OSU Oregon State UNIVERSITY

Open Access Articles

Effect of high pressure processing of Atlantic mackerel (Scomber scombrus) on biochemical changes during commercial frozen storage

The Faculty of Oregon State University has made this article openly available.
Please share how this access benefits you. Your story matters.

Citation	Plazos, M., Méndez, L., Fidalgo, L., Vázquez, M., Torres, J. A., Aubourg, S. P., & Saraiva, J. A. (2015). Effect of high pressure processing of Atlantic mackerel (Scomber scombrus) on biochemical changes during commercial frozen storage. [Article in Press]. Food and Bioprocess Technology.
DOI	
Publisher	Springer
Version	Accepted Manuscript
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsofuse

Oregon State UNIVERSITY OSU

1

2

3

4

5 # Effect of high pressure processing of Atlantic mackerel

6 # (*Scomber scombrus*) on biochemical changes during

7 # commercial frozen storage

8

9

10

11 Manuel Pazos[1], Lucía Méndez[1], Liliana Fidalgo[2], Manuel Vázquez[3],

12 J. Antonio Torres[4], Santiago P. Aubourg[1,*], and Jorge A. Saraiva[2]

13

14

15

16

17 [1] Department of Food Technology, Instituto de Investigaciones Marinas (CSIC), 36208-

18 Vigo, Spain

19 [2] Research Unit of Organic Chemistry, Natural and Agro-food Products (QOPNA),

20 Chemistry Department, Aveiro University, Campus Universitário de Santiago,

21 3810-193 Aveiro, Portugal.

22 [3] Department of Analytical Chemistry, Faculty of Veterinary Science, University of

23 Santiago de Compostela, 27002-Lugo, Spain

24 [4] Food Processing Engineering Group, Department of Food Science & Technology,

25 Oregon State University, Corvallis, OR 97331, USA

26 * Corresponding author. Phone: +34986231930; Fax: +34986292762; e-mail:

27 saubourg@iim.csic.es

28

ABSTRACT

This research focuses on biochemical changes related to quality losses observed in Atlantic mackerel (*Scomber scombrus*) muscle stored under commercial frozen storage conditions (9 months, -18 ºC) when subjected to high-hydrostatic pressure (HHP) treatments (125, 150, 175 and 200 MPa for 0 min) before freezing. After freezing, free fatty acid (FFA) formation (lipid hydrolysis assessment) showed a marked inhibition in HHP-treated fish and during frozen storage of samples treated at 175 MPa. Fluorescence ratio (FR) assessment of tertiary lipid oxidation showed a partial inhibitory effect during the 0-9-month period for samples treated at 175 and 200 MPa. After 3 months storage of samples treated at these pressure levels, one-dimensional SDS-PAGE analysis of the sarcoplasmic protein fraction revealed the disappearance of a band; while samples treated at 150 MPa showed the same effect at month 9. After gel excision, trypsin digestion, tandem mass spectrometry (MS/MS) and sequence database analysis, the band was identified as phosphoglycerate mutase 2 (28.7 kDa). On the other hand, HHP processing did not show a significant effect on trimethylamine (TMA) values, primary and secondary lipid oxidation, PUFA levels, 1-D myofibril protein pattern and the activity of acid phosphatase and cathepsins B and D. Biochemical quality indices such as FFA, TMA, and FR, and the activity of acid phosphatase and cathepsin B showed a progressive increase throughout the frozen storage of all samples.

Running Title: High-pressure mackerel treatment before freezing and commercial frozen storage

Keywords: *Scomber scombrus*; high pressure processing; frozen storage; lipids; proteins; enzymes

INTRODUCTION

During frozen fish storage, changes in chemical constituents may lead to marked quality losses as a result of texture, flavor and color deterioration and the breakdown of nutritional components. Factors such as the freezing and frozen storage conditions and the quality of the raw material have been reported as responsible for such quality losses. Several hypotheses have been proposed to explain the degradation of fish proteins caused by freezing and frozen storage including partial dehydration, interaction with deteriorative molecules (oxidized lipids, free fatty acids, formaldehyde, etc.), and alteration of the microenvironment (Mackie 1993; Sikorski and Kolakowska 1994). Lipid oxidation is a most important quality loss factor in fatty fish species since the presence of highly unsaturated fatty acids and pro-oxidant molecules can lead to substantial enzymatic and non-enzymatic rancidity (Harris and Tall 1994; Sikorski and Kolakowski 2000).

High-hydrostatic pressure (HHP) processing is a non-thermal technology applied commercially in the 100-700 MPa range allowing sensory and nutritional retention of food while inactivating microbial populations and endogenous enzymes (Ashie et al. 1996; Norton and Sun 2008; Mújica-Paz et al. 2011). Although covalent bonds are not broken by HHP processing, weak hydrogen and hydrophobic bonds can be irreversibly modified. Consequently, low-molecular weight food components such as vitamins are not affected, whereas high-molecular weight molecules such as enzymes and other functional proteins can be modified (Groß et al. 1993; Campus 2010). Previous research has shown a wide range of practical benefits when HHP is applied prior to subsequent processing or storage such as products to be further refrigerated (Chéret et al. 2006), chilled (Ortea et al. 2010) or cold-smoked stored (Lakshmanan et al. 2005). In the case of fish freezing, frozen storage and thawing, pressure-shift technology has shown

80 improvements in protein denaturation, water-holding capacity and toughening
81 (Chevalier et al. 2000; Tironi et al. 2010). In the case of frozen fish storage, recent
82 studies have focused on the potential of HHP treatment prior to freezing. Thus, sensory,
83 physical (Aubourg et al. 2013), chemical (Vázquez et al. 2013) and enzymatic (Fidalgo
84 et al. 2014) changes were analyzed in Atlantic mackerel (*Scomber scombrus*) muscle
85 HHP-treated (150, 300 and 450 MPa for 0, 2.5 and 5 min) prior to freezing and then
86 stored under accelerated frozen storage conditions (-10 ºC). As a result, sensory
87 acceptance and nutritional quality retention throughout the frozen storage showed to be
88 improved when the treatment applied was 150-MPa for 0 min.

89 Atlantic mackerel is a small pelagic fish species captured in large amounts
90 during periods of relatively low demand. Previous research has shown an important
91 endogenous pro-oxidant activity and significant quality loss during its frozen storage
92 (Saeed and Howell 2001; Aubourg et al. 2005). Consequently, Atlantic mackerel
93 remains underutilized reflecting mainly its poor frozen shelf-life. HHP treatment of
94 Atlantic mackerel has been reported to inactivate *Anisakis simplex* larvae in raw fish
95 (Brutti et al. 2010). Gelification conditions of mackerel muscle by HHP processing
96 were optimized by addition of natural antioxidants (Montero et al. 2005). The effect on
97 the physico-chemical quality parameters of refrigerated (4 ºC) mackerel subjected to
98 HHP processing (200, 300 and 400 MPa for 5 and 15 min at 5, 10 and 15 ºC) was
99 recently evaluated also (Senturk and Alpas 2013). This research focuses on the quality
100 retention of Atlantic mackerel under commercial frozen conditions (9-month period at -
101 18 ºC). Based on previous work (Aubourg et al. 2013), HHP treatments at 125, 150, 175
102 and 200 MPa for 0 min were applied prior to sample freezing. Changes in lipids
103 (hydrolysis and oxidation), proteins (myofibril and sarcoplasmic fractions), and

104 enzymes activity (acid phosphatase, cathepsin B and cathepsin D) were evaluated
105 during frozen storage of mackerel muscle.

106 **MATERIALS AND METHODS**

107 **Chemicals**

108 Bicinchoninic acid (BCA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), Tris-
109 HCl and the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) were purchased
110 from Sigma-Aldrich Corp. (St. Louis, MO). Acrylamide and bis N,N´-methylene-bis-
111 acrylamide were provided by Bio-Rad Laboratories, Inc. (Hercules, CA). Glycerol was
112 obtained from Merck KGaA (Darmstadt, Germany). Ammonium persulfate (APS),
113 N,N,N',N'-tetramethylethylenediamine (TEMED) and bromophenol blue were
114 purchased from GE Healthcare Science (Uppsala, Sweden). Sequencing grade bovine
115 trypsin was purchased from Promega Corp. (Madison, WI). Trizma hydrochloride (Tris-
116 HCl), acetic acid, sodium hydroxide, citric acid, 2-bis-(2-hydroxyethyl)-amino-2-
117 (hydroxymethyl)-1,3-propanediol (Bis-Tris), ethylenediamine-tetraacetic acid (EDTA),
118 *p*-nitrophenol, thymolphtalein, trichloracetic acid (TCA), trisodium citrate and L-
119 tyrosine were also obtained from Sigma-Aldrich Co. LLC (Steinheim, Germany). The
120 enzymes substrates *p*-nitrophenylphosphate (*p*-NPP), *p*-nitrophenol (*p*-NP), Z-arginine-
121 arginine-7-amido-4-methylcoumarin hydrochloride (Z-Arg-Arg-7-AMC HCl, #C5429),
122 hemoglobin from bovine blood and olive oil were purchased from Sigma-Aldrich Co.
123 LLC. All other chemicals were of analytical grade (Panreac Quimica S.L.U., Barcelona,
124 Spain) and water was purified using a Millipore Milli-Q system (Billerica, MA).

125

126 **Raw fish, processing, frozen storage and sampling**

127 The response to HHP treatments of marine species has been reported to vary
128 with species, composition and size at capture (Ortea et al. 2010; Campus 2010).

129 Previous research on HHP-treated Atlantic mackerel stored under accelerated storage
130 conditions (-10 ºC) had shown that 150 MPa for 0 min yielded the highest sensory
131 scores (Aubourg et al. 2013), while lowering lipid damage development (Vázquez et al.
132 2013) and enzyme activity (Fidalgo et al. 2014). Accordingly, this pressure level and a
133 lower (125 MPa) and two higher (175 and 200 MPa) values were included in this study.
134 Atlantic mackerel (156 individuals) caught near the Bask coast in Northern Spain was
135 obtained at the Ondarroa harbor (Bizkaia, Spain) and transported under ice to the AZTI
136 Tecnalia (Derio, Spain) pilot plant for HHP treatment within 6 h after catch. Whole
137 mackerel individuals were placed in flexible polyethylene bags (three individuals per
138 bag) and vacuum sealed at 400 mbar. The length and weight of the specimens ranged
139 30-34 cm and 245-295 g, respectively. Samples were treated in a 55-L high pressure
140 unit (WAVE 6000/55HT; NC Hyperbaric, Burgos, Spain) at 125, 150, 175 and 200
141 MPa and 0 min holding time. Water used to pressurize samples at a rate of 3 MPa/s
142 yielded 41.7, 50, 58.3 and 66.7 s as the corresponding come up times while
143 decompression time was less than 3 s in all cases. Cold pressurizing water was used to
144 maintain temperature conditions during HHP treatment at room temperature (20 ºC).
145 After HHP treatment, mackerel (120 individuals) were kept at -18 ºC and analyzed after
146 48 hours (month 0) and after storage for 1, 3, 6 and 9 months at -18 ºC. Fish without
147 HHP treatment and subjected to the same freezing and frozen storage conditions (30
148 individuals) were used as 0.1 MPa controls. Three batches or replicates (n=3) for each
149 processing condition were analyzed independently. Analyses were carried out on the
150 fish white muscle pooled from two individual fishes. Similarly, six initial individuals
151 were distributed into three batches (two individuals per batch) and analyzed as initial
152 fish material.
153

154 **Assessment of lipid and trimethylamine contents**

155 Lipids in fish muscle were extracted with a chloroform-methanol (1:1) mixture
156 following the Bligh and Dyer (1959) method and expressed as g lipid/100 g muscle.
157 Trimethylamine-nitrogen (TMA-N) values expressed as mg TMA-N/100 g muscle were
158 obtained following the picrate method (Tozawa et al. 1971) requiring the preparation of
159 a 5% (w/v) trichloroacetic acid extract of the mackerel muscle.

160

161 **Lipid damage analysis**

162 Free fatty acids (FFA) in lipid extracts, determined by the Lowry and Tinsley (1976)
163 method, were expressed as mg FFA/100 g muscle and as g FFA/100 g lipids. Peroxide
164 value (PV) in lipid extracts, determined following the Chapman and McKay (1949)
165 method, were expressed as meq active oxygen/kg lipids. The thiobarbituric acid index
166 (TBA-i) was determined as described by Vyncke (1970). Content of TBA reactive
167 substances (TBARS) spectrophotometrically measured at 532 nm was expressed as
168 mg malondialdehyde/kg muscle. Formation of fluorescent compounds was determined
169 in the aqueous phase obtained during the lipid extraction by measurements at 393/463
170 nm and 327/415 nm (Aubourg 1999). A relative fluorescence (RF) was defined as the
171 F/F_{st} ratio where F is the fluorescence measured at each excitation and emission
172 maximum, and F_{st} is the fluorescence intensity of a quinine sulfate solution (1 μg/mL in
173 0.05 M H_2SO_4) at the corresponding wavelength. To quantify the formation of
174 fluorescent compounds, a fluorescence ratio (FR) was calculated as the ratio between
175 the two RF values: $FR = RF_{393/463\ nm} / RF_{327/415\ nm}$. Lipid extracts were converted into
176 fatty acid methyl esters (FAME) and then analyzed using a Perkin-Elmer 8700 gas
177 chromatograph (Waltham, MA) equipped with a fused silica capillary column SP-2330
178 (0.25 mm i.d. x 30 m, 0.20 μm film, Supelco Inc., Bellefonte, PA) and using nitrogen at

179 10 psi as carrier gas (linear flow rate of 1.0 mL/min), a flame ionization detector (FID)
180 set at 250 ºC, and 19:0 fatty acid as internal standard (Aubourg et al. 1996). Peaks
181 corresponding to fatty acids were identified by comparison of the retention times of two
182 standards mixtures (Qualmix Fish, Larodan, Malmo, Sweden; FAME Mix, Supelco
183 Inc.). Content of each fatty acid was calculated as g/100 g total fatty acids. The polyene
184 index (PI) was calculated as the following fatty acids ratio: (C 20:5ω3 + C 22:6ω3)/C
185 16:0.

186

187 **<u>Protein changes: analysis and identification</u>**

188 Sarcoplasmic (low-salt-soluble) and myofibril (high-salt-soluble) protein extractions
189 from the mackerel muscle were performed by modification of the protocol developed
190 by Pazos et al. (2011). Briefly, 0.5 g muscle were homogenized using an Ultra-Turrax
191 high-performance disperser in ten volumes of Tris buffer (10 mM Tris-HCl, pH 7.2),
192 containing 5 mM protease inhibitor (PMFS). The sarcoplasmic protein fraction was
193 obtained from the supernatant after centrifugation (IKA®-Werke GmbH & Co.,
194 Staufen, Germany) of the homogenate at 40,000*g* (4 ºC, 20 min). The pellet was then
195 homogenized with a saline solution (0.6 M NaCl, 10 mM Tris buffer, 5 mM PMFS, pH
196 7.2), and myofibril proteins were isolated from the supernatant obtained after
197 centrifugation at 4,500*g* (4 ºC, 20 min). Sarcoplasmic and myofibril proteins were
198 stored at -80 ºC until use. Protein concentrations were determined in both fractions by
199 the BCA assay (Smith et al. 1985) and expressed as g/100 g muscle.

200 One-dimensional (1-D) laboratory-made 10% polyacrylamide gels (v/v;
201 acrylamide:N,N´-ethylene-bis-acrylamide, 200:1) with an upper stacking gel (4 %
202 polyacrylamide) were loaded with 20 or 30 mg of protein per lane. 1-D gels were run in
203 a Mini-PROTEAN 3 cell (Bio-Rad) with an aqueous running buffer containing 1.44%

204 (w/v) glycine, 0.67% Tris-base, and 0.1% SDS (Laemmly 1970). Gels were stained
205 overnight with the Coomassie dye PhastGel Blue R-350 (GE Healthcare). Protein bands
206 of interest were manually excised from 1-D gels, and in-gel digested with trypsin as
207 previously described (Pazos et al. 2014). Tryptic peptides were analyzed by liquid
208 chromatography (LC) (Model 1260, Agilent, Palo Alto, CA) coupled to a linear ion trap
209 (LIT) mass spectrometer model LTQ Velos Pro with electrospray interface (Thermo
210 Fisher Scientific Inc., Rockford, IL). Nitrogen was used as nebulizing and drying gas
211 and helium as collision gas. The chromatographic separation was performed on a
212 BioBasic-18 column (5 μm particle size, 150 x 0.18 mm RP; Thermo Scientific,
213 Waltham, MA) at a flow rate of 1.5-1.7 μL/min with a 90 min linear gradient of mobile
214 phases A (0.5% acetic acid in water) and B (0.5% acetic acid in 100% acetonitrile) from
215 5 to 35% B. Peptides were monitored using MS survey scans from 350 to 1600 Da (2
216 μscans), followed by MS/MS scans (2 μscans) of the six more intense *m/z* peaks using a
217 1 Da isolation width, and a normalized collision energy of 35%. Singly charged ions
218 were directly excluded from MS/MS analysis, and a dynamic exclusion was set at 30 s
219 after the second fragmentation event of the same *m/z* peak. Protein identification was
220 performed using the database searching function (PEAKS 7, Bioinformatics Solutions
221 Inc, Waterloo, Ontario, Canada), to compare experimental MS/MS spectra with those
222 on the UniProtKB/Swiss-Prot database, which included their respective decoy
223 sequences. The following search limitations were used: tryptic cleavage, up to 2 missed
224 cleavage sites, and tolerances ±1.2 Da for precursor ions and ±0.8 Da for MS/MS
225 fragments ions. The variable modifications allowed were cysteine
226 carbamidomethylation and methionine oxidation. The false discovery rate (FDR) was
227 kept below 1%.
228

229 **Enzymatic activity analysis**

230 Enzymatic extracts were prepared by homogenization of fish muscle (10 g) with 50 mL
231 of ice-cold distilled water for 2 min, using an IKA Ultra-Turrax T25 homogenizer for 2
232 min at 8,000*g* and then kept in ice with occasional stirring during 30 min. Thereafter,
233 extracts were centrifuged at 14,600*g* for 20 min at 4 ºC (Laboratory Centrifuge 3K30,
234 Sigma Laborzentrifugen GmbH, Osterode, Germany). Supernatant were filtered through
235 a Whatman nº 1 filter and stored at -20 ºC prior to enzymatic activity quantifications.

236 Acid phosphatase activity was determined following the methodology described
237 by Ohmory et al. (1992) with only minor modifications. Enzymatic extracts (0.250 mL)
238 were mixed with 0.225 mL of substrate solution (4 mM *p*-NPP in 0.1 mM acetate buffer
239 and 1 mM EDTA, pH 5.5). After 15 min incubation at 37 ºC, the reaction was stopped
240 by adding 1 mL of 100 mM KOH, and measuring spectrophotometrically at 400 nm
241 (Lambda 35 UV/vis spectrometer, Perkin-Elmer Instruments, Waltham, MA) the release
242 of *p*-nitrophenol (*p*-NP). Enzyme activity was expressed as nmol *p*-NP/min/g muscle.

243 Cathepsin B activity evaluated by the method described by Lakshmanan et al.
244 (2005) was expressed as fluorescence units (FU)/min/g muscle. With few modifications,
245 cathepsin D activity was determined following the protocol described by Buckow et al.
246 (2010). Enzyme extracts (0.2 mL) were mixed with 0.6 mL of substrate solution (2%
247 denatured hemoglobin (w/v) in 200 mM citrate buffer, pH 3.7). After 3h incubation at
248 37 ºC, the reaction was stopped by adding 0.6 mL of 10% TCA (w/v). After vigorous
249 stirring, the precipitate was removed by 15 min centrifugation at 18,000*g* (Elmi Micro
250 Centrifuge CM-50, Porvoo, Finland). Soluble peptides were spectrophotometrically
251 measured at 280 nm (Lambda 35 UV/vis spectrometer, Perkin-Elmer Instruments) and
252 converted to tyrosine equivalents using a previously built calibration curve. Cathepsin D
253 activity was expressed as μg tyrosine/min/g muscle.

254

255 **Statistical analysis**

256 Biochemical measurements for fish samples after 0, 1, 3, 6 and 9 months of frozen
257 storage time were subjected to one-way ANOVA ($p<0.05$) to explore differences in
258 pressure level and storage time effects. Comparison of means was performed using a
259 least-squares difference (LSD) method (Statsoft, Statistica, version 6.0, Tulsa, OK).
260 Correlation analysis indicating the type of fitting was also performed.

261

262 **RESULTS AND DISCUSSION**

263 **Assessment of lipid hydrolysis**

264 Lipid hydrolysis determined as FFA formation in mackerel muscle (Figure 1A, mg/100
265 g muscle) were also expressed on the basis of lipid content values (6.5-9.0 g/100 g
266 muscle). After freezing (month-0 frozen samples), a marked FFA content increase was
267 observed in all samples; however, this increase was lower in samples treated at 175 and
268 200 MPa. During frozen storage, FFA formation in all samples showed a progressive
269 increase (r^2 = 0.88-0.94). Compared with control fish, all HHP-treated samples showed
270 lower values at time 0 and 3 month and thereafter the 175-MPa treatment yielded the
271 lowest mean values. Comparison among HHP-treated samples did not provide evidence
272 of a definite effect of pressure level throughout the frozen storage period under study.
273 Similar conclusions were obtained when FFA content was expressed on the basis of
274 lipid content (g/100g lipids) showing also a good correlation with storage time for all
275 treatments (r^2 = 0.86-0.94).

276 Previous research has detected an important FFA formation in Atlantic mackerel
277 throughout frozen storage (Aubourg et al. 2005; Vázquez et al. 2013). Accumulation of
278 FFA in fish muscle has no nutritional significance but undesirable secondary effects

279 include muscle texture changes (Sikorski and Kolakowska 1994), lipid oxidation
280 acceleration (Mackie 1993), and off-odor development (Refsgaard et al. 2000).
281 Research reported concerning the effect on FFA formation after HHP treatment
282 followed by a frozen storage is scarce. Ohshima et al. (1992) found that enzymatic
283 degradation of phospholipids in cod (*Gadus morhua*) muscle was successfully inhibited
284 during storage at -2 ºC for 6 days when previously treated at pressures above 400 MPa
285 for 15 and 30 min; however, no effect was observed when applying a pressure of 200
286 MPa. Chevalier et al. (2000) showed that FFA formation during storage at -20 ºC for 75
287 days did not show differences between turbot (*Scophthalmus maximus*) fillets subjected
288 to pressure shift freezing at 140 MPa or air-blast freezing (-20 ºC). Finally, Vázquez et
289 al. (2013) observed a marked reduction in FFA content in Atlantic mackerel (*Scomber*
290 *scombrus*) subjected to HHP treatments (150, 300, 450 MPa for 0.0, 2.5, and 5.0 min)
291 prior to freezing at -20 ºC and frozen storage at -10 ºC for 3 months. The reduction
292 effect increased with the pressure level and holding time employed.
293

294 **Assessment of lipid oxidation development**

295 The formation of primary and secondary lipid oxidation compounds was evaluated by
296 means of peroxide and TBA values, respectively (Tables 1 and 2). Values for both
297 parameters showed a relatively low lipid oxidation development as reflected by values
298 in the 0.07-4.99 meq active oxygen/kg lipids and 0.35-1.08 mg malondialdehyde/kg
299 muscle range, respectively. Such low values are in agreement with previous research on
300 frozen (-10 ºC for 3 months) mackerel previously treated under HHP conditions
301 (Vázquez et al. 2013). An inhibitory effect on peroxide formation was observed in
302 HHP-treated fish after freezing (comparison of month-0 samples), particularly at 200
303 MPa. Although some significant differences were detected during frozen storage, a

304 definite pressure level effect on the presence of primary oxidation compounds was not
305 identified.

306 No effect of pressure treatment on TBARS values was observed after freezing
307 and during frozen storage. Samples of Atlantic mackerel muscle previously subjected to
308 more intense HHP treatments than the ones here reported (200, 300 and 400 MPa for 5
309 and 15 min at 5, 10 and 15 ºC) were recently analyzed by Senturk and Alpas (2013)
310 showing in TBA-i values an increase and decrease with pressure level and holding time,
311 respectively. In the present study, although PV and TBA-i scores showed some
312 significant differences effect of frozen storage time, definite trends could not be
313 determined. For 175 and 200 MPa treated samples, the highest peroxide mean values
314 were obtained at month 6, while control and 125-MPa and 150-MPa fish samples
315 showed the highest mean values at month 9. All samples yielded the highest mean
316 TBARS values at the end of storage. In addition, PV and TBA-i values showed poor
317 correlations with frozen storage time.

318 Data on the formation of tertiary lipid oxidation compounds (namely, fluorescent
319 interaction molecules) is presented in Figure 1B. Immediately after freezing, a higher
320 mean value was obtained for control samples when compared with HHP-treated fish.
321 This inhibitory effect on fluorescent compound formation was observed throughout
322 frozen storage, i.e., a higher mean value was observed for control fish for all sampling
323 times. Values corresponding to samples treated at 175 MPa were significantly lower
324 than control fish when sampled at months 1-9, while 200-MPa treated samples were
325 also significantly lower than controls after 1, 3 and 9 months of frozen storage. For 125
326 and 150 MPa treated samples, lower values were also found for 1 and 3 months when
327 compared with control samples. These results show that HHP treatments caused an
328 inhibitory effect on lipid oxidation development (tertiary lipid oxidation compounds).

329 Concerning the effect of storage time, a progressive increase with time was observed in
330 most cases showing correlation values in the 0.88-0.93 range (quadratic fitting). Lipid
331 oxidation is a complex process involving the formation of different classes of
332 compounds, most of them unstable, and thus susceptible to breakdown and formation of
333 lower molecular weight compounds or to react with other molecules, mostly
334 nucleophiles, present in fish muscle. This is the case of peroxides and TBARS, widely
335 reported to breakdown and give rise to tertiary lipid oxidation compounds after
336 interacting with protein-type molecules (Aubourg 1999; Tironi et al. 2002). According
337 to data obtained in this study, formation of fluorescent compounds seems to be the most
338 adequate index to follow the progress of lipid oxidation.

339 Two opposite mechanisms can be considered to explain lipid changes observed
340 in this study, i.e., the inactivation effect of pressure on endogenous enzyme activity
341 (Torres et al. 2013; Vázquez et al. 2013) and the pressure-denaturation of iron-
342 containing proteins which has been reported to increase free metal iron (Lakshmanan et
343 al. 2003). These two effects would account for the opposite effects of HHP treatment
344 observed when lipid oxidation development is evaluated. During 6 d storage at -2 ºC,
345 Ohshima et al. (1992) found a TBARS formation increase in frozen cod (*Gadus*
346 *morhua*) and mackerel (*Scomber scombrus*) muscle previously pressure treated at 616,
347 408 and 204 MPa for 15 and 30 min. A comparison of TBARS values observed during a
348 75-day storage at -20 ºC of turbot (*Scophthalmus maximus*) fillets subjected to pressure
349 shift freezing at 140 MPa or to air-blast freezing at -20 ºC could not identify a definite
350 advantage of the pressure t (Chevalier et al. 2000). In work closely related to this study,
351 Vázquez et al. (2013) observed a marked inhibition of tertiary lipid oxidation
352 compounds formation in frozen (-10 ºC up to 3 months) mackerel (*Scomber scombrus*)

353 previously subjected to 150, 300, 450 MPa for 0.0, 2.5, and 5.0 min. The effect
354 observed was greater when increasing the pressure level or the holding time.

355 Interaction of lipid oxidation and lipolysis is a particularly intriguing area of
356 study, as triglyceride hydrolysis has been shown to lead to an oxidation increase, while
357 phospholipid hydrolysis would produce the opposite effect (Shewfelt 1981; Sikorski
358 and Kolakowski 2000). In the present study, correlation values in the 0.76-0.95 range
359 were observed between the FR score and the development of lipid hydrolysis, while
360 poor correlation values were determined for PV and TBA-i scores with FFA content.

361 The effect of pressure level and subsequent frozen storage on the PUFA content
362 was also analyzed in this study. PI values (data not shown) were in the 0.85-1.03 range
363 for all pressure treatments in agreement with previous research on frozen mackerel also
364 pressure treated before freezing and frozen storage (Vázquez et al. 2013). In spite of
365 changes observed in lipid hydrolysis (FFA content) and oxidation (FR values), pressure
366 treatment and frozen storage had no significant effect on PI scores. Previous research
367 (Ortiz et al. 2009; Tironi et al. 2010) has shown an important detrimental effect of lipid
368 oxidation on the PUFA content expressed as a PI decrease.

369

370 **<u>Trimethylamine formation</u>**

371 TMA is one of the most commonly employed quality methods to assess microbial
372 activity in marine species kept under refrigerated conditions. However, if freezing and
373 frozen storage are encountered, microbial activity is expected to be mostly inhibited so
374 that TMA formation would not be expected. Consequently, the conversion of TMAO
375 into TMA observed in this study may be caused by non-enzymatic processes, native
376 tissue enzymes, or enzymes produced by microorganisms before the HHP and freezing
377 process. Values summarized in Table 3 showed that after freezing, TMA-N content

378 increased for all samples with significant differences ($p<0.05$) resulting from the
379 pressure treatment prior to freezing. Although some significant differences among
380 samples can be pointed out throughout frozen storage, a definite effect of the pressure
381 pre-treatment could not be determined during the 1-9-month fish-sampling period.
382 TMA-N content showed a progressive formation during frozen storage (r^2 = 0.84-0.93).
383 Additionally, good correlation values were obtained for TMA values with FFA values
384 (r^2 = 0.90-0.93) and FR scores (r^2 = 0.86-0.93). These results show that significant
385 conversion of trimethylamine oxide (TMAO) to TMA occurred during the frozen
386 storage of all samples. In previous research by Senturk and Alpas (2013), the combined
387 effect on TMA formation of pressure level (200, 300 and 400 MPa), pressure holding
388 time (5 and 15 min) and temperature (5, 10 and 15 ºC) was determined in mackerel
389 (*Scomber scombrus*) muscle; as a result, a marked increase in most cases after the HHP
390 treatment was observed, but with no significant differences among the different HHP
391 condition tested.
392

393 **Analysis of protein changes**

394 Figure 2 shows the SDS-PAGE profile of myofibril proteins for all frozen
395 mackerel samples. After freezing, myofibril proteins from pressure-treated and control
396 samples exhibited the same 1-D SDS-PAGE profile pattern (Figure 2A). Moreover, the
397 protein pattern obtained throughout frozen storage (months 3 and 9; Figures 2B and 2C,
398 respectively) did not reveal differences with the protein profile observed after freezing.
399 This means that HHP processing in the 150-200 MPa range and frozen storage for up to
400 9 months at -18 ºC did not significantly affect the 1-D electrophoretic pattern of
401 myofibril proteins. In previous work by Pazos et al. (2014), the effect on Atlantic
402 mackerel myofibril proteins of treatments at 150, 300 and 450 MPa for 0, 2.5 and 5 min

403 was analyzed after frozen storage at -10 ºC for 3 months. In agreement with this study,
404 myofibril proteins showed no solubility and electrophoretic gel profiles changes
405 throughout the whole frozen storage period. The SDS-PAGE profile of sarcoplasmic
406 proteins showed no differences among frozen fish samples at month 0 (Figure 3A).
407 However, a band of approximately 30 kDa disappeared during frozen storage. This
408 band, labeled as 1S, could not be detected after 3 months of frozen storage in samples
409 treated at 175 and 200 MPa (Figure 3B). Fish treated under 150 MPa showed the loss of
410 this band at month 9 (Figure 3C). It can be concluded that the HHP treatments studied
411 in this work induce this targeted degradation only in combination with frozen storage.
412 Values of total sarcoplasmic protein content in the 2.74-4.22 g/100 g muscle range
413 showed no effect of pressure treatment and frozen storage time. Previous research
414 concerning mackerel showed a decrease on sarcoplasmic protein content when higher
415 pressures (300 and 450 MPa) than in the present case were applied (Pazos et al. 2014).
416 Over the last decade, proteomics has been successfully applied to evaluate
417 quality in food systems including meat, fish, milk and transgenic plants (Gallardo et al.
418 2013). Proteomics analysis based on one- and two-dimensional PAGE and tandem mass
419 spectrometry (MS/MS) is a particularly powerful technology to identify global changes
420 in protein constituents (Han and Wang 2008). In spite of its potential, proteomics tools
421 applications to HHP-treated fish are still very limited. A marked content decrease in 94-
422 , 50- and 43-kDa bands was noticed in mackerel (*Scomber japonicus*) muscle treated at
423 200 MPa or higher, although the identity of these proteins was not reported (Ohshima et
424 al. 1992). Chevalier et al. (1999) also found a disappearance of an unidentified 48-kDa
425 protein band in turbot (*Scophthalmus maximus*) muscle when treated at pressures higher
426 than 150 MPa. More recently, SDS-PAGE analysis of Coho salmon (*Oncorhynchus*
427 *kisutch*) sarcoplasmic fraction showed a partial loss of a band corresponding to 29 kDa

428 that was identified by MS/MS analysis as phosphoglycerate mutase (Ortea et al. 2010),
429 in agreement with the identification carried out in the present research. Finally, Pazos et
430 al. (2014) analyzed the selective-targeted effect of HHP processing (150, 300 and 450
431 MPa for 0.0, 2.5 and 5.0 min) on proteins by identifying sarcoplasmic protein bands of
432 frozen (-10 ºC) Atlantic mackerel that were modified by the treatment. In agreement
433 with their work, the present proteomics study showed that sarcoplasmic proteins are
434 more liable to HHP-freezing-frozen storage processing than the myofibril fraction. The
435 1S band excised from the gel was digested with trypsin and the resulting peptides were
436 subjected to MS analysis by means of an electrospray ion trap mass detector. Peptides
437 were fragmented and non-interpreted fragmentation spectra (MS/MS) and de novo-
438 inferred sequences were searched against the protein sequence databases using the
439 SEQUEST software and the BLAST tool, respectively. As a result, the protein band was
440 assigned to the glycolytic enzyme phosphoglycerate mutase 2 (PGAM2) based on the
441 following identification parameters: UniProtKB/Swiss-Prot Code (Q32DV0), Mass
442 (28,685 Da), -10IgP Score (106.99), #Peptides/#Unique Peptides (6/5), Sequence
443 (K.AMEAVAAQGK.A*; R.KAMEAVAAQGK.A*; R.ALPFWNDEIAPQIK.A*;
444 R.HYGGLTGLNK.A*; R.FCGWFDADLSEK.G*; K.HGEEQVK.I) and Sequence
445 Coverage (21%). Phophoglycerate mutase is known to be a transferase enzyme
446 responsible for transferring a phosphate group from the C-3 carbon of 3-
447 phosphoglycerate to the C-2 carbon forming 2-phosphoglycerate. In agreement with
448 previously mentioned results (Ortea et al. 2010), results from this study show that the
449 assessment of this enzyme could be an effective tool to study the fish quality loss
450 during storage.
451

452 **Enzymatic activity analysis**

453 A general behavior of the acid phosphatase activity data summarized in Table 4 showed
454 for all samples a progressive increase with storage time (r^2 = 0.79-0.87; quadratic
455 fitting). An inhibitory effect of HHP treatments at 150 and 200 MPa, was observed in
456 month-0 samples by comparison with their counterpart controls. Comparisons
457 throughout frozen storage of pressure treatments did not show a general pattern;
458 however, HHP-treated samples showed a lower activity at the end of the experiment
459 when compared with their counterpart control samples. In previous work, Fidalgo et al.
460 (2014) observed that acid phosphatase from mackerel (*Scomber scombrus*) was strongly
461 affected by frozen storage time (3 months at -10 ºC) in samples untreated or HHP-
462 treated (150, 300 and 450 MPa; 0.0, 2.5 and 5.0 min). An important effect of pressure
463 could also be observed with lower values obtained by increasing the pressure level. Fair
464 correlation values were obtained in the present study for the acid phosphatase activity
465 when compared with chemical quality indices such as FFA content (0.79-0.89), TMA
466 formation (0.75-0.94), PV (0.74-0.90) and FR value (0.76-0.93).

467 Cathepsin B activity assessments (Table 4) showed also a progressive increase
468 (r^2 = 0.88-0.91; quadratic fitting) during the frozen storage of all samples. The highest
469 values ($p<0.05$) for each sample type were also observed at the end of storage. A
470 comparison of samples after freezing showed an inhibitory effect of treatments at 125,
471 150 and 175 MPa but not at 200 MPa. Although some significant differences were
472 observed during frozen storage, a definite effect of the HHP treatment was not possible
473 to conclude. It should be mentioned that in the 3-9-month period, a lower mean activity
474 was observed in samples previously treated at 150-200 MPa when compared with their
475 counterpart control samples. In previous related research by Fidalgo et al. (2014),
476 cathepsin B activity in frozen mackerel (3 months at -10 ºC) was also affected by HHP

477 treatments (150, 300 and 450 MPa for 0.0, 2.5 and 5.0 min) before freezing and frozen
478 storage, being the pressure effect higher than that for frozen storage and pressure
479 holding time. A decrease in cathepsin B activity was evident with a pressure level
480 increase, although a recovery effect was observed during frozen storage. In the present
481 study, cathepsin B activity showed fair correlation values with chemical quality indices
482 such as FFA content (0.77-0.89), TMA formation (0.67-0.88), PV score (0.42-0.94) and
483 FR value (0.78-0.94).

484 A higher cathepsin D activity (Table 4) was observed at month 0 in 125-MPa
485 samples. Throughout the 3-6-month period, controls showed higher mean values than
486 HHP-treated fish samples. Finally, 200-MPa-treated samples had the highest ($p<0.05$)
487 cathepsin D activity at the end of storage. A progressive activity increase in control
488 samples was observed throughout frozen storage reaching 71% activity increase. In
489 previous work by Fidalgo et al. (2014), 300-MPa treatment before freezing caused an
490 activity increase when compared with control samples while 150-MPa and 450-MPa
491 treated samples showed the highest activity value after 3 months of storage at -10 ºC.
492 Cathepsin D activity showed poor correlation values with any of the chemical quality
493 indices studied in this work.

494 Although HHP treatment may inhibit the activity of hydrolytic enzymes, the
495 effects on fish muscle of 125-200 MPa treatments depend on several factors causing
496 activation or inactivation of muscle enzymes. Consequently, the enzyme activity values
497 observed in this study can be considered the result of different and opposite effects.
498 While HHP treatments may inactivate enzymes by disrupting intramolecular bonds
499 determining their secondary, tertiary and quaternary conformation (Ashie et al. 1996;
500 Campus 2010), they can also disrupt lysosomal membranes releasing proteases leading
501 to an increase in hydrolytic activity (Ohmori et al. 1992; Chéret et al. 2005). Cathepsins

502 B (a cysteine protease) and D (an aspartic acidic protease) have been reported to be
503 released from the lysosomal matrix into both the cytoplasm and the intracellular spaces
504 as a consequence of the breakdown of lysosomes (Chéret et al. 2006).

505

506 ## CONCLUSIONS

507 Significant biochemical changes were observed in commercially frozen Atlantic
508 mackerel (-18 ºC for 9 months) when previously subjected to 125-200 MPa pressure
509 treatments. After freezing, all HHP-treated fish showed a marked inhibition in FFA
510 formation; this inhibition was also observed during frozen storage of samples treated at
511 175 MPa. Although no effect on primary and secondary lipid oxidation compounds
512 formation was observed, tertiary lipid oxidation showed an inhibitory effect for the 0-9-
513 month period in samples subjected to 175 and 200 MPa. One-dimensional SDS-PAGE
514 analysis of the sarcoplasmic protein fraction revealed the disappearance after 3 month
515 frozen storage of a band in samples treated to 175 and 200 MPa while those treated at
516 150 MPa showed the loss of this band at month 9. Proteomics analysis showed that this
517 protein band was the glycolytic enzyme phosphoglycerate mutase 2 (MW: 28.7 kDa). In
518 agreement with previous research, this finding confirms that monitoring this enzyme
519 could be an effective alternative to study the fish quality loss during frozen or
520 refrigerated storage. Finally, no effect of the HHP treatments was observed on other
521 biochemical indicators of fish quality loss including TMA and PUFA content, 1-D
522 myofibril protein fraction, and the activity of acid phosphatase, and cathepsins B and D.

523

524 **Acknowledgments**

525 The Xunta de Galicia and the European Social Fund are thankfully recognized for the
526 financial support of the postdoctoral "Isidro Parga Pondal" contract to M. P. The
527 Spanish Ministry of Science and Innovation is also gratefully acknowledged for the
528 doctoral fellowship to L. M. The authors thank Dr. María Lavilla (AZTI Tecnalia,
529 Derio, Spain), Dr. Barbara Teixeira (IPMA, Lisbon, Portugal), Mr. Marcos Trigo and
530 Mrs. Lorena Barros for their help in carrying out the present study. This work was
531 supported by the Secretaría Xeral de I+D from the Xunta de Galicia (Galicia, Spain)
532 through the Research Project 10TAL402001PR (2010-2012), by Fundação para a
533 Ciência e a Tecnologia (FCT Portugal), European Union, QRN, FEDER, COMPETE
534 through founding of the Organic Chemistry Research Unit (QOPNA) (project PEst-
535 C/QUI/UI0062/2013; FCOMP-01-0124-FEDER-037296), and by Formula Grants no.
536 2011-31200-06041 and 2012-31200-06041 from the USDA National Institute of Food
537 and Agriculture.

538

539

540

541

FIGURE LEGENDS

Figure 1: Free fatty acid content (mg/100 g muscle) (Panel A) and fluorescence ratio (Panel B) assessment in frozen mackerel muscle previously processed under different high-pressure conditions. Mean values of three replicates (n=3) with standard deviations 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 pressure treatment prior to freezing and frozen storage at -18 ^{o}C for up to 9 months. Initial values: 11.99±5.88 mg/100 g muscle (free fatty acid content) and 0.56±0.10 (fluorescence ratio).

Figure 2: Effect of high-pressure level and frozen storage time on the 1-D SDS-PAGE profile of myofibril proteins (MW, molecular weight). Atlantic mackerel was treated at 150, 175 and 200 MPa for 0 min and subsequently stored at -18 ^{o}C for 0 (Panel A), 3 (Panel B) and 9 (Panel C) months. Profiles for control fish (0.1-MPa condition) are also expressed.

Figure 3: Effect of high-pressure level and frozen storage on the 1-D SDS-PAGE profile of sarcoplasmic proteins (MW, molecular weight). Atlantic mackerel was treated at 150, 175 and 200 MPa for 0 min and subsequently stored at -18 ^{o}C for 0 (Panel A), 3 (Panel B) and 9 (Panel C) months. The band labeled 1S denotes the protein band affected by high-pressure processing and frozen storage. Profiles for control fish (0.1-MPa condition) are also expressed.

568 **REFERENCES**

569 Ashie, I., Smith, J., & Simpson, B. (1996). Spoilage and shelf-life extension of fresh
570 fish and shellfish. *Critical Reviews in Food Science and Nutrition, 36*, 87-121.
571 Aubourg, S. (1999). Review: Recent advances in assessment of marine lipid oxidation
572 by using fluorescence. *Journal of the American Oil Chemists' Society, 76*, 409-
573 419.
574 Aubourg, S, Medina, I., & Pérez-Martín, R. (1996). Polyunsaturated fatty acids in tuna
575 phospholipids: Distribution in the sn-2 location and changes during cooking.
576 *Journal of Agricultural and Food Chemistry, 44*, 585-589.
577 Aubourg, S., Rodríguez, A., & Gallardo, J. (2005). Rancidity development during
578 frozen storage of mackerel (*Scomber scombrus*): Effect of catching season and
579 commercial presentation. *European Journal of Lipid Science and Technology,*
580 *107*, 316-323.
581 Aubourg, S., Torres, J. A., Saraiva, J., Guerra-Rodríguez, E., & Vázquez, M. (2013).
582 Effect of high-pressure pretreatments applied before freezing and frozen storage
583 on the functional and sensory properties of Atlantic mackerel (*Scomber*
584 *scombrus*). *Food Science and Technology (LWT), 53*, 100-106.
585 Bligh, E., & Dyer, W. (1959). A rapid method of total lipid extraction and purification.
586 *Canadian Journal of Biochemistry and Physiology, 37*, 911-917.
587 Brutti, A., Rovere, P., Cavallero, S., D'Amelio, S., Danesi, P., & Arcangeli, G. (2010).
588 Inactivation of *Anisakis simplex* larvae in raw fish using high hydrostatic
589 pressure treatments. *Food Control, 21*, 331-333.
590 Buckow, R., Truong, B., & Versteeg, C. (2010). Bovine cathepsin D activity under high
591 pressure. *Food Chemistry, 120*, 474-481.

592 Campus, M. (2010). High pressure processing of meat, meat products and seafood. *Food Engineering Reviews, 2*, 256-273.

594 Chapman, R., & McKay, J. (1949). The estimation of peroxides in fats and oils by the ferric thiocyanate method. *Journal of the American Oil Chemists' Society, 26*, 360-363.

597 Chéret, R., Chapleau, N., Delbarre-Ladrat, C., Vérrez-Bagnis, V., & De Lamballerie, M. (2005). Effects of high pressure on texture and microstructure of sea bass (*Dicentrarchus labrax* L.) fillets. *Journal of Food Science, 70*, E477-E483.

600 Chéret, R., Hernández-Andrés, A., Delbarre-Ladrat, C., de Lamballerie, M., & Vérrez-Bagnis, V. (2006). Proteins and proteolytic activity changes during refrigerated storage in sea bass (*Dicentrarchus labrax* L.) muscle after high-pressure treatment. *European Food Research and Technology, 222*, 527-535.

604 Chevalier, D., Le Bail, A., Chourot, J., & Chantreau, P. (1999). High pressure thawing of fish (whiting): Influence of the process parameters on drip losses. *Lebensmittel-Wissenschaft und -Technologie, 32*, 25-31.

607 Chevalier, D., Sequeira-Muñoz, A., Le Bail, A., Simpson, B., & Ghoul, M. (2000). Effect of pressure shift freezing, air-blast freezing and storage on some biochemical and physical properties of turbot (*Scophthalmus maximus*). *Lebensmittel-Wissenschaft und -Technologie, 33*, 570-577.

611 Fidalgo, L. G., Saraiva, J. A., Aubourg, S. P., Vázquez, M., & Torres, J. A. (2014). Effect of high-pressure pre-treatments on enzymatic activities of Atlantic mackerel (*Scomber scombrus*) during frozen storage. *Innovative Food Science & Emerging Technologies, 23*, 18-24.

615 Gallardo, J. M., Carrera, M., & Ortea, I. (2013). Proteomics in Food Science. In A. Cifuentes (Ed.), *Foodomics: Advanced mass spectrometry in modern food*

617 *science and Nutrition* (pp. 125-165). Hoboken, NJ, USA: John Wiley & Sons,
618 Inc.
619 Groβ, M., Auerbach, G., & Jaenicke, R. (1993). The catalytic activities of monomeric
620 enzymes show complex pressure dependence. *FEBS Letters, 321*, 256-260.
621 Han, J.-Z., & Wang, Y.-B. (2008). Proteomics: present and future in food science and
622 technology. *Trends in Food Science and Technology, 19*, 26-30.
623 Harris, P., & Tall, J. (1994). Rancidity in fish. In J. Allen, & R. Hamilton (Eds.),
624 *Rancidity in foods* (pp. 256-272). London, UK: Chapman and Hall.
625 Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of
626 bacteriophage T4. *Nature, 227*, 680-685.
627 Lakshmanan, R., Miskin, D., & Piggott, J. R. (2005). Quality of vacuum packed cold-
628 smoked salmon during refrigerated storage as affected by high-pressure
629 processing. *Journal of the Science of Food and Agriculture, 85*, 655-661.
630 Lakshmanan, R., Pigott, J., & Paterson, A. (2003). Potential applications of high
631 pressure for improvement in salmon quality. *Trends in Food Science and*
632 *Technology, 14*, 354-363.
633 Lowry, R., & Tinsley, I. (1976). Rapid colorimetric determination of free fatty acids.
634 *Journal of the American Oil Chemists' Society, 53*, 470-472.
635 Mackie, I. M. (1993). The effects of freezing on flesh proteins. *Food Reviews*
636 *International, 9*, 575-610.
637 Montero, P., Giménez, B., Pérez-Mateos, M., & Gómez-Guillén, MªC. (2005).
638 Oxidation stability of muscle with quecetin and rosemary during thermal and
639 high-pressure gelation. *Food Chemistry, 93*, 17-23.

640 Mújica-Paz, H., Valdez-Fragoso, A., Tonello Samson, C., Welti-Chanes, J. & Torres,
641 J.A. (2011). High-pressure processing technologies for the pasteurization and
642 sterilization of foods. *Food and Bioprocess Technology, 4*, 969-985.
643 Norton, T., & Sun D.-W. (2008). Recent advances in the use of high pressure as an
644 effective processing technique in the food industry. *Food and Bioprocess*
645 *Technology, 1*, 2-34.
646 Ohmori, T., Shigehisa, T., Taji, S., & Hayashi, R. (1992). Biochemical effects of high
647 hydrostatic pressure on the lysosome and proteases involved in it. *Bioscience,*
648 *Biotechnology, and Biochemistry, 56*, 1285-1288.
649 Ohshima, T., Nakagawa, T., & Koizumi, C. (1992). Effect of high hydrostatic pressure
650 on the enzymatic degradation of phospholipids in fish muscle during storage. In
651 E. Bligh (Ed.), *Seafood Science and Technology*, Chapter 8 (pp. 64-75). Oxford,
652 UK: Fishing News Books.
653 Ortea, I., Rodríguez, A., Tabilo-Munizaga, G., Pérez-Won, M., & Aubourg, S. (2010).
654 Effect of hydrostatic high-pressure treatment on proteins, lipids and nucleotides
655 in chilled farmed salmon (*Oncorhynchus kisutch*) muscle. *European Food*
656 *Research and Technology, 230*, 925-934.
657 Ortiz, J., Larraín, MªA., Vivanco, J., & Aubourg, S. (2009). Rancidity development
658 during the frozen storage of farmed coho salmon (*Oncorhynchus kisutch*): Effect
659 of antioxidant composition supplied in the diet. *Food Chemistry, 115*, 143-148.
660 Pazos, M., Méndez, L., Gallardo, J. M., & Aubourg, S. (2014). Selective-targeted effect
661 of high pressure processing on proteins related to quality: A proteomics evidence
662 in Atlantic mackerel (*Scomber scombrus*). *Food and Bioprocess Technology, 7*,
663 2342-2353.
664 Pazos, M., Pereira da Rocha, A., Roepstorff, P., & Rogowska-Wrzesinska, A. (2011).

665 Fish Proteins as Targets of Ferrous-Catalyzed Oxidation: Identification of
666 Protein Carbonyls by Fluorescent Labeling on Two-Dimensional Gels and
667 MALDI-TOF/TOF Mass Spectrometry. *Journal of Agricultural and Food*
668 *Chemistry, 59,* 7962-7977.
669 Refsgaard, H., Brockhoff, P., & Jensen, B. (2000). Free polyunsaturated fatty acids
670 cause taste deterioration of salmon during frozen storage. *Journal of*
671 *Agricultural and Food Chemistry, 48*, 3280-3285.
672 Saeed, S., & Howell, N. (2001). 12-lipoxygenase activity in the muscle tissue of
673 Atlantic mackerel (*Scomber scombrus*) and its prevention by antioxidants.
674 *Journal of the Science of Food and Agriculture, 81*, 745-750.
675 Senturk, T., & Alpas, H. (2013). Effect of high hydrostatic pressure treatment (HHPT)
676 on quality and shelf life of Atlantic mackerel (*Scomber scombrus*). *Food and*
677 *Bioprocess Technology, 6*, 2306-2318.
678 Sikorski, Z., & Kolakowska, A. (1994). Changes in protein in frozen stored fish. In Z.
679 Sikorski, B. Sun Pan, & F. Shahidi (Eds.), *Seafood proteins* (pp. 99-112). New
680 York, USA: Chapman and Hall.
681 Sikorski, Z., & Kolakowski, E. (2000). Endogenous enzyme activity and seafood
682 quality: Influence of chilling, freezing, and other environmental factors. In N.
683 Haard, & B. Simpson (Eds.), *Seafood enzymes* (pp. 451-487). New York, USA:
684 Marcel Dekker.
685 Smith, P., Krohn, R., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M.,
686 Fujimoto, E., Goeke, N., Olson, B., & Klenk, D. (1985). Measurement of protein
687 using bicinchoninic acid. *Analytical Biochemistry, 150*, 76–85.
688 Shewfelt, R. (1981). Fish muscle lipolysis - A review. *Journal of Food Biochemistry, 5*,
689 79-100.

690 Tironi, V., de Lamballerie, M., & Le Bail, A. (2010). Quality changes during the frozen
691 storage of sea bass (*Dicentrarchus labrax*) muscle after pressure shift freezing
692 and pressure assisted thawing. *Innovative Food Science and Emerging*
693 *Technologies, 11*, 565-573.
694 Tironi, V., Tomás, M., & Añón, MªC. (2002). Structural and functional changes in
695 myofibrillar proteins of sea salmon (*Pseudopercis semifasciata*) by interaction
696 with malondialdehyde (RI). *Journal of Food Science, 67*, 930-935.
697 Torres, A., Vázquez, M., Saraiva, J., Gallardo, J., & Aubourg, S. (2013). Lipid damage
698 inhibition by previous high pressure processing in white muscle of frozen horse
699 mackerel. *European Journal of Lipid Science and Technology, 115*, 1454-1461.
700 Tozawa, H., Erokibara, K., & Amano, K. (1971). Proposed modification of Dyer's
701 method for trimethylamine determination in codfish. In R. Kreuzer (Ed.), *Fish*
702 *Inspection and Quality Control* (pp. 187-190). London, UK: Fishing News
703 Books Ltd.
704 Vázquez, M., Torres, J. A., Gallardo, J., Saraiva, J., & Aubourg, S. (2013). Lipid
705 hydrolysis and oxidation development in frozen mackerel (*Scomber scombrus*):
706 Effect of a high hydrostatic pressure pre-treatment. *Innovative Food Science and*
707 *Emerging Technologies, 18*, 24-30.
708 Vyncke, W. (1970). Direct determination of the thiobarbituric acid value in
709 trichloracetic acid extracts of fish as a measure of oxidative rancidity. *Fette*
710 *Seifen Anstrichmittel, 72,* 1084-1087.

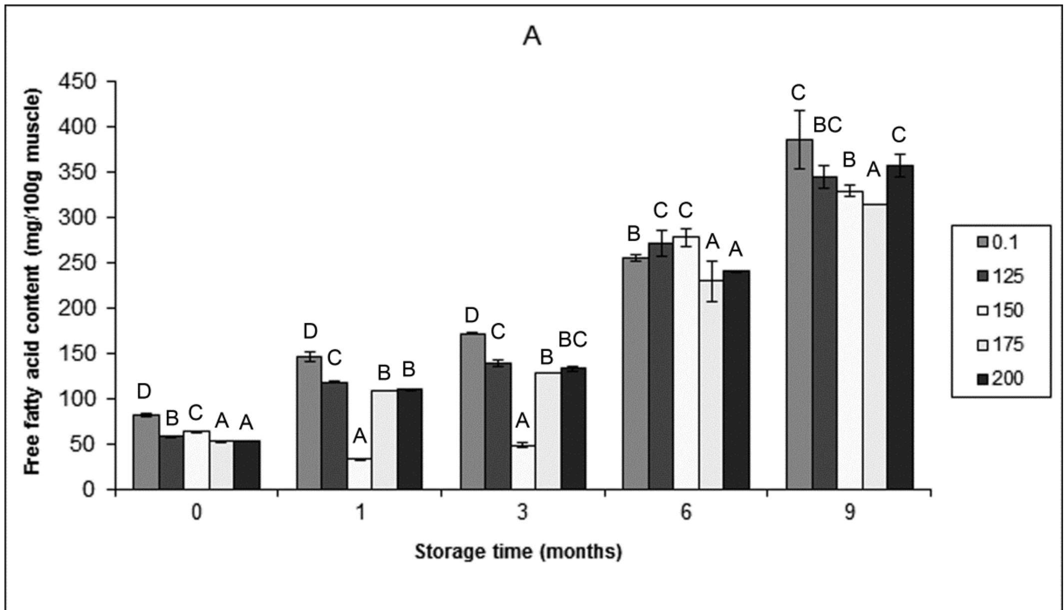
711

712

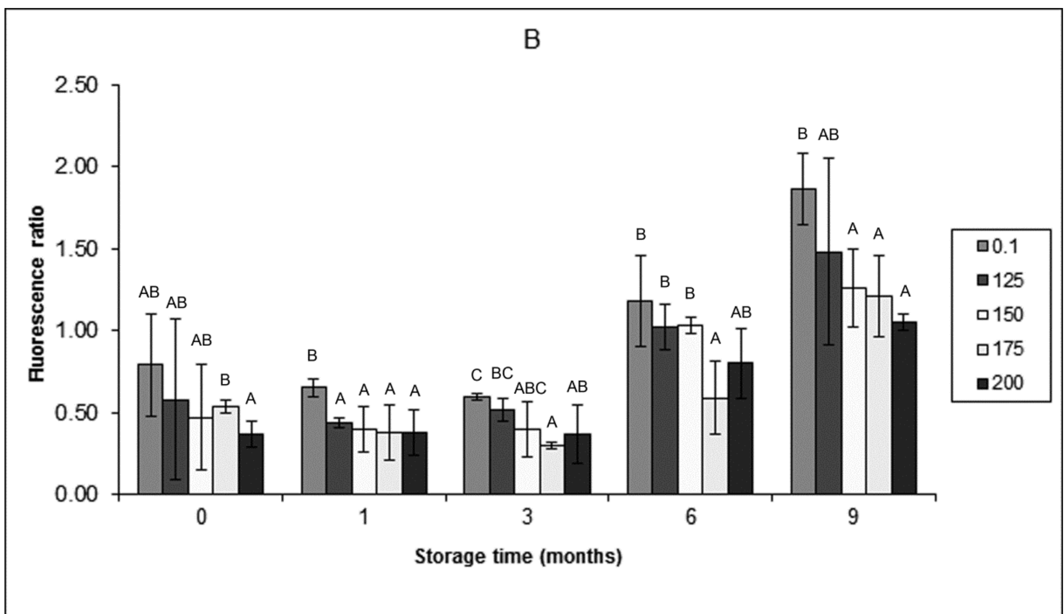
713 FIGURE 1

714

715

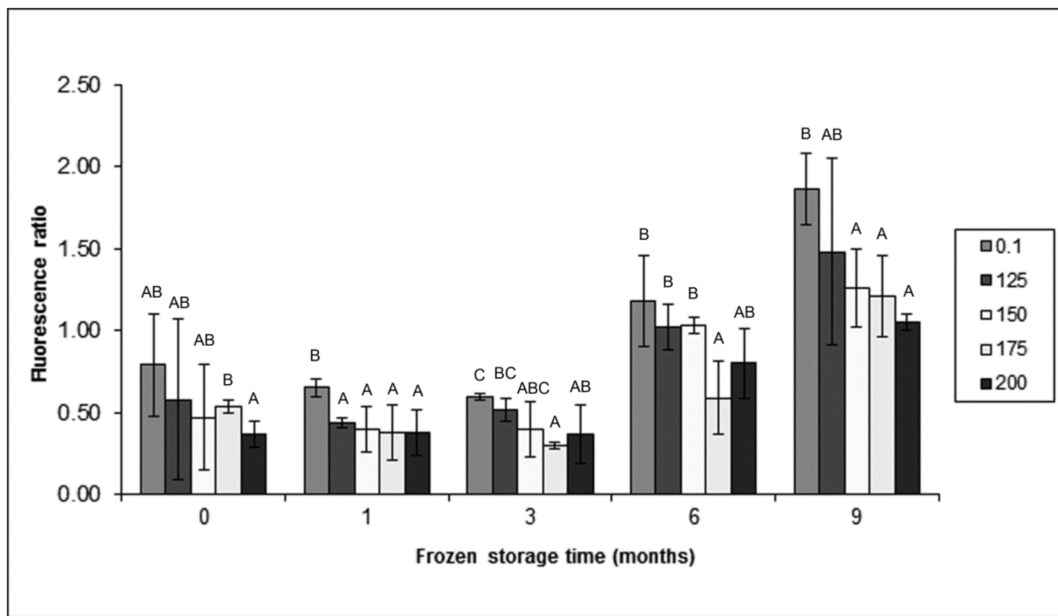


716
717
718
719
720
721
722
723
724
725



726
727
728
729
730
731
732

733 **Figure 2**

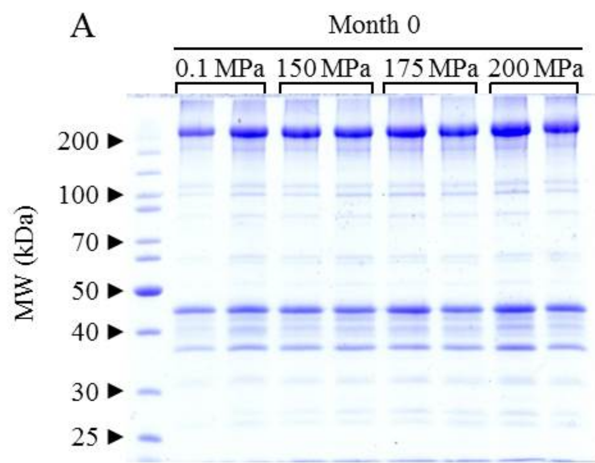


734

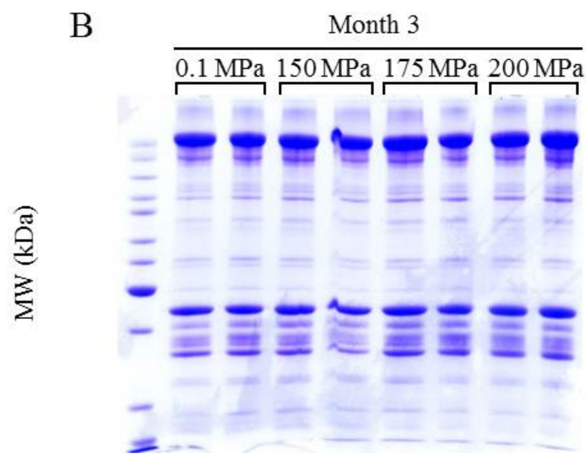
735

736 **Figure 3**

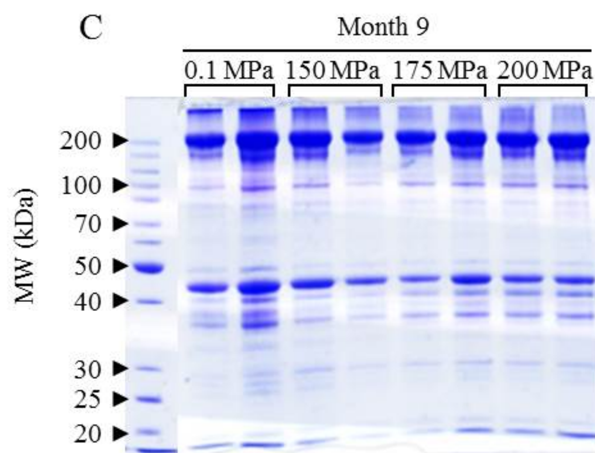
737



738



739



740

741

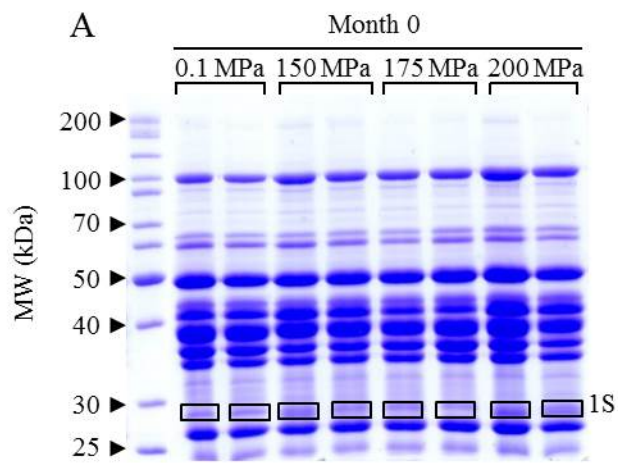
742

743

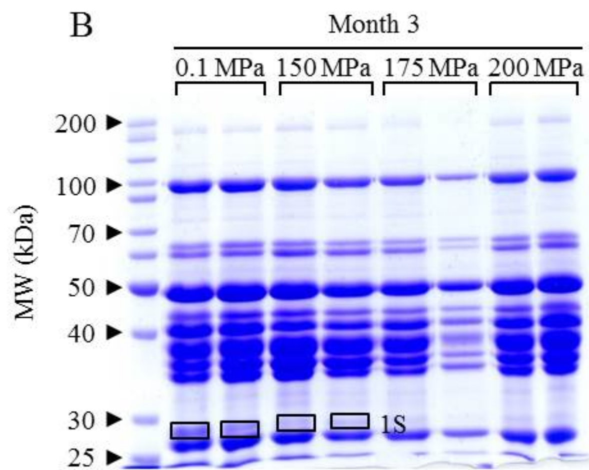
744

745 **Figure 4**

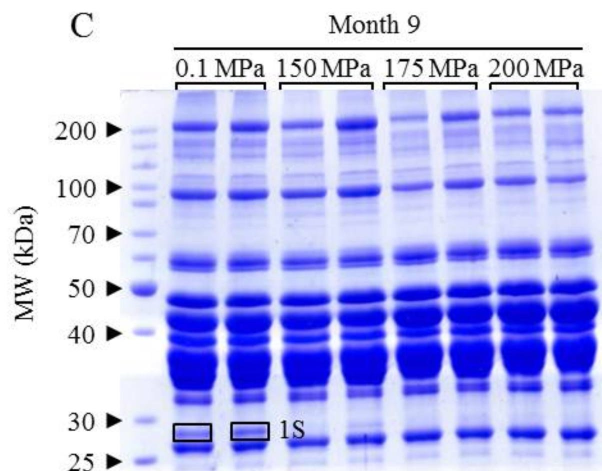
746



747



748



749

750

751

752

<u>TABLE 1</u>

Peroxide values* (meq active oxygen/kg lipids) in frozen mackerel treated by high-pressure prior to freezing and subsequent storage at -18ºC for up to 9 months

Storage time (months)	Pressure (MPa)				
	0.1	**125**	**150**	**175**	**200**
Initial fish	0.36±0.23^{a}	0.36±0.23^{ab}	0.36±0.23^{a}	0.36±0.23^{a}	0.36±0.23^{ab}
0	1.72±0.22^{cC}	0.47±0.26^{abcB}	0.36±0.18^{aB}	0.36±0.21^{aB}	0.07±0.04^{aA}
1	0.290.06^{aA}	0.140.10^{aA}	0.260.12^{aA}	0.240.07^{aA}	0.60±0.17^{bB}
3	0.570.31^{ab}	0.340.21^{ab}	0.450.19^{a}	0.440.25^{a}	0.74±0.41^{b}
6	1.62±0.74^{bcA}	0.7±80.40^{bcA}	3.11±0.69^{bB}	4.90±1.73^{bBC}	4.99±0.72^{dC}
9	3.01±0.88^{d}	2.33±1.70^{c}	3.24±1.28^{b}	2.94±1.07^{b}	1.99±0.61^{c}

* Mean values of three replicates (n=3) ± standard deviations. 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 condition, means followed by different low-case letters (a-d) indicate significant differences ($p<0.05$) as a result of the frozen storage time. No letters are included when no differences ($p>0.05$) are found.

779 **TABLE 2**

780
781
782
783 **Thiobarbituric acid index* (mg malondialdehyde/kg muscle) in frozen mackerel**
784 **treated by high-pressure prior to freezing and subsequent storage at -18ºC for up**
785 **to 9 months**

786

787

Storage time (months)	Pressure (MPa)				
	0.1	**125**	**150**	**175**	**200**
Initial fish	0.37±0.16^{a}	0.37±0.16^{a}	0.3±70.16	0.37±0.16	0.37±0.16^{ab}
0	0.48±0.01^{a}	0.54±0.05^{ab}	0.56±0.11	0.50±0.17	0.46±0.06^{b}
1	0.52±0.10^{a}	0.52±0.13^{ab}	0.62±0.11	0.55±0.22	0.62±0.12^{bc}
3	1.08±0.23^{bB}	0.64±0.06^{bA}	0.62±0.07^{A}	0.75±0.19^{AB}	0.64±0.03^{cA}
6	0.48±0.09^{a}	0.42±0.16^{ab}	0.44±0.10	0.46±0.09	0.55±0.05^{bc}
9	0.37±0.15^{aAB}	0.39±0.11^{aAB}	0.55±0.03^{B}	0.47±0.07^{B}	0.35±0.03^{aA}

788

789

790
791
792
793
794
795 * Mean values of three replicates (n=3) ± standard deviations. For each frozen storage
796 time, mean values followed by different capital letters (A-C) indicate significant
797 differences ($p<0.05$) as a result of the pressure treatment. For each pressure
798 condition, means followed by different low-case letters (a-d) indicate significant
799 differences ($p<0.05$) as a result of the frozen storage time. No letters are
800 included when no differences ($p>0.05$) are found.

801

TABLE 3

Trimethylamine (TMA) values* (mg TMA-N/100 g muscle) in frozen mackerel treated by high-pressure prior to freezing and subsequent storage at -18ºC for up to 9 months

Storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
Initial fish	0.02±0.01^{a}	0.02±0.01^{a}	0.02±0.01^{a}	0.02±0.01^{a}	0.02±0.01^{a}
0	0.06±0.01^{b}	0.06±0.01^{b}	0.07±0.01^{b}	0.06±0.01^{b}	0.06±0.01^{b}
1	0.12±0.02^{cAB}	0.14±0.02^{cAB}	0.11±0.02^{bA}	0.10±0.02^{cA}	0.14±0.02^{cB}
3	0.11±0.01^{c}	0.12±0.03^{c}	0.10±0.03^{b}	0.12±0.02^{c}	0.13±0.03^{c}
6	0.28±0.01^{dA}	0.29±0.02^{dA}	0.47±0.06^{cB}	0.31±0.01^{dA}	0.39±0.02^{dB}
9	0.54±0.05^{eB}	0.52±0.11^{eAB}	0.40±0.04^{cA}	0.52±0.10^{eAB}	0.49±0.04^{dAB}

* Mean values of three replicates (n=3) ± standard deviations. 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 condition, means followed by different low-case letters (a-d) indicate significant differences ($p<0.05$) as a result of the frozen storage time. No letters are included when no differences ($p>0.05$) are found.

TABLE 4

Enzymes activity* in frozen mackerel treated by high-pressure prior to freezing and subsequent storage at -18ºC for up to 9 months

Storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
Acid phosphatase (mmol p-NP.min^{-1}.g^{-1})					
0	218.2±4.7^{aB}	228.3±5.7^{abB}	194.0±6.9^{aA}	207.5±7.2^{bAB}	187.7±5.8^{aA}
1	241.1±0.3^{bB}	206.7±5.4^{aA}	202.0±8.9^{aA}	199.1±2.7^{bA}	189.6±6.4^{aA}
3	205.1±2.9^{aBC}	249.0±4.0^{bcD}	227.4±5.4^{bCD}	170.4±5.4^{aA}	191.4±6.8^{aAB}
6	245.8±9.7^{bA}	255.2±8.9^{cdAB}	267.5±9.9^{cAB}	272.5±5.9^{cB}	255.1±2.7^{bAB}
9	331.8±12.6^{cB}	276.2±9.8^{dA}	268.5±9.0^{cA}	277.0±3.9^{cA}	265.8±11.7^{bA}
Cathepsin B (10^5 FU.min^{-1}.g^{-1})					
0	9.46±0.77^{b}	9.51±0.81^{a}	8.17±0.85^{a}	8.76±0.40^{a}	10.37±1.21^{ab}
1	6.88±0.73^{aA}	7.71±0.34^{aAB}	8.68±1.33^{aAB}	9.81±0.53^{aB}	9.38±0.37^{aB}
3	12.48±0.66c^{C}	9.87±0.59^{aB}	12.22±1.23^{bBC}	11.77±1.08^{bABC}	10.05±0.15^{aAB}
6	13.59±0.29^{cB}	11.10±0.42^{bA}	12.27±0.26^{bAB}	11.29±0.07^{bA}	12.57±0.81^{bAB}
9	18.88±0.48^{dBC}	19.84±0.81^{cC}	17.25±0.49^{cAB}	17.58±0.12^{cB}	15.22±0.57^{cA}
Cathepsin D (µg tyrosine.min^{-1}.g^{-1})					
0	2.54±0.24^{aA}	3.80±0.52^{bB}	2.24±0.45^{A}	2.62±0.40^{abcA}	2.36±0.19^{aA}
1	3.10±0.33^{abB}	3.84±0.76^{bB}	2.78±0.49^{AB}	2.04±0.22^{aA}	2.91±0.20^{abAB}
3	3.64±0.41^{bcB}	3.10±0.42^{abAB}	3.18±0.22^{AB}	2.55±0.13^{abA}	2.77±0.06^{abAB}
6	3.98±0.48^{bc}	3.66±0.14^{b}	3.15±0.18	3.61±0.37^{c}	3.49±0.30^{b}
9	4.34±0.03^{cC}	2.17±0.26^{aA}	2.8±70.13^{AB}	3.40±0.05^{bcBC}	5.53±0.30^{cD}

* Mean values of three replicates (n=3) ± standard deviations. For each frozen storage time, mean values followed by different capital letters (A-D) indicate significant differences ($p<0.05$) as a result of the pressure treatment. For each pressure condition, means followed by different low-case letters (a-d) indicate significant differences ($p<0.05$) as a result of the frozen storage time. No letters are included when no differences ($p>0.05$) are found.