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

Manuel Pazos[1], Lucía Méndez[1], Liliana Fidalgo[2], Manuel Vázquez[3], J. Antonio Torres[4], Santiago P. Aubourg[1,*], and Jorge A. Saraiva[2]

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

[2] Research Unit of Organic Chemistry, Natural and Agro-food Products (QOPNA), Chemistry Department, Aveiro University, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal.

[3] Department of Analytical Chemistry, Faculty of Veterinary Science, University of Santiago de Compostela, 27002-Lugo, Spain

[4] Food Processing Engineering Group, Department of Food Science & Technology, Oregon State University, Corvallis, OR 97331, USA

* Corresponding author. Phone: +34986231930; Fax: +34986292762; e-mail: saubourg@iim.csic.es

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; additionally, 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

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

Atlantic mackerel is a small pelagic fish species captured in large amounts during periods of relatively low demand. Previous research has shown an important endogenous pro-oxidant activity and significant quality loss during its frozen storage (Saeed and Howell 2001; Aubourg et al. 2005). Consequently, Atlantic mackerel remains underutilized reflecting mainly its poor frozen shelf-life. HHP treatment of Atlantic mackerel has been reported to inactivate *Anisakis simplex* larvae in raw fish (Brutti et al. 2010). Gelification conditions of mackerel muscle by HHP processing were optimized by addition of natural antioxidants (Montero et al. 2005). The effect on the physico-chemical quality parameters of refrigerated (4 ºC) mackerel subjected to HHP processing (200, 300 and 400 MPa for 5 and 15 min at 5, 10 and 15 ºC) was recently evaluated also (Senturk and Alpas 2013). This research focuses on the quality retention of Atlantic mackerel under commercial frozen conditions (9-month period at -18 ºC). Based on previous work (Aubourg et al. 2013), HHP treatments at 125, 150, 175 and 200 MPa for 0 min were applied prior to sample freezing. Changes in lipids (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
107 ## MATERIALS AND METHODS
108 ### Chemicals
109 Bicinchoninic acid (BCA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), Tris-
110 HCl and the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) were purchased
111 from Sigma-Aldrich Corp. (St. Louis, MO). Acrylamide and bis N,N´-methylene-bis-
112 acrylamide were provided by Bio-Rad Laboratories, Inc. (Hercules, CA). Glycerol was
113 obtained from Merck KGaA (Darmstadt, Germany). Ammonium persulfate (APS),
114 N,N,N',N'-tetramethylethylenediamine (TEMED) and bromophenol blue were
115 purchased from GE Healthcare Science (Uppsala, Sweden). Sequencing grade bovine
116 trypsin was purchased from Promega Corp. (Madison, WI). Trizma hydrochloride (Tris-
117 HCl), acetic acid, sodium hydroxide, citric acid, 2-bis-(2-hydroxyethyl)-amino-2-
118 (hydroxymethyl)-1,3-propanediol (Bis-Tris), ethylenediamine-tetraacetic acid (EDTA),
119 *p*-nitrophenol, thymolphtalein, trichloracetic acid (TCA), trisodium citrate and L-
120 tyrosine were also obtained from Sigma-Aldrich Co. LLC (Steinheim, Germany). The
121 enzymes substrates *p*-nitrophenylphosphate (*p*-NPP), *p*-nitrophenol (*p*-NP), Z-arginine-
122 arginine-7-amido-4-methylcoumarin hydrochloride (Z-Arg-Arg-7-AMC HCl, #C5429),
123 hemoglobin from bovine blood and olive oil were purchased from Sigma-Aldrich Co.
124 LLC. All other chemicals were of analytical grade (Panreac Quimica S.L.U., Barcelona,
125 Spain) and water was purified using a Millipore Milli-Q system (Billerica, MA).
126
127

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

129 The response to HHP treatments of marine species has been reported to vary
130 with species, composition and size at capture (Ortea et al. 2010; Campus 2010).
131 Previous research on HHP-treated Atlantic mackerel stored under accelerated storage
132 conditions (-10 ºC) had shown that 150 MPa for 0 min yielded the highest sensory
133 scores (Aubourg et al. 2013), while lowering lipid damage development (Vázquez et al.
134 2013) and enzyme activity (Fidalgo et al. 2014). Accordingly, this pressure level and a
135 lower (125 MPa) and two higher (175 and 200 MPa) values were included in this study.
136 Atlantic mackerel (156 individuals) caught near the Bask coast in Northern Spain was
137 obtained at the Ondarroa harbor (Bizkaia, Spain) and transported under ice to the AZTI
138 Tecnalia (Derio, Spain) pilot plant for HHP treatment within 6 h after catch. Whole
139 mackerel individuals were placed in flexible polyethylene bags (three individuals per
140 bag) and vacuum sealed at 400 mbar. The length and weight of the specimens ranged
141 30-34 cm and 245-295 g, respectively. Fish individuals were randomly distributed.
142 Samples were treated in a 55-L high pressure unit (WAVE 6000/55HT; NC Hyperbaric,
143 Burgos, Spain) at 125, 150, 175 and 200 MPa and 0 min holding time. Water used to
144 pressurize samples at a rate of 3 MPa/s yielded 41.7, 50, 58.3 and 66.7 s as the
145 corresponding come up times while decompression time was less than 3 s in all cases.
146 Cold pressurizing water was used to maintain temperature conditions during HHP
147 treatment at room temperature (20 ºC). After HHP treatment, mackerel (120
148 individuals) were kept at -18 ºC and analyzed after 48 hours (month 0) and after storage
149 for 1, 3, 6 and 9 months at -18 ºC. Fish without HHP treatment and subjected to the
150 same freezing and frozen storage conditions (30 individuals) were used as 0.1 MPa
151 controls. Three batches or replicates (n=3) for each processing condition were analyzed
152 independently. Analyses were carried out on the fish white muscle pooled from two

153 individual fishes. Similarly, six initial individuals were distributed into three batches
154 (two individuals per batch) and analyzed as initial fish material.
155
156 **Assessment of lipid and trimethylamine contents**
157 Lipids in fish muscle were extracted with a chloroform-methanol (1:1) mixture
158 following the Bligh and Dyer (1959) method and expressed as g lipid/100 g muscle.
159 Trimethylamine-nitrogen (TMA-N) values expressed as mg TMA-N/100 g muscle were
160 obtained following the picrate method (Tozawa et al. 1971) requiring the preparation of
161 a 5% (w/v) trichloroacetic acid extract of the mackerel muscle.
162
163 **Lipid damage analysis**
164 Free fatty acids (FFA) in lipid extracts, determined by the Lowry and Tinsley (1976)
165 method, were expressed as mg FFA/100 g muscle and as g FFA/100 g lipids. Peroxide
166 value (PV) in lipid extracts, determined following the Chapman and McKay (1949)
167 method, were expressed as meq active oxygen/kg lipids. The thiobarbituric acid index
168 (TBA-i) was determined as described by Vyncke (1970). Content of TBA reactive
169 substances (TBARS) spectrophotometrically measured at 532 nm was expressed as
170 mg malondialdehyde/kg muscle. Formation of fluorescent compounds was determined
171 in the aqueous phase obtained during the lipid extraction by measurements at 393/463
172 nm and 327/415 nm (Aubourg 1999). A relative fluorescence (RF) was defined as the
173 F/F_{st} ratio where F is the fluorescence measured at each excitation and emission
174 maximum, and F_{st} is the fluorescence intensity of a quinine sulfate solution (1 µg/mL in
175 0.05 M H_2SO_4) at the corresponding wavelength. To quantify the formation of
176 fluorescent compounds, a fluorescence ratio (FR) was calculated as the ratio between
177 the two RF values: $FR = RF_{393/463\ nm} / RF_{327/415\ nm}$. Lipid extracts were converted into

178 fatty acid methyl esters (FAME) and then analyzed using a Perkin-Elmer 8700 gas
179 chromatograph (Waltham, MA) equipped with a fused silica capillary column SP-2330
180 (0.25 mm i.d. x 30 m, 0.20 μm film, Supelco Inc., Bellefonte, PA) and using nitrogen at
181 10 psi as carrier gas (linear flow rate of 1.0 mL/min), a flame ionization detector (FID)
182 set at 250 ºC, and 19:0 fatty acid as internal standard (Aubourg et al. 1996). Peaks
183 corresponding to fatty acids were identified by comparison of the retention times of two
184 standards mixtures (Qualmix Fish, Larodan, Malmo, Sweden; FAME Mix, Supelco
185 Inc.). Content of each fatty acid was calculated as g/100 g total fatty acids. The polyene
186 index (PI) was calculated as the following fatty acids ratio: (C 20:5ω3 + C 22:6ω3)/C
187 16:0.
188
189 **Protein changes: analysis and identification**
190 Sarcoplasmic (low-salt-soluble) and myofibril (high-salt-soluble) protein extractions
191 from the mackerel muscle were performed by modification of the protocol developed by
192 Pazos et al. (2011). Briefly, 0.5 g muscle were homogenized using an Ultra-Turrax
193 high-performance disperser in ten volumes of Tris buffer (10 mM Tris-HCl, pH 7.2),
194 containing 5 mM protease inhibitor (PMFS). The sarcoplasmic protein fraction was
195 obtained from the supernatant after centrifugation (IKA®-Werke GmbH & Co.,
196 Staufen, Germany) of the homogenate at 40,000*g* (4 ºC, 20 min). The pellet was then
197 homogenized with a saline solution (0.6 M NaCl, 10 mM Tris buffer, 5 mM PMFS, pH
198 7.2), and myofibril proteins were isolated from the supernatant obtained after
199 centrifugation at 4,500*g* (4 ºC, 20 min). Sarcoplasmic and myofibril proteins were
200 stored at -80 ºC until use. Protein concentrations were determined in both fractions by
201 the BCA assay (Smith et al. 1985) and expressed as g/100 g muscle.

One-dimensional (1-D) laboratory-made 10% polyacrylamide gels (v/v; acrylamide:N,N´-ethylene-bis-acrylamide, 200:1) with an 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 containing 1.44% (w/v) glycine, 0.67% Tris-base, and 0.1% SDS (Laemmly 1970). Gels were stained overnight with the Coomassie dye PhastGel Blue R-350 (GE Healthcare). Protein bands of interest were manually excised from 1-D gels, and in-gel digested with trypsin as previously described (Pazos et al. 2014). Tryptic peptides were analyzed by liquid chromatography (LC) (Model 1260, Agilent, Palo Alto, CA) coupled to a linear ion trap (LIT) mass spectrometer model LTQ Velos Pro with electrospray interface (Thermo Fisher Scientific Inc., Rockford, IL). Nitrogen was used as nebulizing and drying gas and helium as collision gas. The chromatographic separation was performed on a BioBasic-18 column (5 µm particle size, 150 x 0.18 mm RP; Thermo Scientific, Waltham, MA) at a flow rate of 1.5-1.7 µL/min with a 90 min linear gradient of mobile phases A (0.5% acetic acid in water) and B (0.5% acetic acid in 100% acetonitrile) from 5 to 35% B. Peptides were monitored using MS survey scans from 350 to 1600 Da (2 µscans), followed by MS/MS scans (2 µscans) of the six more intense *m/z* peaks using a 1 Da isolation width, and a normalized collision energy of 35%. Singly charged ions were directly excluded from MS/MS analysis, and a dynamic exclusion was set at 30 s after the second fragmentation event of the same *m/z* peak. Protein identification was performed using the database searching function (PEAKS 7, Bioinformatics Solutions Inc, Waterloo, Ontario, Canada), to compare experimental MS/MS spectra with those on the UniProtKB/Swiss-Prot database, which included their respective decoy sequences. The following search limitations were used: tryptic cleavage, up to 2 missed cleavage sites, and tolerances ±1.2 Da for precursor ions and ±0.8 Da for MS/MS

227 fragments ions. The variable modifications allowed were cysteine
228 carbamidomethylation and methionine oxidation. The false discovery rate (FDR) was
229 kept below 1%.
230
231 **Enzymatic activity analysis**
232 Enzymatic extracts were prepared by homogenization of fish muscle (10 g) with 50 mL
233 of ice-cold distilled water for 2 min, using an IKA Ultra-Turrax T25 homogenizer for 2
234 min at 8,000*g* and then kept in ice with occasional stirring during 30 min. Thereafter,
235 extracts were centrifuged at 14,600*g* for 20 min at 4 ºC (Laboratory Centrifuge 3K30,
236 Sigma Laborzentrifugen GmbH, Osterode, Germany). Supernatant were filtered through
237 a Whatman nº 1 filter and stored at -20 ºC prior to enzymatic activity quantifications.
238 Acid phosphatase activity was determined following the methodology described
239 by Ohmory et al. (1992) with only minor modifications. Enzymatic extracts (0.250 mL)
240 were mixed with 0.225 mL of substrate solution (4 mM *p*-NPP in 0.1 mM acetate buffer
241 and 1 mM EDTA, pH 5.5). After 15 min incubation at 37 ºC, the reaction was stopped
242 by adding 1 mL of 100 mM KOH, and measuring spectrophotometrically at 400 nm
243 (Lambda 35 UV/vis spectrometer, Perkin-Elmer Instruments, Waltham, MA) the release
244 of *p*-nitrophenol (*p*-NP). Enzyme activity was expressed as nmol *p*-NP/min/g muscle.
245 Cathepsin B activity evaluated by the method described by Lakshmanan et al.
246 (2005) was expressed as fluorescence units (FU)/min/g muscle. With few modifications,
247 cathepsin D activity was determined following the protocol described by Buckow et al.
248 (2010). Enzyme extracts (0.2 mL) were mixed with 0.6 mL of substrate solution (2%
249 denatured hemoglobin (w/v) in 200 mM citrate buffer, pH 3.7). After 3h incubation at
250 37 ºC, the reaction was stopped by adding 0.6 mL of 10% TCA (w/v). After vigorous
251 stirring, the precipitate was removed by 15 min centrifugation at 18,000*g* (Elmi Micro

252 Centrifuge CM-50, Porvoo, Finland). Soluble peptides were spectrophotometrically
253 measured at 280 nm (Lambda 35 UV/vis spectrometer, Perkin-Elmer Instruments) and
254 converted to tyrosine equivalents using a previously built calibration curve. Cathepsin D
255 activity was expressed as μg tyrosine/min/g muscle.
256
257 **Statistical analysis**
258 Biochemical measurements for fish samples after 0, 1, 3, 6 and 9 months of frozen
259 storage time were subjected to one-way ANOVA ($p<0.05$) to explore differences in
260 pressure level and storage time effects. Comparison of means was performed using a
261 least-squares difference (LSD) method (Statsoft, Statistica, version 6.0, Tulsa, OK).
262 Correlation analysis indicating the type of fitting was also performed.
263
264 **RESULTS AND DISCUSSION**
265 **Assessment of lipid hydrolysis**
266 Lipid hydrolysis determined as FFA formation in mackerel muscle (Figure 1A, mg/100
267 g muscle) was calculated on the basis of lipid content values (6.5-9.0 g lipids/100 g
268 muscle; *data not shown*). After freezing (month-0 frozen samples), a marked FFA
269 content increase was observed in all samples; however, this increase was lower in
270 samples treated at 175 and 200 MPa. During frozen storage, FFA formation in all
271 samples showed a progressive increase (r^2 = 0.88-0.94). Compared with control fish, all
272 HHP-treated samples showed lower values at time 0 and 3 month and thereafter the
273 175-MPa treatment yielded the lowest mean values. Comparison among HHP-treated
274 samples did not provide evidence of a definite effect of pressure level throughout the
275 frozen storage period under study. Similar conclusions were obtained when FFA

276 content was expressed on the basis of lipid content (g/100g lipids) showing also a good
277 correlation with storage time for all treatments (r^2 = 0.86-0.94).

278 Previous research has detected an important FFA formation in Atlantic mackerel
279 throughout frozen storage (Aubourg et al. 2005; Vázquez et al. 2013). Accumulation of
280 FFA in fish muscle has no nutritional significance but undesirable secondary effects
281 include muscle texture changes (Sikorski and Kolakowska 1994), lipid oxidation
282 acceleration (Mackie 1993), and off-odor development (Refsgaard et al. 2000).
283 Research reported concerning the effect on FFA formation after HHP treatment
284 followed by a frozen storage is scarce. Ohshima et al. (1992) found that enzymatic
285 degradation of phospholipids in cod (*Gadus morhua*) muscle was successfully inhibited
286 during storage at -2 ºC for 6 days when previously treated at pressures above 400 MPa
287 for 15 and 30 min; however, no effect was observed when applying a pressure of 200
288 MPa. Chevalier et al. (2000) showed that FFA formation during storage at -20 ºC for 75
289 days did not show differences between turbot (*Scophthalmus maximus*) fillets subjected
290 to pressure shift freezing at 140 MPa or air-blast freezing (-20 ºC). Finally, Vázquez et
291 al. (2013) observed a marked reduction in FFA content in Atlantic mackerel (*Scomber*
292 *scombrus*) subjected to HHP treatments (150, 300, and 450 MPa for 0.0, 2.5, and 5.0
293 min) prior to freezing at -20 ºC and frozen storage at -10 ºC for 3 months. The reduction
294 effect increased with the pressure level and holding time employed.
295
296 **Assessment of lipid oxidation development**
297 The formation of primary and secondary lipid oxidation compounds was evaluated by
298 means of peroxide and TBA values, respectively (Tables 1 and 2). Values for both
299 parameters showed a relatively low lipid oxidation development as reflected by values
300 in the 0.07-4.99 meq active oxygen/kg lipids and 0.35-1.08 mg malondialdehyde/kg

301 muscle range, respectively. Such low values are in agreement with previous research on
302 frozen (-10 ºC for 3 months) mackerel previously treated under HHP conditions
303 (Vázquez et al. 2013). An inhibitory effect on peroxide formation was observed in
304 HHP-treated fish after freezing (comparison of month-0 samples), particularly at 200
305 MPa. Although some significant differences were detected during frozen storage, a
306 definite pressure level effect on the presence of primary oxidation compounds was not
307 identified.

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

320 Data on the formation of tertiary lipid oxidation compounds (namely, fluorescent
321 interaction molecules) is presented in Figure 1B. Immediately after freezing, a higher
322 mean value was obtained for control samples when compared with HHP-treated fish.
323 This inhibitory effect on fluorescent compound formation was observed throughout
324 frozen storage, i.e., a higher mean value was observed for control fish for all sampling
325 times. Values corresponding to samples treated at 175 MPa were significantly lower

than control fish when sampled at months 1-9, while 200-MPa treated samples were also significantly lower than controls after 1, 3 and 9 months of frozen storage. For 125 and 150 MPa treated samples, lower values were also found for 1 and 3 months when compared with control samples. These results show that HHP treatments caused an inhibitory effect on lipid oxidation development (tertiary lipid oxidation compounds). Concerning the effect of storage time, a progressive increase with time was observed in most cases showing correlation values in the 0.88-0.93 range (quadratic fitting). Lipid oxidation is a complex process involving the formation of different classes of compounds, most of them unstable and thus susceptible to breakdown and formation of lower molecular weight compounds or to react with other molecules, mostly nucleophiles, present in fish muscle. This is the case of peroxides and TBARS, widely reported to breakdown and give rise to tertiary lipid oxidation compounds after interacting with protein-type molecules (Aubourg 1999; Tironi et al. 2002). According to data obtained in this study, formation of fluorescent compounds seems to be the most adequate index to follow the progress of lipid oxidation.

Two opposite mechanisms can be considered to explain lipid changes observed in this study, i.e., the inactivation effect of pressure on endogenous enzyme activity (Torres et al. 2013; Vázquez et al. 2013) and the pressure-denaturation of iron-containing proteins which has been reported to increase free metal iron (Lakshmanan et al. 2003). These two effects would account for the opposite effects of HHP treatment observed when lipid oxidation development is evaluated. During 6 d storage at -2 ºC, Ohshima et al. (1992) found a TBARS formation increase in frozen cod (*Gadus morhua*) and mackerel (*Scomber scombrus*) muscle previously pressure treated at 616, 408 and 204 MPa for 15 and 30 min. A comparison of TBARS values observed during a 75-day storage at -20 ºC of turbot (*Scophthalmus maximus*) fillets subjected to pressure

shift freezing at 140 MPa or to air-blast freezing at -20 ºC could not identify a definite advantage of the pressure treatment (Chevalier et al. 2000). In work closely related to this study, Vázquez et al. (2013) observed a marked inhibition of tertiary lipid oxidation compounds formation in frozen (-10 ºC up to 3 months) mackerel (*Scomber scombrus*) previously subjected to 150, 300, 450 MPa for 0.0, 2.5, and 5.0 min. The effect observed was greater when increasing the pressure level or the holding time.

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

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

Trimethylamine formation

TMA is one of the most commonly employed quality methods to assess microbial activity in marine species kept under refrigerated conditions. However, if freezing and frozen storage are encountered, microbial activity is expected to be mostly inhibited so

376 that TMA formation would not be expected. Consequently, the conversion of
377 trimethylamine oxide (TMAO) into TMA observed in this study may be caused by non-
378 enzymatic processes, native tissue enzymes, or enzymes produced by microorganisms
379 before the HHP and freezing process. Values summarized in Table 3 showed that after
380 freezing, TMA-N content increased for all samples with significant differences ($p<0.05$)
381 resulting from the pressure treatment prior to freezing. Although some significant
382 differences among samples can be pointed out throughout frozen storage, a definite
383 effect of the pressure pre-treatment could not be determined during the 1-9-month fish-
384 sampling period. TMA-N content showed a progressive formation during frozen storage
385 (r^2 = 0.84-0.93). Additionally, good correlation values were obtained for TMA values
386 with FFA values (r^2 = 0.90-0.93) and FR scores (r^2 = 0.86-0.93). These results show that
387 significant conversion of TMAO to TMA occurred during the frozen storage of all
388 samples. In previous research by Senturk and Alpas (2013), the combined effect on
389 TMA formation of pressure level (200, 300 and 400 MPa), pressure holding time (5 and
390 15 min) and temperature (5, 10 and 15 ºC) was determined in mackerel (*Scomber*
391 *scombrus*) muscle; as a result, a marked increase in most cases after the HHP treatment
392 was observed, but with no significant differences among the different HHP condition
393 tested.
394
395 **Analysis of protein changes**
396 Figure 2 shows the SDS-PAGE profile of myofibril proteins for all frozen
397 mackerel samples. After freezing, myofibril proteins from pressure-treated and control
398 samples exhibited the same 1-D SDS-PAGE profile pattern (Figure 2A). Moreover, the
399 protein pattern obtained throughout frozen storage (months 3 and 9; Figures 2B and 2C,
400 respectively) did not reveal differences with the protein profile observed after freezing.

401 This means that HHP processing in the 150-200 MPa range and frozen storage for up to
402 9 months at -18 ºC did not significantly affect the 1-D electrophoretic pattern of
403 myofibril proteins. In previous work by Pazos et al. (2014), the effect on Atlantic
404 mackerel myofibril proteins of treatments at 150, 300 and 450 MPa for 0, 2.5 and 5 min
405 was analyzed after frozen storage at -10 ºC for 3 months. In agreement with this study,
406 myofibril proteins showed no solubility and electrophoretic gel profiles changes
407 throughout the whole frozen storage period. The SDS-PAGE profile of sarcoplasmic
408 proteins showed no differences among frozen fish samples at month 0 (Figure 3A).
409 However, a band of approximately 30 kDa disappeared during frozen storage. This
410 band, labeled as 1S, could not be detected after 3 months of frozen storage in samples
411 treated at 175 and 200 MPa (Figure 3B). Fish treated under 150 MPa showed the loss of
412 this band at month 9 (Figure 3C). It can be concluded that the HHP treatments studied
413 in this work induce this targeted degradation only in combination with frozen storage.
414 Values of total sarcoplasmic protein content in the 2.74-4.22 g/100 g muscle range
415 showed no effect of pressure treatment and frozen storage time. Previous research
416 concerning mackerel showed a decrease on sarcoplasmic protein content when higher
417 pressures (300 and 450 MPa) than in the present case were applied (Pazos et al. 2014).
418 Over the last decade, proteomics has been successfully applied to evaluate
419 quality in food systems including meat, fish, milk and transgenic plants (Gallardo et al.
420 2013). Proteomics analysis based on one- and two-dimensional PAGE and tandem mass
421 spectrometry (MS/MS) is a particularly powerful technology to identify global changes
422 in protein constituents (Han and Wang 2008). In spite of its potential, proteomics tools
423 applications to HHP-treated fish are still very limited. A marked content decrease in 94-
424 , 50- and 43-kDa bands was noticed in mackerel (*Scomber japonicus*) muscle treated at
425 200 MPa or higher, although the identity of these proteins was not reported (Ohshima et

426 al. 1992). Chevalier et al. (1999) also found a disappearance of an unidentified 48-kDa
427 protein band in turbot (*Scophthalmus maximus*) muscle when treated at pressures higher
428 than 150 MPa. More recently, SDS-PAGE analysis of Coho salmon (*Oncorhynchus*
429 *kisutch*) sarcoplasmic fraction showed a partial loss of a band corresponding to 29 kDa
430 that was identified by MS/MS analysis as phosphoglycerate mutase (Ortea et al. 2010),
431 in agreement with the identification carried out in the present research. Finally, Pazos et
432 al. (2014) analyzed the selective-targeted effect of HHP processing (150, 300 and 450
433 MPa for 0.0, 2.5 and 5.0 min) on proteins by identifying sarcoplasmic protein bands of
434 frozen (-10 ºC) Atlantic mackerel that were modified by the treatment. In agreement
435 with their work, the present proteomics study showed that sarcoplasmic proteins are
436 more liable to HHP-freezing-frozen storage processing than the myofibril fraction. The
437 1S band excised from the gel was digested with trypsin and the resulting peptides were
438 subjected to MS analysis by means of an electrospray ion trap mass detector. Peptides
439 were fragmented and non-interpreted fragmentation spectra (MS/MS) and de novo-
440 inferred sequences were searched against the protein sequence databases using the
441 SEQUEST software and the BLAST tool, respectively. As a result, the protein band was
442 assigned to the glycolytic enzyme phosphoglycerate mutase 2 (PGAM2) based on the
443 following identification parameters: UniProtKB/Swiss-Prot Code (Q32DV0), Mass
444 (28,685 Da), -10IgP Score (106.99), #Peptides/#Unique Peptides (6/5), Sequence
445 (K.AMEAVAAQGK.A*; R.KAMEAVAAQGK.A*; R.ALPFWNDEIAPQIK.A*;
446 R.HYGGLTGLNK.A*; R.FCGWFDADLSEK.G*; K.HGEEQVK.I) and Sequence
447 Coverage (21%). Phophoglycerate mutase is a transferase enzyme responsible for
448 transferring a phosphate group from the C-3 carbon of 3-phosphoglycerate to the C-2
449 carbon forming 2-phosphoglycerate, acting in the final part of the glycolysis pathway.
450 In agreement with previously mentioned research (Ortea et al. 2010), results from this

451 study show that the assessment of this enzyme could be an effective tool to study the
452 fish quality loss during storage.
453
454 **Enzymatic activity analysis**
455 A general behavior of the acid phosphatase activity data summarized in Table 4 showed
456 for all samples a progressive increase with storage time (r^2 = 0.79-0.87; quadratic
457 fitting). An inhibitory effect of HHP treatments at 150 and 200 MPa, was observed in
458 month-0 samples by comparison with their counterpart controls. Comparisons
459 throughout frozen storage of pressure treatments did not show a general pattern;
460 however, HHP-treated samples showed a lower activity at the end of the experiment
461 when compared with their counterpart control samples. In previous work, Fidalgo et al.
462 (2014) observed that acid phosphatase from mackerel (*Scomber scombrus*) was strongly
463 affected by frozen storage time (3 months at -10 ºC) in samples untreated or HHP-
464 treated (150, 300 and 450 MPa; 0.0, 2.5 and 5.0 min). An important effect of pressure
465 could also be observed with lower values obtained by increasing the pressure level. Fair
466 correlation values were obtained in the present study for the acid phosphatase activity
467 when compared with chemical quality indices such as FFA content (0.79-0.89), TMA
468 formation (0.75-0.94), PV (0.74-0.90) and FR value (0.76-0.93).
469 Cathepsin B activity assessments (Table 4) showed also a progressive increase
470 (r^2 = 0.88-0.91; quadratic fitting) during the frozen storage of all samples. The highest
471 values ($p<0.05$) for each sample type were also observed at the end of storage. A
472 comparison of samples after freezing showed an inhibitory effect of treatments at 125,
473 150 and 175 MPa but not at 200 MPa. Although some significant differences were
474 observed during frozen storage, a definite effect of the HHP treatment was not possible
475 to conclude. It should be mentioned that in the 3-9-month period, a lower mean activity

476 was observed in samples previously treated at 150-200 MPa when compared with their
477 counterpart control samples. In previous related research by Fidalgo et al. (2014),
478 cathepsin B activity in frozen mackerel (3 months at -10 ºC) was also affected by HHP
479 treatments (150, 300 and 450 MPa for 0.0, 2.5 and 5.0 min) before freezing and frozen
480 storage, being the pressure effect higher than that for frozen storage and pressure
481 holding time. A decrease in cathepsin B activity was evident with a pressure level
482 increase, although a recovery effect was observed during frozen storage. In the present
483 study, cathepsin B activity showed fair correlation values with chemical quality indices
484 such as FFA content (0.77-0.89), TMA formation (0.67-0.88), PV score (0.42-0.94) and
485 FR value (0.78-0.94).

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

496 Although HHP treatment may inhibit the activity of hydrolytic enzymes, the
497 effects on fish muscle of 125-200 MPa treatments depend on several factors causing
498 activation or inactivation of muscle enzymes. Consequently, the enzyme activity values
499 observed in this study can be considered the result of different and opposite effects.
500 While HHP treatments may inactivate enzymes by disrupting intramolecular bonds

501 determining their secondary, tertiary and quaternary conformation (Ashie et al. 1996;
502 Campus 2010), pressure can also disrupt lysosomal membranes releasing proteases
503 leading to an increase in hydrolytic activity (Ohmori et al. 1992; Chéret et al. 2005).
504 Cathepsins B (a cysteine protease) and D (an aspartic acidic protease) have been
505 reported to be released from the lysosomal matrix into both the cytoplasm and the
506 intracellular spaces as a consequence of the breakdown of lysosomes (Chéret et al.
507 2006).
508

509 ## CONCLUSIONS

510 Research carried out during the latest decades has shown that HHP treatment can lead to
511 an important quality enhancement in seafood on the basis of its ability for inactivating
512 the microbial development and the endogenous enzyme activity. However, if relatively
513 strong conditions are employed, HHP treatment has shown to induce detrimental
514 modifications in valuable nutritional constituents in seafood.

515 On the basis of the results obtained in a previous study, the present research has
516 focused on the biochemical changes produced in frozen Atlantic mackerel stored under
517 commercial conditions (9 months at -18ºC) that was previously treated under optimized
518 HHP conditions (125, 150, 175 and 200 MPa). As a result, previous HHP treatment led
519 to valuable biochemical changes such as inhibition of lipid hydrolysis (FFA formation)
520 and oxidation (tertiary compounds) in the frozen mackerel muscle, that were
521 accompanied by the modification of the sarcoplasmic protein profile (partial
522 disappearance of the phosphoglycerate mutase 2 band) when the strongest pressure
523 conditions (namely, 175 and 200 MPa) were applied. Meantime, no effect of the HHP
524 treatment was observed on other biochemical indicators of quality loss in frozen fish

525 including TMA and PUFA content, 1-D myofibril protein fraction, and the activity of
526 acid phosphatase, and cathepsins B and D.

527 Present research proves the need of optimizing the HHP treatment conditions for
528 each marine species and processing in order to guarantee the highest retention of
529 chemical constituents. Additionally, this work provides the first attempt focused on the
530 quality enhancement of a frozen marine species stored under commercial conditions by
531 means of a previous HHP treatment.

532

533

534

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549

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.

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TABLE 1

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

Frozen storage time (months)	Pressure (MPa)				
	0.1	**125**	**150**	**175**	**200**
Initial fish	0.36 a (0.23)	0.36 ab (0.23)	0.36 a (0.23)	0.36 a (0.23)	0.36 ab (0.23)
0	1.72 cC (0.22)	0.47 abcB (0.26)	0.36 aB (0.18)	0.36 aB (0.21)	0.07 aA (0.04)
1	0.29 aA (0.06)	0.14 aA (0.10)	0.26 aA (0.12)	0.24 aA (0.07)	0.60 bB (0.17)
3	0.57 ab (0.31)	0.34 ab (0.21)	0.45 a (0.19)	0.44 a (0.25)	0.74 b (0.41)
6	1.62 bcA (0.74)	0.78 bcA (0.40)	3.11 bB (0.69)	4.90 bBC (1.73)	4.99 dC (0.72)
9	3.01 d (0.88)	2.33 c (1.70)	3.24 b (1.28)	2.94 b (1.07)	1.99 c (0.61)

* 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 previous 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 2

Measurement of the thiobarbituric acid index (mg malondialdehyde/kg muscle)* in frozen mackerel treated by high-pressure prior to freezing and subsequent storage at -18ºC for up to 9 months

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

* 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 previous pressure treatment. For each pressure condition, means followed by different low-case letters (a-c) 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 3

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

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

* 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 previous pressure treatment. For each pressure condition, means followed by different low-case letters (a-e) 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

Evolution of acid phosphatase activity (mmol p-NP.min^{-1}.g^{-1})* in frozen mackerel treated by high-pressure prior to freezing and subsequent storage at -18ºC for up to 9 months

Frozen storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
0	218.2 aB (4.7)	228.3 abB (5.7)	194.0 aA (6.9)	207.5 bAB (7.2)	187.7 aA (5.8)
1	241.1 bB (0.3)	206.7 aA (5.4)	202.0 aA (8.9)	199.1 bA (2.7)	189.6 aA (6.4)
3	205.1 aBC (2.9)	249.0 bcD (4.0)	227.4 bCD (5.4)	170.4 aA (5.4)	191.4 aAB (6.8)
6	245.8 bA (9.7)	255.2 cdAB (8.9)	267.5 cAB (9.9)	272.5 cB (5.9)	255.1 bAB (2.7)
9	331.8 cB (12.6)	276.2 dA (9.8)	268.5 cA (9.0)	277.0 cA (3.9)	265.8 bA (11.7)

* 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-D) indicate significant differences ($p<0.05$) as a result of the previous 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.

TABLE 5

Evolution of cathepsin B activity (10^5 FU.min $^{-1}$.g^{-1})* in frozen mackerel treated by high-pressure prior to freezing and subsequent storage at -18ºC for up to 9 months

Frozen storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
0	9.46 b (0.77)	9.51 a (0.81)	8.17 a (0.85)	8.76 a (0.40)	10.37 ab (1.21)
1	6.88 aA (0.73)	7.71 aAB (0.34)	8.68 aAB (1.33)	9.81 aB (0.53)	9.38 aB (0.37)
3	12.48 cC (0.66)	9.87 aB (0.59)	12.22 bBC (1.23)	11.77 bABC (1.08)	10.05 aAB (0.15)
6	13.59 cB (0.29)	11.10 bA (0.42)	12.27 bAB (0.26)	11.29 bA (0.07)	12.57 bAB (0.81)
9	18.88 dBC (0.48)	19.84 cC (0.81)	17.25 cAB (0.49)	17.58 cB (0.12)	15.22 cA (0.57)

* 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 previous 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.

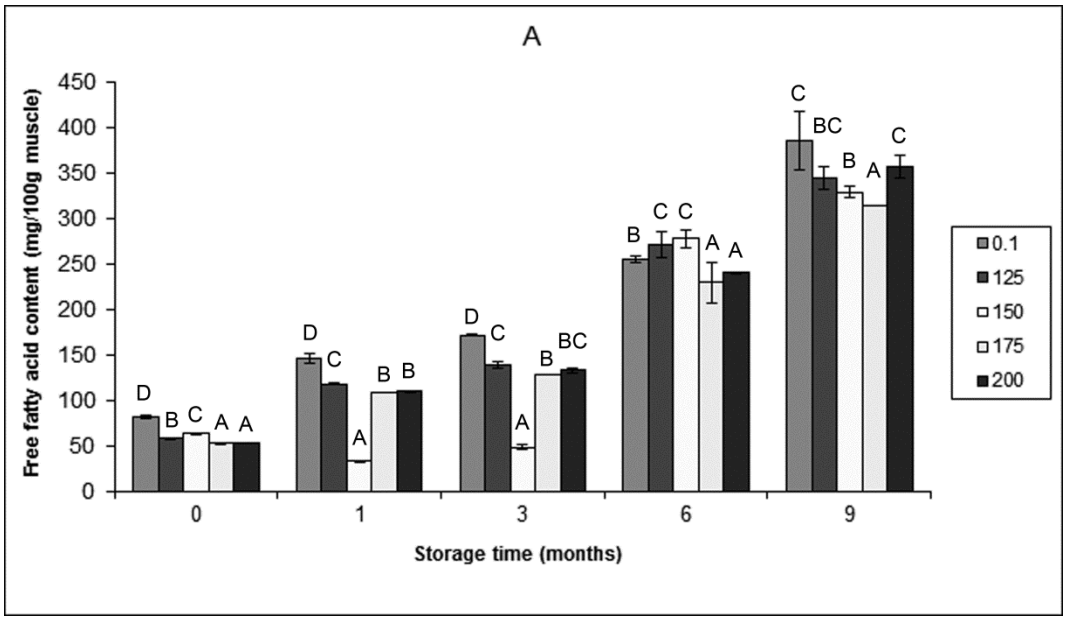
TABLE 6

Evolution of cathepsin D activity (µg tyrosine.min $^{-1}$.g^{-1})* in frozen mackerel treated by high-pressure prior to freezing and subsequent storage at -18ºC for up to 9 months

Frozen storage time (months)	Pressure (MPa)				
	0.1	125	150	175	200
0	2.54 aA (0.24)	3.80 bB (0.52)	2.24 A (0.45)	2.62 abcA (0.40)	2.36 aA (0.19)
1	3.10 abB (0.33)	3.84 bB (0.76)	2.78 AB (0.49)	2.04 aA (0.22)	2.91 abAB (0.20)
3	3.64 bcB (0.41)	3.10 abAB (0.42)	3.18 AB (0.22)	2.55 abA (0.13)	2.77 abAB (0.06)
6	3.98 bc (0.48)	3.66 b (0.14)	3.15 (0.18)	3.61 c (0.37)	3.49 b (0.30)
9	4.34 cC (0.03)	2.17 aA (0.26)	2.87 AB (0.13)	3.40 bcBC (0.05)	5.53 cD (0.30)

* 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-D) indicate significant differences ($p<0.05$) as a result of the previous pressure treatment. For each pressure condition, means followed by different low-case letters (a-c) 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.

FIGURE 1


A
Free fatty acid content (mg/100g muscle)
450
400
350
300
250
200
150
100
50
0
D B C A A
D C A B B
D C A B BC
B C C A A
C BC B A C
0 1 3 6 9
Storage time (months)
0.1
125
150
175
200

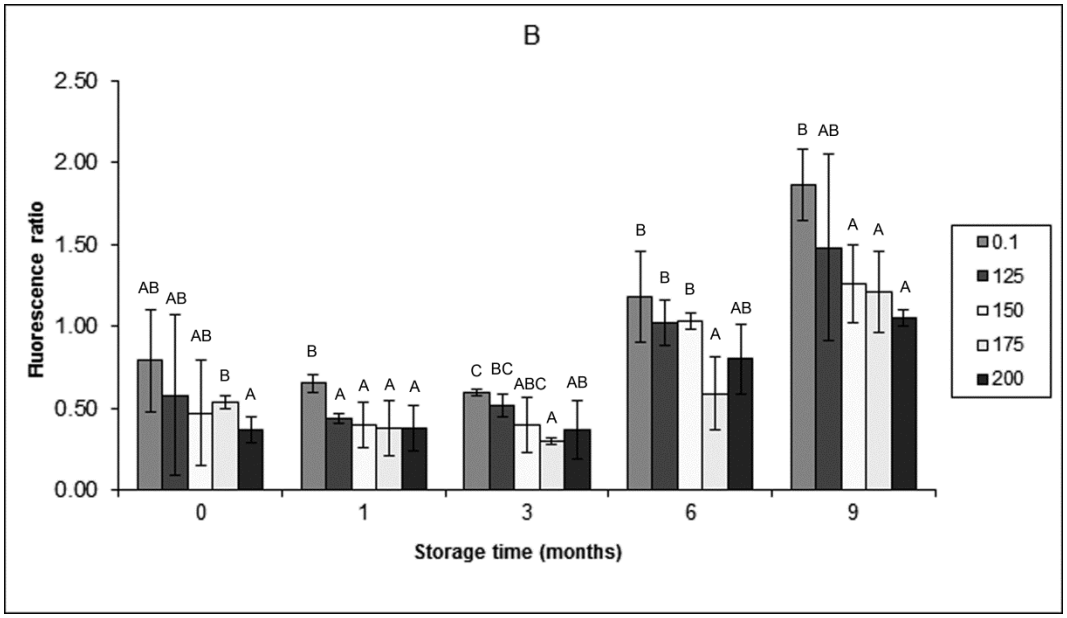

B
Fluorescence ratio
2.50
2.00
1.50
1.00
0.50
0.00
AB AB AB B A
B A A A A
C BC ABC A AB
B B B A AB
B AB A A A
0 1 3 6 9
Storage time (months)
0.1
125
150
175
200

Figure 2

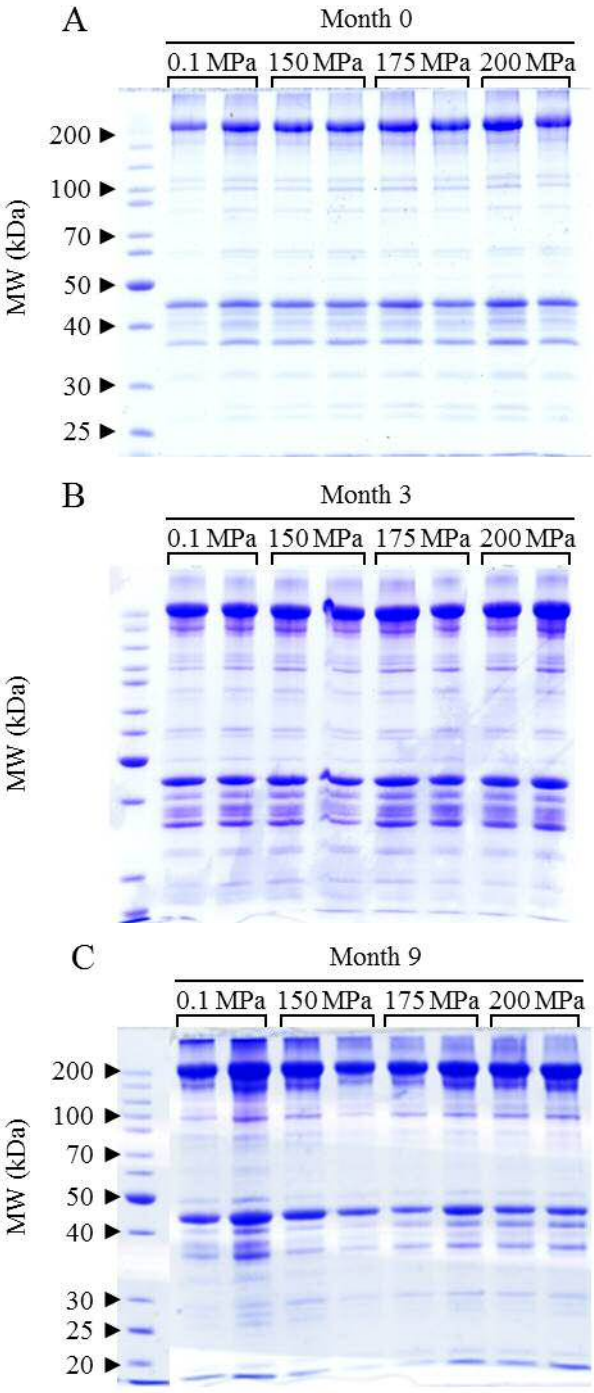
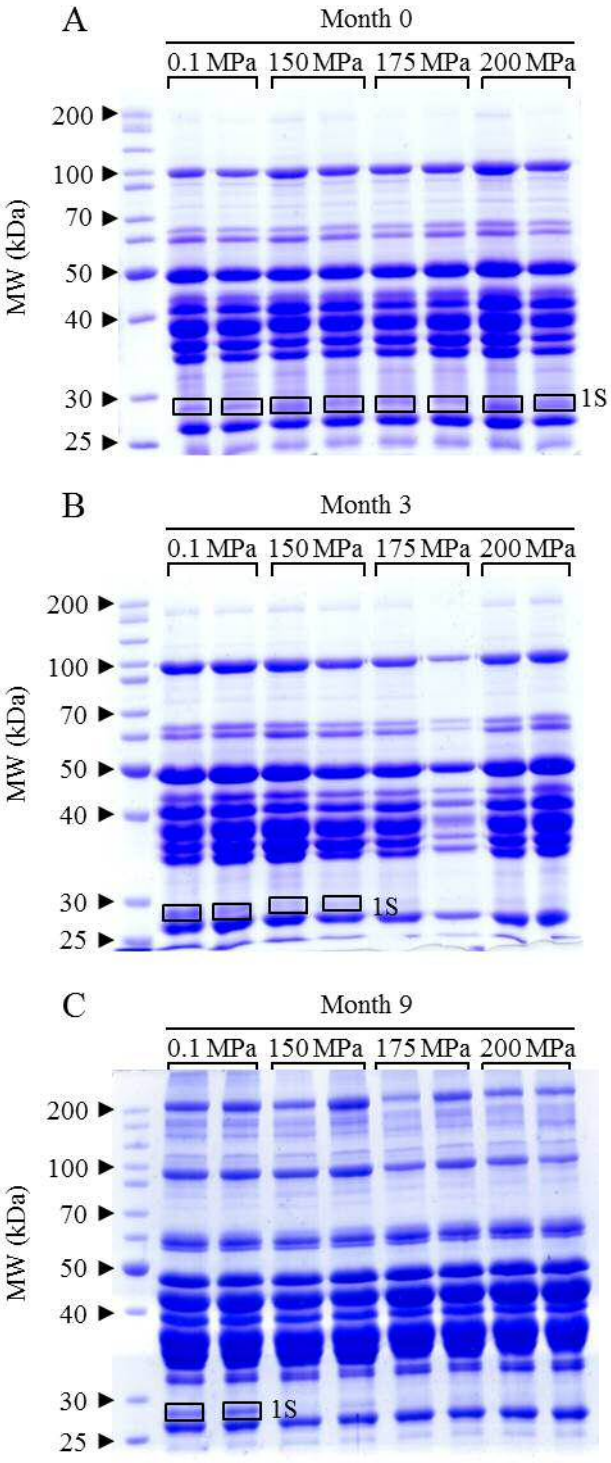

A
Month 0
0.1 MPa
150 MPa
175 MPa
200 MPa
MW (kDa)
200
100
70
50
40
30
25
B
Month 3
0.1 MPa
150 MPa
175 MPa
200 MPa
MW (kDa)
C
Month 9
0.1 MPa
150 MPa
175 MPa
200 MPa
MW (kDa)
200
100
70
50
40
30
25
20

Figure 3


A
Month 0
0.1 MPa
150 MPa
175 MPa
200 MPa
MW (kDa)
200
100
70
50
40
30
25
1S
B
Month 3
0.1 MPa
150 MPa
175 MPa
200 MPa
MW (kDa)
200
100
70
50
40
30
25
1S
C
Month 9
0.1 MPa
150 MPa
175 MPa
200 MPa
MW (kDa)
200
100
70
50
40
30
25
1S