was obtained at months 0, 3 and 6, with mean values lower than
 273 those for the control fish throughout the storage period. Consequently, a pressure
 274 treatment prior to freezing can be concluded to have an inhibitory effect on the FFA
 275 formation. Similar conclusions were obtained when FFA content was expressed on a
 276 lipid content basis (g kg⁻¹ lipids). In this case (data not shown), FFA formation also
 277 showed a good correlation value with the storage time ($r^2 = 0.87-0.91$, linear fitting).

278 Accumulation of FFA via lipid hydrolysis in fish muscle has no nutritional
 279 significance, but it has undesirable secondary effects including muscle texture changes
 280 and lipid oxidation acceleration (Sikorski and Kolakowska 1994), and off-odor and off-
 281 taste development (Refsgaard et al. 2000). In spite of the fact that sardine has shown a

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marked FFA formation throughout the frozen storage (Verma et al. 1995; Aubourg et al. 1998). 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 (*Scomber scombrus*; Vázquez et al. 2013) and horse mackerel (*Trachurus trachurus*; Torres et al. 2013), were subjected to HP treatment (150, 300, 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 reduction of FFA content 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. HP pre-treated samples, with the reduction effect increasing with the previous pressure level and the pressure holding time employed. By checking different frozen storage conditions to the present ones, Ohshima et al. (1992) found that enzymatic degradation of phospholipids (i.e., phospholipases action) in cod (*Gadus morhua*) muscle was successfully 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*) fillets (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.

Previous research showed an important FFA formation throughout the frozen storage of sardine (Verma et al. 1995; Aubourg et al. 1998). Accumulation of FFA in

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fish muscle has no nutritional significance, but it has undesirable secondary effects including muscle texture changes and lipid oxidation acceleration (Sikorski and Kolakowska 1994), and off odor development (Refsgaard et al. 2000). Research reported concerning the effect on FFA formation after HP treatment followed by a frozen storage is scarce. Ohshima et al. (1992) found that enzymatic degradation of phospholipids (i.e., phospholipases action) in cod (*Gadus morhua*) muscle was successfully 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 when applying a pressure of 200 MPa. Later on, freezing by pressure release (i.e., pressure shift freezing; 140 MPa) was compared with air-blast freezing (-20 °C) in turbot (*Scophthalmus maximus*) fillets (Chevalier et al. 2000); however, FFA formation during subsequent storage at -20 °C for 75 days showed no advantages. Finally, Atlantic mackerel (*Scomber scombrus*; Vázquez et al. 2013) and horse mackerel (*Trachurus trachurus*; Torres et al. 2013) were subjected to HP treatment (150, 300, 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 marked reduction of FFA content for all HP pre-treated samples, with the reduction effect increasing with the previous pressure level and the pressure holding time employed.

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 this timemonth 0, a direct relationship ($r^2 = 0.80$) 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 PV 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 PV 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 increase, so that an inverse relationship between TBA-iI and pressure applied was obtained ($r^2 = 0.85$); however, as definite relationship between both parameters could not be implied for the 1-9-month period. This increase was found higher ($p < 0.05$) in control fish when compared to samples corresponding to the two highest pressure conditions. 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 conditiontreatment. 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

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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 this timemonth 0, a poor inverse correlations 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, After this time, 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. At the end of the experiment, the highest mean value was obtained for control 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.

Previous research was in agreement with the lipid oxidation effects observed in the HP treatment of sardine. However, these previous studies had not examined the combination of HP treatment, freezing and frozen storage. Previous research showed an inhibitory effect on TBARS formation at 300 MPa/10 min/7 °C combined with functional gelatin-lignosulphonate film to preserve sardine fillets during chilled storage (Núñez-Flores et al. 2013). Peroxide and TBARS content 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). Lipid oxidation is a complex process producing many different compounds, most of them 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,

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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 compounds 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. 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-lignosulphonate film to preserve sardine fillets during chilled storage (Núñez-Flores et al. 2013). Peroxide and TBARS content 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).

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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 the 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 compounds formation in frozen (−10 °C up to 3 months) mackerel (Vázquez et al. 2013) and horse mackerel (Torres et

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al. 2013) by previous HP treatment (150, 300, 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 (−2°C) storage (−2°C) study, Ohshima et al. (1992) reported an increased TBARS formation in frozen (−2 °C for six days) cod (*Gadus morhua*) and mackerel (*Scomber scombrus*) muscle previously subjected to HP treatments (616, 408 and 204 MPa for 15 and 30 min). Additionally, a On the other hand, a comparison of pressure shift freezing at 140 MPa with air-blast freezing (−20 °C) did not provide differences a definite HP effect on TBARS formation during subsequent storage at −20 °C for 75 days of turbot (*Scophthalmus maximus*) fillets (Chevalier et al. 2000). Closely related to this study, a marked inhibition of tertiary lipid oxidation compounds 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, 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.

Analysis of protein changes

No HP treatment or frozen storage time effects were observed in the analysis of sarcoplasmic protein content (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 the myofibrillar proteins content (Figure 1, Panel B). Thus, an inverse ratio between myofibrillar protein content and pressure applied decrease with storage time ($r^2 = 0.6786-0.94$, quadratic fitting; Figure 1, Panel B) was implied at month 0, being this inverse relationship maintained throughout the whole study. Meantime, a resulted in 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

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432 200-MPa treated samples. Since the content of these proteins decreased by increasing
433 the pressure level applied, lower mean values were observed throughout the whole
434 experiment in fish subjected to the highest HP treatment. Electrophoretic analysis of the
435 sarcoplasmic and myofibrillar protein fractions (data not shown), showed that the
436 pressure treatment and frozen storage time did not modify the 1-D electrophoretic
437 pattern.

438 This higher sensitivity of myofibrillar protein fraction in comparison to the
439 sarcoplasmic one contradicts most previous research (

440 Because of their greater presence in marine species, previous research has shown the
441 greatest attention to changes produced in myofibrillar fraction (Campus 2010; Tabilo-
442 Munizaga et al. 2016). Thus, However, sarcoplasmic proteins have shown to be more

443 sensitive to the HP treatment than any other protein group. Thus, and concerning the
444 combination of HP processing and frozen storage, opposite results to those observed in
445 this study were previously reported for mackerel (Pazos et al. 2015); in it, HP
446 processing (150-200 MPa, 0 min) and frozen storage (−18 °C for 9 months) did not
447 change the electrophoretic pattern of myofibrillar proteins, but a 28.7 kDa band loss was
448 observed in the sarcoplasmic fraction of frozen fish samples previously treated at 175
449 and 200 MPa and identified as phosphoglycerate mutase. Also differing from the
450 present study, a marked decrease in sarcoplasmic protein content was evident in chilled
451 HP treated (170 and 200 MPa, 30 s) Coho salmon (*Oncorhynchus kisutch*); a. As in the
452 study on mackerel, a partial loss of phosphoglycerate mutase was detected and
453 identified by mass spectrometry (Ortea et al. 2010). However, and in agreement with the
454 actual research, Hernández-Andrés et al. (2008) reported a markedn important
455 degradation of myofibrillar proteins (i.e., actin and myosin) in sardine muscle after a
456 300 MPa/20 min/7 °C treatment.

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After freezing (month-0 comparison), the TMA-N content (Table 4) increased strongly ($p < 0.05$) in all kinds of samples and then progressively increased ($p < 0.05$); 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 content and the pressure applied ($r^2 = 0.67$) with frozen storage time in all fish samples ($r^2 = 0.90-0.95$). Thus, a general sharp increase ($p < 0.05$) was observed at the end of storage time. Comparison among fish samples showed an inhibitory effect on TMA formation at month 0 for samples previously treated at the two highest pressure levels. 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 relationship was obtained between the TMA-N value and with FFA ($r^2 = 0.83-0.95$) and TBARS ($r^2 = 0.73-0.95$) formation and with myofibrillar protein content ($r^2 = 0.79-0.91$, inverse ratio).

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 microorganisms 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 increase throughout the 9-month frozen storage. However, when applying higher pressure levels (200, 300, and 400 MPa) and longer

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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

Kinetic ... A marked inverse ratio was observed between lipase activity (Figure 2, Panel A) 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 (Figure 2, Panel A), 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 (Table 1 Figure 2, Panel A) 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 triglycerides 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 HP-treated under stronger HP conditions (150, 300 and 450 MPa for 0, 2.5 and 5 min); in such study, t. 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 (Figure 2, Panel B) were followed by a general increase at months 6 and 9 yielding a good correlation with

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

The analysis of the cathepsin B s activity showed a general decrease at month 1 of cathepsin B, followed by a marked increase at month 3 and then at month 9 (Figure 3, Panel A). 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 and 200-MPa samples, while no effect was observed in other samples (Figure 3, Panel B). 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-month 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

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great dependence on the applied HP conditions. Activity level of enzymes can increase 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, 0-5 min) 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, pPrevious research accounts for different experiments applying higher pressures than in the present research. Thus,

Concerning sardine (*Sardina pilchardus*) muscle, HHernández-Andrés et al. (2008) found an inhibitory effect on proteolytic activity after HP treatment (300 MPa, 10 min, 7 °C). In other fish species, Chéret et al. (2005) observed that after a 500 MPa/5 min treatment, cathepsin B activity in sea bass (*Dicentrarchus labrax*) muscle increased almost 5-fold; 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. ~~In agreement with the~~

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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, 0-5 min) 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).

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 proteins 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 ($p > 0.05$) the development of lipid oxidation. Additionally, the HP treatment did not lead ($p > 0.05$) 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 content 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

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582 treatments with no holding time in the 125-200 MPa range cause only minor
583 modifications in biochemical indicators throughout the subsequent frozen storage of
584 samples for up to 9 months. This study shows the need to optimize HP conditions,
585 particularly in the case of applications combining HP treatments, freezing, frozen
586 storage, and thawing to obtain products with high quality and commercial viability.

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FIGURE LEGENDS

Figure 1: Free fatty acid (FFA, g kg⁻¹ muscle; Panel A) and myofibrillar protein content (g kg⁻¹ muscle; Panel 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 low-case 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 (p>0.05).

Figure 2: Lipase (10⁵ μmol FFA min⁻¹ g⁻¹; Panel A) and acid phosphatase (mmol *p*-NP min⁻¹ g⁻¹; Panel 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 low-case letters (a-d) indicate significant differences (p<0.05) as a result of frozen storage time.

Figure 3: Evolution of cathepsin B (10^5 FU min⁻¹ g⁻¹; Panel A) and cathepsin D (μ g tyrosine min⁻¹ g⁻¹; Panel 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 low-case 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. ($p > 0.05$).

HIGHLIGHTS

- Biochemical changes in frozen (−18 °C for 9 months) sardine (*Sardina pilchardus*)
- Effect of previous high-pressure (HP) processing (125-200 MPa)
- Increasing previous HP treatment led to increasing lipid hydrolysis inhibition
- No effect on lipid oxidation development and proteases activity
- Increased myofibrillar protein content by increasing previous HP processing

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	0.68 ^a (0.11)	0.68 ^a (0.11)	0.68 ^a (0.11)	0.68 ^a (0.11)	0.68 ^a (0.11)
0	1.89 ^{bA} (0.80)	2.31 ^{cA} (0.37)	2.48 ^{bAB} (1.09)	3.63 ^{cAB} (1.00)	3.55 ^{cB} (0.17)
1	5.85 ^{cB} (0.31)	4.49 ^{dB} (1.21)	6.37 ^{cB} (1.50)	1.64 ^{bA} (0.18)	4.29 ^{cB} (1.94)
3	10.61 ^{dB} (2.27)	11.62 ^{eB} (1.24)	13.48 ^{dB} (2.02)	6.28 ^{dA} (1.23)	4.73 ^{cA} (1.15)
6	5.84 ^{cAB} (2.13)	4.21 ^{dAB} (1.37)	7.17 ^{cB} (1.83)	4.39 ^{cdAB} (2.11)	3.26 ^{bcA} (1.08)
9	1.83 ^{abAB} (1.01)	1.30 ^{bA} (0.08)	3.79 ^{bB} (1.17)	3.28 ^{cB} (0.51)	1.54 ^{bA} (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-B) indicate significant differences (p<0.05) as a result of the pressure treatment. For each pressure treatment condition, means followed by different low-case 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	0.18 ^a (0.08)	0.18 ^a (0.08)	0.18 ^a (0.08)	0.18 ^a (0.08)	0.18 ^a (0.08)
0	0.50 ^{bC} (0.07)	0.41 ^{bcBC} (0.10)	0.41 ^{bcABC} (0.13)	0.32 ^{bB} (0.02)	0.23 ^{aA} (0.04)
1	0.53 ^{bB} (0.11)	0.38 ^{bAB} (0.06)	0.51 ^{bcB} (0.09)	0.44 ^{cAB} (0.05)	0.32 ^{aA} (0.05)
3	0.49 ^b (0.02)	0.53 ^c (0.06)	0.52 ^{bc} (0.09)	0.50 ^c (0.05)	0.50 ^b (0.08)
6	0.63 ^B (0.03)	0.58 ^{cAB} (0.10)	0.46 ^{bA} (0.01)	0.54 ^{cdAB} (0.12)	0.52 ^{bAB} (0.11)
9	0.76 ^c (0.03)	0.77 ^d (0.05)	0.66 ^c (0.11)	0.74 ^d (0.11)	0.80 ^c (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 low-case 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.

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	0.30 ^a (0.07)	0.30 ^a (0.07)	0.30 ^a (0.07)	0.30 ^a (0.07)	0.30 (0.07)
0	0.45 ^{bB} (0.03)	0.43 ^{bB} (0.01)	0.41 ^{bAB} (0.06)	0.35 ^{aA} (0.04)	0.42 ^{AB} (0.05)
1	0.38 ^{ab} (0.08)	0.31 ^a (0.05)	0.33 ^a (0.07)	0.40 ^{ab} (0.05)	0.38 (0.01)
3	0.33 ^{aAB} (0.06)	0.30 ^{aA} (0.02)	0.34 ^{aAB} (0.05)	0.36 ^{aAB} (0.03)	0.49 ^B (0.10)
6	0.36 ^{abAB} (0.09)	0.34 ^{aA} (0.04)	0.48 ^{bB} (0.03)	0.43 ^{abAB} (0.07)	0.43 ^{AB} (0.05)
9	0.54 ^{cB} (0.03)	0.38 ^{abA} (0.06)	0.42 ^{abAB} (0.11)	0.45 ^{bA} (0.03)	0.45 ^{AB} (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-B) indicate significant differences ($p < 0.05$) as a result of the pressure treatment. For each pressure treatment condition, means followed by different low-case 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.

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	0.23 ^a (0.04)	0.23 ^a (0.04)	0.23 ^a (0.04)	0.23 ^a (0.04)	0.23 ^a (0.04)
0	2.48 ^{bB} (0.24)	3.31 ^{bcB} (0.83)	2.51 ^{bB} (0.26)	1.58 ^{bA} (0.16)	1.52 ^{bA} (0.42)
1	3.70 ^c (0.18)	3.60 ^b (0.26)	3.64 ^c (0.60)	3.79 ^c (0.40)	3.30 ^c (0.08)
3	3.55 ^{cA} (0.12)	4.41 ^{cB} (0.33)	4.39 ^{cB} (0.28)	4.35 ^{cB} (0.17)	4.52 ^{cB} (0.16)
6	5.88 ^{dAB} (0.48)	5.87 ^{dAB} (0.49)	6.57 ^{dB} (0.18)	5.56 ^{dA} (0.37)	6.08 ^{dAB} (1.04)
9	8.20 ^{eAB} (0.57)	9.36 ^{eB} (0.41)	8.64 ^{eAB} (1.07)	8.64 ^{eAB} (0.07)	8.09 ^{eA} (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-B) indicate significant differences ($p < 0.05$) as a result of the pressure treatment. For each pressure treatment condition, means followed by different low-case 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.

Figure 1

Figure 2

Figure 3