SELECTIVE-TARGETED EFFECT OF HIGH PRESSURE PROCESSING ON PROTEINS RELATED TO QUALITY: A PROTEOMICS EVIDENCE IN ATLANTIC MACKEREL (*Scomber scombrus*)

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ABSTRACT

The effect of high hydrostatic pressure (HHP) treatment (150, 300 and 450 MPa for 0, 2.5 and 5 min) on total sodium dodecyl sulfate (SDS)-soluble and sarcoplasmic proteins in frozen (-10ºC for 3 months) Atlantic mackerel (*Scomber scombrus*) was evaluated. Proteomics tools based on image analysis of SDS-PAGE protein gels and protein identification by tandem mass spectrometry (MS/MS) were applied. Total SDS-soluble proteins, composed in high proportion by myofibrillar proteins, were stable under pressurization treatment in terms of solubility and electrophoretic gel profiles. However, pressurization reduced sarcoplasmic proteins solubility, modified their 1-D/2-D SDS-PAGE patterns in a direct-dependent manner and exerted a selective effect on particular sarcoplasmic proteins depending on processing conditions. Thus, protein bands assigned to creatine kinase, fructose-bisphosphate aldolase A, glycogen phosphorylase and β-enolase were degraded at 300-450 MPa. Additionally, the stability of triosephosphate isomerase B, phosphoglucomutase and phosphoglycerate kinase-1 was found to be HHP-reduced when submitted at 450 MPa. HHP processing (300-450 MPa) also induced a cross-linking product formation of pyruvate kinase and two compounds derived from tropomyosin at 450 MPa. Frozen storage time of pressurized samples induced an additional lessening in protein solubility, but electrophoretic patterns were not modified. The present investigation emphasizes the higher lability of sarcoplasmic proteins under HHP treatment and the important role of these proteins in the sensory quality enhancement provided by milder HHP conditions on frozen mackerel. HHP technology is expected to boost the development of novel tailored processing approaches to tackle food quality challenges.

Key words: *Scomber scombrus*; high pressure; frozen storage; proteomics; sarcoplasmic proteins; SDS-soluble proteins.

Running head: Proteomics and pressurized frozen fish.

INTRODUCTION

Marine species provide valuable components to human nutrition but are known to deteriorate rapidly postmortem due to the effects of a variety of degradation mechanisms. Consequently, the actual increasing consumer's demand for high quality marine food has led to the development of advanced technologies that are able to present attractive, nutritional and safe products (Ashie et al. 1996; Oms-Oliu et al. 2010; Rodríguez et al. 2012). Among such technologies, high hydrostatic pressure (HHP) has shown to maintain sensory and nutritional properties, while inactivating microbial development and leading to a shelf-life extension and a safety enhancement (Torres and Velázquez 2005; Norton and Sun 2008). An additional profitable effect on quality retention, is that HHP technology has shown to inactivate deteriorative hydrolytic (namely, lipases and phospholipases) and oxidative (peroxidases, lipoxygenases, etc.) endogenous enzymes (Teixeira et al. 2013; Vázquez et al. 2013).

Previous research has been focused on changes produced on chemical constituents as a result of HHP processing. Most of it has concerned the ability for protein denaturation and modification, being the intensity and reversibility of this effect strongly dependent on the strength of the HHP conditions. Since HHP treatment has been reported to follow the Le Châtelier principle (Campus 2010), any phenomenon in equilibrium accompanied by a decrease in volume can be enhanced by the HHP treatment, and vice versa. Accordingly, primary structure of proteins (amino acids in a polypeptide chain joined by covalent bonding) would not be modified (Hendrickx et al. 1998; Knorr 1999). However, HHP processing has shown to affect non-covalent bonds (hydrogen, ionic, and hydrophobic bonds) substantially as most of them are very sensitive to pressure. As a result, marked changes are likely to be produced in quaternary (namely, dissociation of oligomers), tertiary (namely, modification of

87 hydrophobic and ionic interactions) and secondary (namely, modification of hydrogen
88 bonds) protein structures.

89 Over the last decade, proteomics has been successfully applied to evaluate
90 quality in food systems including meat, fish, milk and transgenic plants, and food safety
91 aspects as the occurrence of food-derived pathogens and allergens (D'Alessandro and
92 Zolla 2012; Gallardo et al. 2013). In particular, proteomics analysis based on two-
93 dimensional polyacrylamide gel electrophoresis (2D-PAGE) and tandem mass
94 spectrometry (MS/MS) has been found a powerful technology to identify global
95 changes in protein constituents of tissues and subcellular compartments (Han and Wang
96 2008). Despite this outstanding potential of proteomics, the application of proteomics
97 tools to HHP-treated fish is still very limited. Thus, a marked content decrease in 94-,
98 50- and 43-kDa bands was noticed in mackerel (*Scomber japonicus*) muscle when
99 treated at 200 MPa or higher, although their protein identity was not characterized
100 (Ohshima et al. 1992). Chevalier et al. (1999) also found losses in a non-identified 48-
101 kDa protein band in turbot (*Scophthalmus maximus*) muscle when treated at a pressure
102 higher than 150 MPa. More recently, SDS-PAGE analysis of Coho salmon
103 (*Oncorhynchus kisutch*) sarcoplasmic fraction showed a partial loss of a band
104 corresponding to 29 kDa that was identified by MS-MS analysis as phosphoglycerate
105 mutase (Ortea et al. 2010).

106 Atlantic mackerel (*Scomber scombrus*) is an abundant underutilized species
107 belonging to the Scombridae family (FAO 2007). It has shown an important
108 endogenous enzyme activity and significant quality losses during frozen storage (Saeed
109 and Howell 2001; Aubourg et al. 2005), so that different preservative strategies have
110 been applied to inhibit quality loss (Santos et al. 1990; Richards et al. 1998; Saeed and
111 Howell 2002). However, previous research has shown a lipid damage inhibition

(Vázquez et al. 2013) and sensory acceptance enhancement (Aubourg et al. 2013b) of frozen mackerel when a previous HHP processing (150, 300 and 450 MPa for 0.0, 2.5 and 5.0 min) was applied. The present investigation complements these previous studies with the aim of comprehensively understanding quality improvements linked to changes induced by HHP process on either structural myofibrillar or enzymatic sarcoplasmic proteins. For this purpose, protein solubility measurement and a proteomics approach consisting of image analysis of one- and two-dimensional SDS-PAGE profiles, and protein identification by MS/MS analysis and database homology were performed on both total SDS-soluble and sarcoplasmic proteins from frozen Atlantic mackerel (*Scomber scombrus*) previously submitted to HHP processing.

MATERIALS AND METHODS

Materials

Dithiothreitol (DTT), Tris-HCl, bicinchoninic acid (BCA), sodium dodecyl sulfate (SDS) and the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). Immobilized pH-gradient strips covering pH 3-10 (11 cm), pharmalyte 3-10, 1,2-bis-dimethylamine-ethane (TEMED), ammonium persulfate (APS), bromophenol blue and deStreak reagent were purchased from GE Healthcare Science (Uppsala, Sweden). Acrylamide and bis N,N´-methylene-bis-acrylamide were obtained from Bio-rad (Hercules, CA, USA). Glycerol was obtained from Merck (Darmstadt, Germany). Sequencing grade bovine trypsin was purchased from Promega (Madison, WI, USA). All other chemicals were reagent/analytical grade and water was purified using a Milli-Q system (Millipore, Billerica, MA, USA).

137 **Raw fish, HHP processing and frozen storage**

138 Atlantic mackerel (47 kg) caught near the Bask coast in Northern Spain was
139 obtained at the Ondarroa harbor (Bizkaia, Spain) and transported under ice to the AZTI
140 Tecnalia (Derio, Spain) pilot plant for HHP treatment within 6 hours after catch. Whole
141 mackerel individuals were placed in flexible polyethylene bags (three individuals per
142 bag) and vacuum sealed at 400 mbar. The length and weight of the specimens ranged
143 28-33 cm and 230-280 g, respectively.

144 HHP treatments at 150, 300 and 450 MPa and 0.0, 2.5 and 5.0 min pressure
145 holding times were performed in a 55-L high pressure unit (WAVE 6000/55HT; NC
146 Hyperbaric, Burgos, Spain). Water applied as the pressurizing medium at 3 MPa/s
147 yielded 50, 100 and 150 s as the come up times for the 150, 300 and 450 MPa
148 treatments, respectively, while decompression time was less than 3s. Cold pressurizing
149 water was used to maintain temperature conditions during HHP treatment at room
150 temperature (20ºC).

151 After HHP treatments, mackerel individuals were kept at –20ºC for 48 hours and
152 then stored at –10ºC with samples analyzed after 0 and 3 months of storage. Fish
153 without HHP treatment and subjected to the same freezing and frozen storage
154 conditions was used as control. Three batches or replicates (n=3) for each treatment
155 were analyzed independently. Each analysis was based on the whole white muscle of
156 fish pooled from two individual fish.

157 A frozen storage temperature (-10ºC) higher than commercial practice (-18ºC)
158 was chosen as an accelerated test condition since scarce research was available to
159 estimate the extent of the HHP treatment on the protein damage development of a
160 frozen fish species. In addition, the response to the HHP treatment of marine species has
161 been reported to vary with species, chemical composition and size (Erkan et al. 2011;

Aubourg et al. 2013a). Consequently, a preliminary study was undertaken to confirm the above mentioned pressure conditions. Sensory parameters (eyes, external and muscle color, hardness, external odor, blood, skin and gills) were analyzed after testing a wide range of pressure (600, 500, 400, 350, 300, 250, 200 and 100 MPa) values for 5 minutes as pressure holding time and compared to those observed for untreated mackerel. Most attributes showed quality losses increasing with the pressure applied as compared to control samples. On the other, the appearance of blood and gills remained unchanged in the 0-300-MPa range while at higher pressure, blood coagulated and gills color was markedly lighter. Accordingly, 300 MPa was chosen as the mid pressure point in the present study which included also a lower and a higher pressure value of 150 and 450 MPa, respectively.

Extraction of total SDS-soluble and sarcoplasmic protein fractions

Fish were filleted, and whole white muscle was isolated and chopped. Extraction of total SDS-soluble proteins was performed by modification of a previous protocol (Ortea et al., 2010). Briefly, 0.5 g of raw mince muscle were homogenized in ten volumes of Tris buffer (10 mM Tris-HCl, pH 7.2), and supplemented with 2% of the denaturing agent SDS and 5 mM of the protease inhibitor PMSF, in an Ultra-Turrax blender for 2 min. Samples were then boiled for 5 min, re-homogenized by Ultra-Turrax for 2 min, and centrifuged at 40,000g for 2 min at 4ºC (Avanti centrifuge J-25I, Beckman Coulter, Palo Alto, CA, USA). Supernatants were separated and labeled as total SDS-soluble protein fraction. Sarcoplasmic protein fractions were obtained (Ortea et al. 2010) by homogenizing 0.5 g of minced muscle in ten volumes of a non-denaturing low ionic strength solution composed by the above indicated Tris buffer (10 mM Tris-HCl, pH 7.2), supplemented with 5 mM of PMSF, in an Ultra-Turrax blender

for 2 min. Sarcoplasmic proteins were finally isolated from supernatants of homogenates centrifuged at 40,000g for 2 min at 4ºC (Avanti centrifuge J-25I, Beckman Coulter, Palo Alto, CA, USA). Then, supernatants containing sarcoplasmic proteins were separated. Protein fractions were maintained at -80ºC until the electrophoresis analysis was carried out.

Protein solubility

Protein concentrations were determined in the total SDS-soluble and sarcoplasmic protein fractions by the Bradford (1976) assay. Bovine serum albumin was used as standard. Protein solubility was expressed as g of soluble protein in the total SDS-soluble and sarcoplasmic protein fractions per g of fish muscle.

SDS-Polyacrylamide gel electrophoresis

Protein profiles were visualized on both one-dimensional (1-D) and two-dimensional (2-D) laboratory-made 10% (v/v) polyacrylamide gels (acrylamide:N,N´-ethylene-bis-acrylamide, 200:1). 1-D gels with stacking gel (4 % polyacrylamide) were loaded with 20-30 μg of protein per well, and run in a Mini-PROTEAN 3 cell (Bio-Rad) (Laemmly 1970). The running buffer employed consisted of an aqueous solution composed by 1.44% (w/v) glycine, 0.67% Tris-base, and 0.1% SDS. Gels were finally stained overnight with the PhastGel Blue R-350 (GE Healthcare Science, Uppsala, Sweden) Coomassie dye. 2-D gel electrophoresis was performed by adaptation of a protocol previously described (Pazos et al. 2011). Briefly, 400 μg of proteins were applied to IPG 3-10 strips (11 cm) by active rehydration (50 V for 10 hours) at 20ºC on an Ettan IPGphor II isoelectric focusing system (GE Healthcare Science, Uppsala, Sweden). Protein focusing on IPG strips was performed at 15ºC by applying the

voltage/time profiles suggested by the Ettan IPGphor manufacturer (GE Healthcare Science). After focusing, strips were kept frozen at -80ºC until use. Previously to run the second dimension, strips were equilibrated for 15 min in a buffer containing DTT (6 M urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 30% glycerol, 0.075% DTT) and then re-equilibrated with the same buffer but substituting DTT by 4.5% iodoacetamide. The gels were run at a constant current setting in an Ettan Daltsix electrophoresis system (GE Healthcare) at 15ºC. Two 11-cm IPG strips corresponding to pressurized and non-pressurized control samples were simultaneously run in the same 2-DE gel (24-cm wide). The running buffer of the second dimension was an aqueous solution composed by 1.44% (w/v) glycine, 0.67% Tris-base, and 0.1% SDS. Gels were stained overnight with the Coomassie dye PhastGel Blue R-350 (GE Healthcare).

In-gel digestion and protein identification by LC-ESI-IT-MS/MS

Bands and spots containing the proteins of interest were manually excised respectively from 1-D and 2-D gels, in-gel reduced, alkylated and digested with trypsin as previously described (Jensen et al. 1998). Briefly, protein spots were three times washed with water and dehydrated with acetonitrile. The gel plugs were dehydrated in a vacuum centrifuge and rehydrated with a 0.5 μM solution of sequencing grade bovine trypsin in 50 mM ammonium bicarbonate buffer, pH 8.0, for at least 40 min on ice. After rehydration step, samples were digested overnight at 37ºC.

Peptide digests were analyzed online by a liquid chromatography (LC) system model SpectraSystem P4000 (Thermo Scientific, San Jose, CA, USA) coupled to an ion trap (IT) mass spectrometer model LCQ Deca XP Plus with an electrospray ionization (ESI) interface (Thermo Scientific). The chromatographic separation was performed on a 0.18 mm x 150 mm BioBasic-18 RP column (Thermo Scientific) with a particle size

of 5 μm. A 90 min linear gradient of 0.5% of acetic acid in water and in 80% acetonitrile, as respective mobile phases A and B, from 5 to 60% B, at a flow rate of 1.5-1.7 μL/min, was employed. Peptides were monitored using MS survey scans from 400 to 1600 Da (3 μscans), followed by MS/MS scans (3 μscans) of the four more intense *m/z* peaks using an isolation width of 5 Da and a normalized collision energy of 35%. A dynamic exclusion for 3 min was set after the third fragmentation event of the same *m/z* peak, and singly charged ions were excluded from MS/MS analysis. Protein identification was performed using the MASCOT database search engine to compare experimental MS/MS spectra against MS/MS spectra obtained from theoretical digestion of the proteins present in the nr.fasta database (NCBI Resources, NIH, Bethseda, MD, USA). The following constraints were used for the searches: tryptic cleavage, up to 1 missed cleavage sites, and tolerances ±1.5 Da for precursor ions and ±0.8 Da for MS/MS fragments ions. The variable modifications allowed were methionine oxidation and carbamidomethylation of cysteine. In all protein identifications, the probability scores were greater than the score fixed by MASCOT database as significant ($p<0.05$).

Image analysis of SDS-PAGE gels

Scanned coomassie-stained 1-D gels were analyzed with the 1-DE gel analysis software LabImage 1D (Kapelan Bio-Imaging Solutions, Halle, Germany) using the optical intensity (band volume) to quantify protein level and the Rubber band method as background correction method. 2-D electrophoresis gels were processed by the PDQuest software version 7.1 (Bio-Rad) for removal of background and matching protein spots among the gels. The intensity of the spots, expressed as parts per million

(ppm) of the total integrated optical density of the spots in the gel, was used to evaluate changes in the intensity of coomassie-stained protein spots.

Statistical analysis

Six fish individuals were collected for each technological treatment and sampling time, and treated as three independent biological samples (two individuals per sample) during the subsequent analysis. Biological replicates were individually run on 1-D/2-D SDS-PAGE gels. Data were reported as mean ± standard deviation of three samples (n=3). Statistical significance was assessed by one-way analysis of variance (ANOVA) and the means were compared by the post hoc test Fisher least squares difference (LSD). Differences were considered as statistically significant at a confidence level of 95% ($p<0.05$). The software Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA) was employed for the statistical analyses.

RESULTS AND DISCUSSION

Impact of HHP treatment on total SDS-soluble protein fraction

Extractability

The solubility of total SDS-soluble proteins was not found to be dependent on pressure intensity and pressurization holding time. Thus, SDS-soluble protein solubility in month-0 frozen mackerel previously treated at 150, 300 and 450 MPa for 5 min was included in the 7.4-8.6 g/100g muscle range (Table 1). These values of protein solubility in pressurized fish were not significantly different to those observed in the counterpart control mackerel (8.5 g/100 g muscle). However, frozen storage after pressurization showed to induce a significant effect on SDS-soluble protein solubility. Thus, protein solubility was significantly lower in month-3 frozen mackerel, both for

286 pressurized and unpressurized fish, compared to samples corresponding to frozen
287 storage of month 0 (Table 1). In fact, frozen storage for 3 months reduced the solubility
288 of SDS-soluble proteins to levels of 5.5-6.5 g/100 g muscle; similarly to the 0-month
289 results, solubility was not found to be dependent on HHP processing at the end of the
290 frozen storage time.

291 In a related research, turbot (*Scophthalmus maximus)* fillets were frozen either
292 by pressure release (i.e. pressure shift freezing, 140 MPa, -14ºC) or by air-blast freezing
293 (-20ºC) and were then stored at –20ºC for 75 d (Chevalier et al. 2000). Contrary to the
294 present research, a marked extractability decrease of salt-soluble proteins was observed
295 2 d after freezing for pressure-shift freezing samples as compared to non-frozen
296 samples, which was correlated with a decrease in the apparent viscosity of the salt-
297 soluble protein extract; meantime, air-blast freezing induced lower changes in protein
298 extractability. Later on (Tironi et al. 2010), sea bass (*Dicentrarchus labrax*) muscle was
299 stored at –15 and –25ºC for 1, 3 and 5 months after a pressure-shift freezing process
300 (200 MPa; -18ºC) and then thawed by pressure assisted process (200 MPa for 20 min);
301 comparison with samples frozen and thawed using conventional methods was
302 undertaken. As a result, parameters related to protein denaturation, extractability and
303 water-holding capacity did not present a significant effect of HHP treatment; however,
304 conventionally treated samples experienced changes during frozen storage, such as
305 protein denaturation and water-holding capacity modifications.

306

307 *SDS-PAGE profile analysis*

308 The assessment of protein profile on 1-D SDS-PAGE gels indicated no
309 significant changes in the total SDS-soluble protein electrophoretic pattern as a result of
310 neither HHP treatment nor frozen storage (Figure 1). The fraction of total SDS-soluble

311 proteins was mainly composed by myofibrillar proteins such as myosin heavy chain
312 (MHC) and actin, being both proteins stable under pressurization conditions and the
313 subsequent frozen storage at -10ºC. Other proteins contained in the SDS-soluble
314 fraction seemed to be also unaffected by HHP processing and subsequent frozen storage
315 since the formation and/or disappearance of protein bands was not observed. These
316 findings are in agreement with a previous investigation in sea bream (*Sparus aurata*)
317 subjected to HHP treatment (200-400 MPa for 10 min) and subsequently stored under
318 refrigerated (3ºC up to 13 d) conditions (Campus et al. 2010). SDS-PAGE and
319 immunoblot analyses did not reveal degradation of main structural proteins as a result of
320 HHP treatment. In fact, authors noted the capacity of HHP applied at 400 MPa to
321 prevent a refrigeration time-dependent degradation of desmin, an important protein in
322 the muscle cell architecture. In contrast, other previous studies reported significant
323 effects on myofibrillar proteins in other fish species and HHP conditions. Thus, Ashie
324 and Simpson (1998) analyzed protein modifications in refrigerated (4-7ºC) bluefish
325 (*Pomatomus saltatrix*) that was previously submitted to HHP treatment (100-300 MPa)
326 for 30 min; disintegration of myofibrillar structure was evident as a result of HHP
327 treatment, although no observable changes in myofibrillar structure were observed
328 during the subsequent 21-day storage period under refrigerated conditions. Later on,
329 turbot (*Scophthalmus maximus)* fillets were frozen either by pressure release (i.e.
330 pressure shift freezing, 140 MPa, -14ºC) or by air-blast freezing (-20ºC) and were then
331 stored at –20ºC for 75 d (Chevalier et al. 2000); as a result, the electrophoresis analysis
332 led to a significant decrease in the intensity of myosin heavy chains. Effects of pressure-
333 shift freezing and/or pressure assisted thawing on the quality of sea bass (*Dicentrarchus*
334 *labrax*) muscle proteins were evaluated and compared with conventional (air-blast)
335 frozen and thawed samples (Tironi et al. 2007); according to SDS-PAGE results, HHP

336 treatment (200 MPa for 20 min) produced a partial denaturation with aggregation and
337 insolubilization of the myosin and a water-holding capacity decrease.
338
339 **Impact of HHP treatment on sarcoplasmic protein fraction**
340 *Extractability*
341 The solubility of sarcoplasmic proteins was found to be dependent on HHP
342 processing (Table 2). In month-0 frozen control, the solubility of sarcoplasmic proteins
343 was 3.4 g/ 100 g muscle, and that value was similar to that observed in samples
344 pressurized at 150 MPa, whatever the pressure holding time applied was. However, a
345 pronounced decrease of sarcoplasmic protein concentration was registered in month-0
346 frozen mackerel when previously subjected to a higher pressure level (Table 2). Thus,
347 protein concentrations of about 62-70% of the initial values were observed after
348 processing at 300 MPa; meanwhile, samples pressurized at 450 MPa exhibited uniquely
349 32% of initial protein solubility, i.e. 1.1 g protein/ 100 g muscle. A similar trend was
350 observed in mackerel samples stored frozen at -10 ºC for 3 months after pressurization;
351 thus, protein concentration decreased significantly after processing at 300 MPa, and
352 again the most dramatic reduction of sarcoplasmic protein extractability was observed
353 after pressurization at 450 MPa. These results indicate that the solubility of
354 sarcoplasmic proteins was significantly reduced in a straight-dependent manner with
355 HHP intensity in the 300-450 MPa pressure range, being this pressurization effect on
356 solubility maintained throughout the subsequent frozen storage. On the other hand, a
357 low impact on sarcoplasmic solubility in frozen mackerel is concluded for the pressure
358 holding time when pressure levels encountered are 150 and 300 MPa (Table 2).
359 According to the present results, Ohshima et al. (1992) proved that a marked
360 sarcoplasmic content decrease was produced on cod (*Gadus macrocephalus*) and

mackerel (*Scomber japonicus*) muscle after HHP treatment (200, 400 and 600 MPa for 30 min); this decrease showed to be more important by increasing the pressure applied. A marked decrease in sarcoplasmic protein content was also evident in Coho salmon (*Oncorhynchus kisutch*) muscle subjected to HHP treatment (135, 170 and 200 MPa for 30 s) (Ortea et al. 2010); additionally, a marked decrease in sarcoplasmic protein content was evident in samples corresponding to 170- and 200-MPa treatments throughout the chilling storage. Contrary to the above-mentioned results, extraction of water-soluble proteins was barely affected by HHP treatment (50-300 MPa for 1-12 hours) when applied to tilapia (*Orechromis niloticus*) fillets (Ko et al. 2006).

SDS-PAGE profile analysis and MS-MS identification

Figure 2 shows representative 1-D SDS-PAGE gel profiles of frozen mackerel previously treated with different HHP levels (0, 150, 300 and 450 MPa for 5 min). Protein changes were directly analyzed after pressurization and freezing process (month-0 time), and also after a 3-month storage of pressurized samples at -10ºC. The analysis of protein patterns indicated an important impact of HHP processing on sarcoplasmic proteins at both frozen storage times (Figure 2 and Figure 3). An HHP intensity of 150 MPa was apparently weak to provoke modifications on sarcoplasmic proteins. However, important changes on sarcoplasmic proteins were distinguished at 300 MPa, but even higher alterations were achieved by pressurizing at 450 MPa. Technological treatments at 300-450 MPa provoked modifications at least on 8 protein bands that were successfully identified by MS/MS sequencing and database homology (Table 3); those bands were numbered from b1 to b8 in Figure 2, in samples corresponding to 0 and 3 months of frozen storage. Intensity of protein bands b1 (assigned to UNP, unnamed protein products, similar to glycogen phosphorylase muscle

386 form-like), b3 (assigned to UNP similar to β-enolase-like isoform 1), b4 (identified as
387 creatine kinase 3) and b5 (assigned to fructose-bisphosphate aldolase A) significantly
388 decreased in mackerel pressurized at 300 and 450 MPa (Figure 3 and Table 3). The
389 intensity of band b6 (identified as triosephosphate isomerase B) was also reduced under
390 pressurization, but band b6 was found to be less pressure-sensitive than the above
391 mentioned protein bands (b1, b3, b4 and b5). The disruptive effect of processing on
392 band b6 was detected at the highest pressure applied (450 MPa). On the other hand,
393 HHP processing exerted an opposite effect, this leading to an increasing concentration
394 of protein bands b2, b7 and b8. Presence of the protein band b2 successfully assigned to
395 pyruvate kinase showed a significant increase at 300 MPa; meantime, protein bands b7
396 and b8, identified as tropomyosin protein products, were generated by the most elevated
397 pressure tested (450 MPa; Table 3 and Figure 3). Experimental molecular weights of b7
398 (~ 40 KDa) and b8 (~ 38 KDa) bands were more elevated than the corresponding
399 theoretical counterparts of tropomyosin (32.7 KDa), suggesting that b7 and b8 bands are
400 formed by HHP-induced cross-linking of native tropomyosin. However, the increase of
401 b2 band seems to be due to a pressure-induced dissociation of tetramer pyruvate kinase,
402 composed by 58 KDa pyruvate kinase monomers. In agreement with this, a previous
403 investigation showed that HHP treatment causes dissociation and inactivation of
404 pyruvate kinase (De Felice et al. 1999). This effect of the HHP strength on sarcoplasmic
405 proteins was not altered after a 3-month storage at -10 ºC.

406 The influence of different pressure holding times (0, 2.5 and 5 min) on
407 sarcoplasmic profiles was also evaluated. The weakest pressure level tested (150 MPa)
408 was not effective to exert significant changes on sarcoplasmic 1-DE gel profiles after
409 any of the pressure holding times tested, as was exemplified above for the highest
410 holding time (5 min; Figure 2 and Figure 3). However, pressurization at 300 MPa

411 caused significant changes on sarcoplasmic proteins, and that effect was found to be
412 dependent on pressure holding times (Figure 4). Thus, the degradation of proteins
413 identified as UNP similar to β-enolase-like isoform 1 (band b3), creatine kinase 3 (band
414 b4) and fructose-bisphosphate aldolase A (band b5) was more intense in mackerel
415 treated at 300 MPa for 2.5 or 5 min, than in their counterparts corresponding to a 0-min
416 pressure holding time. However, band b1, identified as UNP similar to glycogen
417 phosphorylase muscle form-like, showed analogous decomposition degrees for the
418 different pressure holding times when 300-MPa pressure is considered (Figure 4). The
419 formation of protein band b2, assigned to pyruvate kinase, was observed if pressure was
420 maintained at 300 MPa for at least 2.5-5 min. The protein bands assigned to
421 triosephosphate isomerase B (protein band b6) and protein products of tropomyosin
422 (protein bands b7 and b8) were not significantly influenced by the time of pressurization
423 holding at 300 MPa (Figure 4), and that observation is in agreement with the fact that
424 changes in those proteins were exclusively detected under the highest pressure level
425 tested (450 MPa) (Figure 2 and Figure 3).

426 To deepen the selective impact of HHP processing on sarcoplasmic proteins,
427 protein fractions from mackerel pressurized at 450 MPa for 5 min and from mackerel
428 corresponding to 0-month controls were mapped on 2-D SDS-PAGE gels (Figure 5);
429 protein spots with differential expression were identified by MS/MS analysis and
430 database homology (Table 4). 2-DE gels displayed marked differences in the patterns of
431 pressurized and unpressurized mackerel samples. Pressurization at 450 MPa exerted a
432 strong impact on eleven protein spots identified as glycogen phophorylase muscle form-
433 like isoform (spots 1 and 2), phosphoglucomutase-1 (spots 3 and 4), pyruvate kinase
434 muscle isozyme (spots 5 and 6), beta-enolase-like isoform 1 (spots 7), UNP similar to
435 phosphoglycerate (spot 8), creatine kinase isoforms (spots 9 and 10) and fructose-

436 bisphosphate aldolase A (spot 11). According to the 1-DE profiles formerly exposed, 2-
437 DE patterns pointed out a significant presence reduction of glycogen phophorylase
438 (spots 1 and 2), pyruvate kinase (spots 5 and 6), beta-enolase 1 (spot 7), creatine kinase
439 (spots 9 and 10) and fructose-bisphosphate aldolase A (spot 11) in mackerel HHP-
440 treated at 450 MPa. Furthermore, 2-DE mapping indicated a drastic disrupting effect of
441 HHP on protein spots identified as phosphoglucomutase-1 (spots 3 and 4) and UNP
442 similar to phosphoglycerate kinase 1 (spot 8) (Figure 5 and Table 4).

443 An important effect of HHP treatment on the electrophoresis profile of
444 sarcoplasmic proteins has already been reported, although MS identification of protein
445 bands modified has been very scarce. Thus, Ohshima et al. (1992) analyzed the
446 electrophoresis profiles changes produced on cod (*Gadus macrocephalus*) and mackerel
447 (*Scomber japonicus*) muscle after HHP treatment (200, 400 and 600 MPa for 30 min). It
448 could be observed that the SDS-PAGE analysis showed that most of the sarcoplasmic
449 components in cod samples were not detected in the pressurized samples when 400 and
450 600 MPa conditions were applied; for mackerel, a marked content decrease could also
451 be observed for 94, 50 and 43 kDa components when pressurized at 200 MPa. Rather
452 than being degraded, such disappeared components were expected to be covalently
453 linked together and be thus resistant to extraction with SDS. Pressure-assisted thawing
454 was applied to frozen whiting (*Gadus merlangus*) fillets (Chevalier et al. 1999). The
455 influence of different factors such as pressure applied (0-200 MPa), freezing rate,
456 pressurization rate, and pressure holding time (15-60 min) was studied in comparison
457 with thawing process at atmospheric pressure. The electrophoresis analysis of
458 sarcoplasmic protein fraction showed a modification of the 48 kDa band, whose
459 intensity decreased when applying a pressure higher than 150 MPa. Later on, sea bream
460 (*Sparus aurata*) was subjected to HHP treatment (200-400 MPa for 10 min) and

461 subsequently stored under refrigerated (3ºC up to 13 d) conditions (Campus et al. 2010);
462 SDS and immunoblot analyses revealed that a refrigeration time-dependent degradation
463 of desmin took place, which could be prevented if pressure applied was 400 MPa. In sea
464 bass (*Dicentrarchus labrax*) muscle HHP-treated (100-300 MPa for 5 min),
465 electrophoresis profiles showed an increase of some sarcoplasmic protein (21.5, 51 and
466 97 kDa molecular weight) presence with the pressure treatment (Chéret et al. 2006).
467 Other study in sea bass fillets suggested that HHP causes lysosomes disruption and also
468 denaturation, fragmentation and aggregation of sarcoplasmic proteins, together with a
469 decrease in enzyme activity (Teixeira et al. 2013). Recently, Ortea et al. (2010) analyzed
470 the sarcoplasmic proteins modifications of Coho salmon (*Oncorhynchus kisutch*)
471 subjected to HHP treatment (135, 170 and 200 MPa for 30 s); as a result, the SDS-
472 PAGE analysis of such protein fraction showed a partial loss of a band corresponding to
473 29 kDa, which after excision, digestion and mass spectrometry analysis was identified
474 as phosphoglycerate mutase. Therefore, the present investigation comes to bring light to
475 the identification of sarcoplasmic proteins that are found labile to HHP treatment;
476 among them, inhibition of enzymatic activity (protein and lipid hydrolysis and lipid
477 oxidation) during frozen storage should play an important role in quality retention of
478 fish muscle (Aubourg et al., 2013a).

479 In a recent investigation (Aubourg et al. 2013b), we reported that the application
480 of HHP at 150 MPa (holding time 0.0, 2.5 and 5 min) as a pre-treatment to frozen
481 mackerel storage kept lightness and odor similar to fresh muscle, and most importantly,
482 the sensorial acceptability of oven cooked HHP-treated samples was better than that of
483 frozen unpressurized controls and similar to that of fresh mackerel HHP-treated. Beside,
484 HHP at 150 MPa improved the water holding capacity of frozen fish meat by
485 maintaining expressible water lower than 40%, value similar to the optimum expressible

486 water value considered for low-salt restructured fish (Martelo-Vidal et al. 2012);
487 meanwhile pressurization at 300 and 450 MPa led to a significant expressible water
488 increase, worsening then water holding capacity.

489 The present investigation emphasizes the higher susceptibility of certain
490 mackerel sarcoplasmic proteins to be disrupted by HHP compared to myofibrillar
491 proteins. Thus, HHP-susceptibility for particular sarcoplasmic protein was dependent on
492 pressure conditions. These observed alterations of sarcoplasmic proteins may coherently
493 explain HHP-induced changes on sensory and water holding capacity. Early
494 investigations have proved that denaturation of sarcoplasmic proteins have an important
495 impact on meat and fish quality parameters such as color and water holding capacity
496 (Sayd et al. 2006). Accordingly, tilapia (*Oreochromis niloticus*) sarcoplasmic proteins
497 have been suggested as ingredient for enhancing textural properties of fish surimi
498 (Godiksen et al. 2009). Stability of specific sarcoplamic proteins such as creatine
499 kinase, glycogen phophorylase and triosephosphate isomerase B were found to be
500 correlated with firmness and texture parameters of rainbow trout (Godiksen et al. 2009).

501 In the present research, samples corresponding to 300 MPa showed a significant
502 disappearance of the sarcoplasmic proteins creatine kinase, glycogen phosphorylase,
503 fructose-bisphosphate aldolase A and β-enolase, as well as the generation of a HHP-
504 induced product of pyruvate kinase. Further, the highest pressure intensity tested (450
505 MPa) was required to effectively decompose triosephosphate isomerase B,
506 phosphoglucomutase and phosphoglycerate kinase 1, and to lead to the generation of
507 two products derived from tropomyosin. These changes induced on sarcoplasmic
508 proteins, mainly involved in energy homeostasis, glycolysis and carbohydrate
509 metabolism, as well as on the contractile tropomyosin protein seem to be behind the
510 decline of textural properties of mackerel treated at 300-450 MPa. Conversely, no

protein alterations at 150 MPa were observed in this study; under such pressure condition, water holding capacity of mackerel muscle was found to be improved (Aubourg et al. 2013b). This observation is in agreement with a previous investigation that reports a toughness increase in fish treated at 100-200 MPa, probably due to a stronger protein-protein interaction as a result of the volume decrease and compaction, and a tissue softening under stronger pressurization conditions (Ashie and Simpson 1996).

CONCLUSIONS

High pressure treatment is being increasingly used by the fish industry as a pre-processing technology to extend the shelf life and to improve quality. However, a marked lack of available information can be pointed out concerning the behavior during HHP processing of protein components that are known to be relevant for fish quality. In this scenario, the present investigation puts into relevance the elevated impact of HHP treatment on sarcoplasmic proteins.

Thus, HHP processing reduced the solubility of sarcoplasmic proteins in a higher extent than in total SDS-soluble proteins. The study of electrophoretic profiles on 1-D and 2-D SDS-PAGE indicated important changes in specific sarcoplasmic proteins, so that a targeted effect on proteins was found to be critically dependent on pressurization conditions. The stability of creatine kinase, fructose-bisphosphate aldolase A, glycogen phosphorylase and β-enolase was found to be pressure-sensitive starting from 300 MPa, whereas triosephosphate isomerase B, phosphoglucomutase and phosphoglycerate kinase 1 were decomposed at 450 MPa. HHP processing also induced the generation of a protein product of pyruvate kinase at 300-450 MPa, and two products derived from tropomyosin at 450 MPa. As a result, a marked inhibitory effect

on such enzymes is to be expected during the frozen storage, this leading to a higher quality retention in the fish muscle.

Such results agree to previous parallel research where sensory and physical properties of frozen Atlantic mackerel were studied. In it, pressure-treated fish showed a higher quality retention than its counterpart frozen fish without previous HHP treatment; additionally, a lower water holding ability was observed in fish treated under 300- and 450-MPa conditions when compared to 150-MPa treated fish.

The present research provides for the first time an extensive study concerning the application of proteomics tools to analyze the effect of HHP treatment, followed by a freezing step with or without a subsequent frozen storage, on changes produced in fish proteins. Assessment of modified bands and identified proteins can be considered a profitable tool to be employed as a quality biomarker in mackerel during its frozen storage.

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560 **FIGURE CAPTIONS**

561

562 **Figure 1.** 1-D SDS-PAGE profiles of total SDS-soluble protein fractions in control (0
563 MPa) and pressurized (150, 300 and 450 MPa for 5 min) mackerel that was
564 subsequently stored under frozen (0 and 3 months) conditions. MHC: Myosin heavy
565 chains.

566 **Figure 2.** 1-D SDS-PAGE profiles of sarcoplasmic protein fractions in control (0 MPa)
567 and pressurized (150, 300 and 450 MPa for 5 min) mackerel that was subsequently
568 stored under frozen (0 and 3 months) conditions. Protein bands labeled from b1 to b8
569 denote proteins with differential susceptibility towards HHP processing.

570 **Figure 3.** Quantitative evaluation of sarcoplasmic protein bands in frozen (months 0
571 and 3, Figures 3A and 3B, respectively) mackerel that was previously submitted to
572 different pressure levels (0, 150, 300 and 450 MPa for 5 min). Intensity of Coomassie-
573 stained protein bands was calculated by using the software Labimage, as indicated in the
574 experimental section**.** Protein bands b1-b8 refer to bands labeled in Figure 2. For each
575 protein band, bars with different letters (a, b, c) indicate significant ($p<0.05$) differences
576 as a result of pressure applied.

577 **Figure 4.** Effect of the pressure holding time (0, 2.5 and 5 min) at 300 MPa on the
578 sarcoplasmic proteins of mackerel that was subsequently stored under frozen (months 0
579 and 3, Figures 4A and 4B, respectively) conditions. The 1-D SDS-PAGE patterns of
580 sarcoplasmic proteins were obtained in frozen mackerel after 0 and 3 months at -10ºC.

581 **Figure 5.** 2-D SDS-PAGE gels of sarcoplasmic protein fractions from control (0-month
582 samples) and pressurized (450 MPa for 5 min; 0-month frozen time) mackerel that was
583 subsequently submitted to freezing process. Protein spots marked as s1-s11 correspond

584 to those showing differential abundance in unpressurized and pressurized samples, and
585 successfully identified by MS/MS analysis and database homology search (Table 5).

586 **REFERENCES**

587 Ashie, I., & Simpson, B. (1996). Application of high hydrostatic pressure to control
588 enzyme related fresh seafood texture deterioration. *Food Research International, 29*, 569-575.

590 Ashie, I., & Simpson, B. (1998). Effects of pressurization and refrigerated storage on
591 microstructure of fish muscle tissue. *Journal of Muscle Foods, 9*, 193-199.

592 Ashie, I., Smith, J., & Simpson, B. (1996). Spoilage and shelf-life extension of fresh
593 fish and shellfish. *Critical Reviews in Food Science and Nutrition, 36*, 87-121.

594 Aubourg, S., Rodríguez, A., & Gallardo, J. (2005). Rancidity development during
595 frozen storage of mackerel (*Scomber scombrus*): Effect of catching season and
596 commercial presentation. *European Journal of Lipid Science and Technology, 107*, 316-323.

598 Aubourg, S., Rodríguez, A., Sierra, Y., Tabilo-Munizaga, G., & Pérez-Won, M.
599 (2013a). Sensory and physical changes in chilled farmed Coho salmon
600 (*Oncorhynchus kisutch*): Effect of previous optimised hydrostatic high-pressure
601 conditions. *Food and Bioprocess Technology, 6*, 1539-1549.

602 Aubourg, S., Torres, J. A., Saraiva, J., Guerra-Rodríguez, E., & Vázquez, M. (2013b).
603 Effect of high-pressure pretreatments applied before freezing and frozen storage
604 on the functional and sensory properties of Atlantic mackerel (*Scomber*
605 *scombrus*). *Food Science and Technology (LWT), 53*, 100-106.

606 Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram
607 quantities of protein utilizing the principle of protein-dye binding. *Analytical*
608 *Biochemistry, 72,* 248-254.

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

611 Campus, M., Addis, MªF., Cappuccinelli, R., Porcu, MªC., Pretti, L., Tedde, V., Secchi,
612 N., Stara, G., & Roggio, T. (2010). Stress relaxation behaviour and structural
613 changes of muscle tissues from gilthead bream (*Sparus aurata* L.) following
614 high pressure treatment. *Journal of Food Engineering, 96*, 192-198.
615 Chéret, R., Hernández-Andrés, A., Delbarre-Ladrat, C., de Lamballerie, M., & Vérrez-
616 Bagnis, V. (2006). Proteins and proteolytic activity changes during refrigerated
617 storage in sea bass (*Dicentrarchus labrax* L.) muscle after high-pressure
618 treatment. *European Food Research and Technology, 222*, 527-535.
619 Chevalier, D., Le Bail, A., Chourot, J., & Chantreau, P. (1999). High pressure thawing
620 of fish (whiting): Influence of the process parameters on drip losses.
621 *Lebensmittel-Wissenschaft und –Technologie, 32*, 25-31.
622 Chevalier, D., Sequeira-Muñoz, A., Le Bail, A., Simpson, B., & Ghoul, M. (2000).
623 Effect of pressure shift freezing, air-blast freezing and storage on some
624 biochemical and physical properties of turbot (*Scophthalmus maximus*).
625 *Lebensmittel-Wissenschaft und –Technologie, 33*, 570-577.
626 D'Alessandro, A., & Zolla, L. (2012). Food Safety and Quality Control: Hints from
627 Proteomics. *Food Technology and Biotechnology, 50*, 275-285
628 De Felice, F., Soares, V., & Ferreira, S. (1999). Subunit dissociation and inactivation of
629 pyruvate kinase by hydrostatic pressure oxidation of sulfhydryl groups and
630 ligand effects on enzyme stability. *European Journal of Biochemistry, 266*, 163-
631 169.
632 Erkan, N., Üretener, G., Alpas, H., Selçuk, A., Özden Ö., & Buzrul, S. (2011). Effect of
633 high hydrostatic pressure (HHP) treatment on physicochemical properties of
634 horse mackerel (*Trachurus trachurus*). *Food and Bioprocess Technology, 4*,
635 1322-1329.

636 FAO (2007) Fishery statistics. *Capture Production. Yearbook 2005*, 100/1, p. 262. Food
637 and Agriculture Organization of the United Nations, Rome, Italy.
638 Gallardo, J. M., Carrera, M., & Ortea, I. (2013). Proteomics in Food Science. In A.
639 Cifuentes (Ed.), *Foodomics: Advanced mass spectrometry in modern food*
640 *science and Nutrition* (pp. 125-165). Ciudad Hoboken, NJ, USA: John Wiley &
641 Sons, Inc.
642 Godiksen, H., Morzel, M., Hyldig, G., & Jessen, F. (2009). Contribution of cathepsins
643 B, L and D to muscle protein profi les correlated with texture in rainbow trout
644 (*Oncorhynchus mykiss*). *Food Chemistry, 113*, 889–896.
645 Han, J.-Z., & Wang, Y.-B. (2008). Proteomics: present and future in food science and
646 technology. *Trends in Food Science and Technology, 19*, 26-30.
647 Hendrickx, M., Ludikhuyze, L., van den Broeck, I., & Weemaes, C. (1998). Effects of
648 high pressure on enzymes related to food quality. *Trends in Food Science and*
649 *Technology, 9*, 197-203.
650 Jensen, O., Wilm, M., Shevchenko, A., & Mann, M. (1998). Sample preparation
651 methods for mass spectrometric peptide mapping directly from 2-DE gels. In A.
652 J. Link, (Ed.) *2-D Proteome Analysis Protocols* (pp. 513–530). Totowa, NJ,
653 USA: Humana Press.
654 Knorr, D. (1999). Novel approaches in food-processing technology: New technologies
655 for preserving foods and modifying function. *Current Opinion in Biotechnology,*
656 *59*, 81-116.
657 Ko, W-C., Jao, C-L., Hwang, J-S., & Hsu, K-C. (2006). Effect of high-pressure
658 treatment on processing quality of tilapia meat fillets. *Journal of Food*
659 *Engineering, 77*, 1007-1011.

660 Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of
661 bacteriophage T4. Nature 227: 680-685.
662 Martelo-Vidal, M., Mesas, J., & Vázquez, M. (2012). Low-salt restructured fish
663 products from Atlantic mackerel (*Scomber scombrus*) with texture resembling
664 turkey breast. *Food Science and Technology International, 18,* 251-259.
665 Norton, T., & Sun D.-W. (2008). Recent advances in the use of high pressure as an
666 effective processing technique in the food industry. *Food and Bioprocess*
667 *Technology, 1*, 2-34.
668 Ohshima, T., Nakagawa, T., & Koizumi, C. (1992). Effect of high hydrostatic pressure
669 on the enzymatic degradation of phospholipids in fish muscle during storage. In
670 E. Bligh (Ed.), *Seafood Science and Technology*, Chapter 8 (pp. 64-75). Oxford,
671 UK: Fishing News Books.
672 Oms-Oliu, G., Martín-Belloso, O., & Soliva-Fortuny, R. (2010). Pulsed light treatments
673 for food preservation. A review. *Food and Bioprocess Technology, 3*, 13-23.
674 Ortea, I., Rodríguez, A., Tabilo-Munizaga, G., Pérez-Won, M., & Aubourg, S. (2010).
675 Effect of hydrostatic high-pressure treatment on proteins, lipids and nucleotides
676 in chilled farmed salmon (*Oncorhynchus kisutch*) muscle. *European Food*
677 *Research and Technology, 230*, 925-934.
678 Pazos, M., Pereira da Rocha, A., Roepstorff, P., & Rogowska-Wrzesinska, A. (2011).
679 Fish Proteins as Targets of Ferrous-Catalyzed Oxidation: Identification of
680 Protein Carbonyls by Fluorescent Labeling on Two-Dimensional Gels and
681 MALDI-TOF/TOF Mass Spectrometry. *Journal of Agricultural and Food*
682 *Chemistry, 59,* 7962-7977.

683 Richards, M., Kelleher, S., & Hultin, H. (1998). Effect of washing with or without
684 antioxidants on quality retention of mackerel fillets during refrigerated and
685 frozen storage. *Journal of Agricultural and Food Chemistry, 46*, 4363-4371.
686 Rodríguez, A., Cruz, J., Paseiro, P., & Aubourg, S. (2012). Effect of a polyphenol-
687 vacuum packaging on lipid deterioration during an 18-months frozen storage of
688 coho salmon (*Oncorhynchus kisutch*). *Food and Bioprocess Technology. 5*,
689 2602-2611.
690 Saeed, S., & Howell, N. (2001). 12-lipoxygenase activity in the muscle tissue of
691 Atlantic mackerel (*Scomber scombrus*) and its prevention by antioxidants.
692 *Journal of the Science of Food and Agriculture, 81*, 745-750.
693 Saeed, S., & Howell, N. (2002). Effect of lipid oxidation and frozen storage on muscle
694 proteins of Atlantic mackerel (*Scomber scombrus*). *Journal of the Science of*
695 *Food and Agriculture, 82*, 579-586.
696 Santos, S., & Regenstein, J. (1990). Effects of vacuum packaging, glazing and
697 erythorbic acid on the shelf-life of frozen white hake and mackerel. *Journal of*
698 *Food Science, 55*, 64-70.
699 Sayd, T., Morzel, M., Chambon, C., Franck, M., Figwer, P., Larzul, C., Le Roy, P.,
700 Monin, G., Chérel, P., & Laville, E. (2006). Proteome analysis of the
701 sarcoplasmic fraction of pig Semimembranosus muscle: Implications on meat
702 color development. *Journal of Agricultural and Food Chemistry, 54*, 2732–
703 2737.
704 Teixeira, B., Fidalgo, L., Mendes, R., Costa, G., Cordeiro, C., Marques, A., Saraiva, J.,
705 & Nunes, Mª L. (2013). Changes of Enzymes Activity and Protein Profiles
706 Caused by High-Pressure Processing in Sea Bass (*Dicentrarchus labrax*) Fillets.
707 *Journal of Agricultural and Food Chemistry, 61*, 2851-2860.

Tironi, V., de Lamballerie, M., & Le Bail, A. (2010). Quality changes during the frozen storage of sea bass (*Dicentrarchus labrax*) muscle after pressure shift freezing and pressure assisted thawing. *Innovative Food Science and Emerging Technologies, 11*, 565-573.

Tironi, V., Le Bail, A., & de Lamballerie, M. (2007). Effects of pressure-shift freezing and pressure-assisted thawing on sea bass (*Dicentrarchus labrax*) quality. *Journal of Food Science, 72*, C381-C387.

Torres, J. A., & Velázquez, G. (2005). Commercial opportunities and research challenges in the high pressure processing of foods. *Journal of Food Engineering, 67*, 95-112.

Vázquez, M., Torres, J. A., Gallardo, J., Saraiva, J., & Aubourg, S. (2013). Lipid hydrolysis and oxidation development in frozen mackerel (*Scomber scombrus*): Effect of a high hydrostatic pressure pre-treatment. *Innovative Food Science and Emerging Technologies, 18*, 24-30.

TABLE 1

Effect of high hydrostatic pressure (HHP) processing on the solubility (g/100 g muscle)* of total SDS-soluble proteins in frozen mackerel**

HHP treatment	Frozen storage time (months)	
	0	3
Control	y 8.5±0.4	z 5.5±0.6
150 MPa-5 min	y 7.8±0.5	z 6.5±0.6
300 MPa-5 min	y 8.6±0.9	z 6.1±0.2
450 MPa-5 min	y 7.4±0.5	z 6.4±0.5

* Mean values of three (n = 3) replicates ± standard deviations. Control samples correspond to frozen fish without previous HHP treatment.

** For each HHP treatment, mean values preceded by different letters (z, y) indicate significant ($p<0.05$) differences as a result of frozen storage time. No significant ($p>0.05$) differences were obtained as a result of the pressure applied.

TABLE 2

Effect of high hydrostatic pressure (HHP) processing on the solubility (g/100 g muscle)* of sarcoplasmic proteins in frozen mackerel**

HHP treatment	Frozen storage time (months)	
	0	3
Control	3.4±0.2 c	3.1±0.4 c
150 MPa-0 min	3.1±0.2 c	2.7±0.4 c
150 MPa-2.5 min	3.0±0.3 c	2.7±0.3 c
150 MPa-5 min	3.0±0.2 c	2.5±0.1 c
300 MPa-0 min	2.4±0.3 b	2.6±0.2 c
300 MPa-2.5 min	2.0 ±0.3 b	2.1±0.1 b
300 MPa-5 min	2.1±0.1 b	2.1±0.1 b
450 MPa-5 min	1.1±0.1 a	0.9±0.1 a

* Mean values of three (n = 3) replicates ± standard deviations. Control samples correspond to frozen fish without previous HHP treatment.

** For each frozen storage time, values followed by different letters (a, b, c) indicate significant ($p<0.05$) differences as a result of HHP applied. No significant ($p>0.05$) differences were obtained as a result of the frozen storage time.

TABLE 3

Proteins identified from 1-D SDS-PAGE gels of sarcoplasmic protein fractions from control and HHP-treated frozen mackerel. Bands of interest were identified by LC-ESI-IT-MS/MS as described in the experimental procedure. Protein identification number refers to numbered protein band on Figures 2 and 4. For each protein band, different parameters supporting protein identification by MS are indicated: NCBI accession number, number of matching peptides and sequences*, Mascot score, % sequence coverage (SC) and theoretical protein mass (MWt)

Id No.	Identification	Species	Acc. No NCBI	Matches	Sequences	Mascot score	% SC	MWt (kDa)
b1	UNP** similar to glycogen phosphorylase, muscle form-like	*Tetraodon nigroviridis*	gi|47227171	21(3)	8(3)	353	9	97.2
b2	Pyruvate kinase muscle isozyme-like isoform 1	*Oryzias latipes*	gi|432861319	21(3)	8(2)	422	15	58.4
b3	UNP** similar to beta-enolase-like isoform 1	*Tetraodon nigroviridis*	gi|47210809	14(5)	6(3)	335	23	47.1
b4	Creatine kinase 3	*Salmo salar*	gi|197632385	27(8)	7(3)	410	23	42.9
b5	Fructose-bisphosphate aldolase A	*Oreochromis niloticus*	gi|348501948	51(27)	10(5)	543	28	39.6
b6	Triosephosphate isomerase B	*Xiphophorus maculatus*	gi|15149252	9(4)	3(2)	174	8	26.5
b7	Tropomyosin	*Thunnus thynnus*	gi|301017128	27(17)	3(3)	257	13	32.7
b8	Tropomyosin alpha-1 chain	*Liza aurata*	gi|60390740	25(5)	6(3)	355	25	32.7

* The number of peptides and sequences with individual ions scores above the significance threshold score is indicated in parentheses for the matching peptides and sequences values.

** UNP: Unnamed protein product

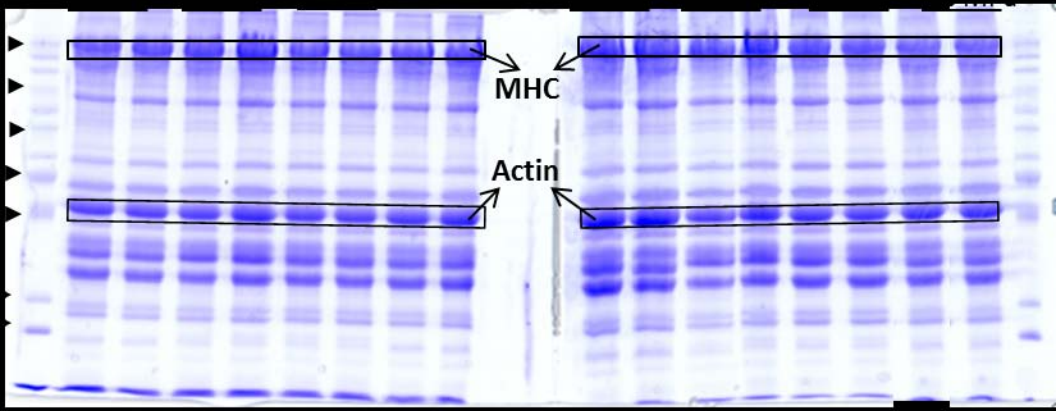
TABLE 4

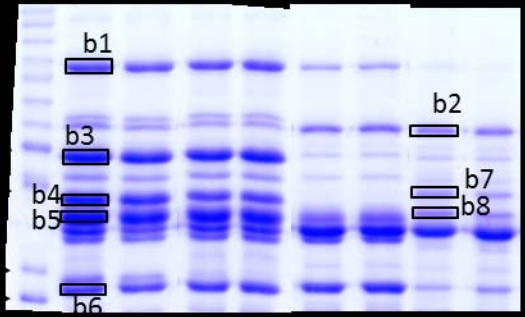
Proteins spots identified from 2-D SDS-PAGE gels of sarcoplasmic protein fraction from control and HHP-treated frozen mackerel. Protein spots of interest were identified by LC-ESI-IT-MS/MS as described in the experimental procedure. Protein identification number refers to numbered protein spots on Figure 5. For each protein spot, different parameters supporting the protein identification by MS are indicated: NCBI accession number, number of matching peptides and sequences*, Mascot score, % sequence coverage (SC) and theoretical protein mass (MWt)

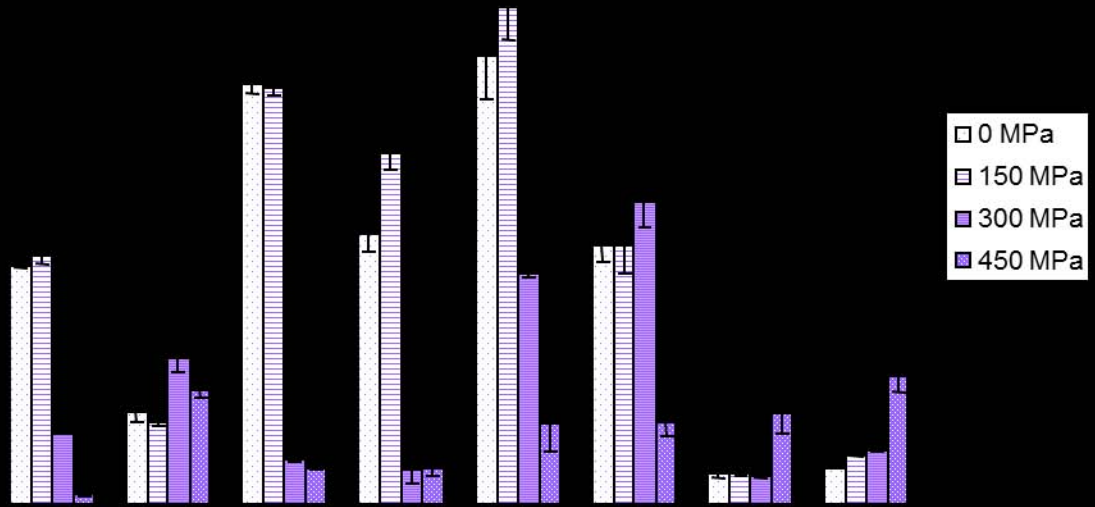
Id No.	Identification	Species	Acc. No NCBI	Matches	Sequences	Mascot score	% SC	MWt (kDa)
s1	Glycogen phosphorylase, muscle form-like isoform	*Oryzias latipes*	gi|432897329	17(1)	5(1)	252	7	96.9
s2	Glycogen phosphorylase, muscle form-like isoform	*Oryzias latipes*	gi|432897329	19(1)	4(1)	220	5	96.9
s3	Phosphoglucomutase-1	*Salmo salar*	gi|213512248	33(3)	6(2)	343	14	60.8
s4	Phosphoglucomutase-1	*Oreochromis niloticus*	gi|348529784	34(12)	9(5)	605	20	68.5
s5	Pyruvate kinase muscle isozyme	*Salmo salar*	gi|213513314	6(1)	3(1)	154	6	58.3
s6	Pyruvate kinase muscle isozyme-like isoform 1	*Oryzias latipes*	gi|432861319	12(2)	6(1)	305	12	58.4
s7	Beta-enolase-like isoform 1	*Takifugu rubripes*	gi|410925068	26(7)	7(2)	413	23	45.0
s8	UNP** similar to phosphoglycerate kinase 1	*Tetraodon nigroviridis*	gi|47221390	6(2)	4(2)	215	13	44.1
S9	Creatine kinase muscle-type	*Pagrus major*	gi|268308331	52(17)	10(5)	636	28	42.9
S10	Creatine kinase M-type-like isoform 1	*Takifugu rubripes*	gi|410910532	36(15)	10(7)	678	32	42.4
S11	Fructose-bisphosphate aldolase A	*Oreochromis niloticus*	gi|348501948	20(2)	4(2)	262	17	39.6

* The number of peptides and sequences with individual ions scores above the significance threshold score is indicated in parentheses for the matching peptides and sequences values.

** UNP: Unnamed protein product


MHC
Actin


b1
b2
b3
b4
b5
b6
b7
b8


0 MPa
150 MPa
300 MPa
450 MPa

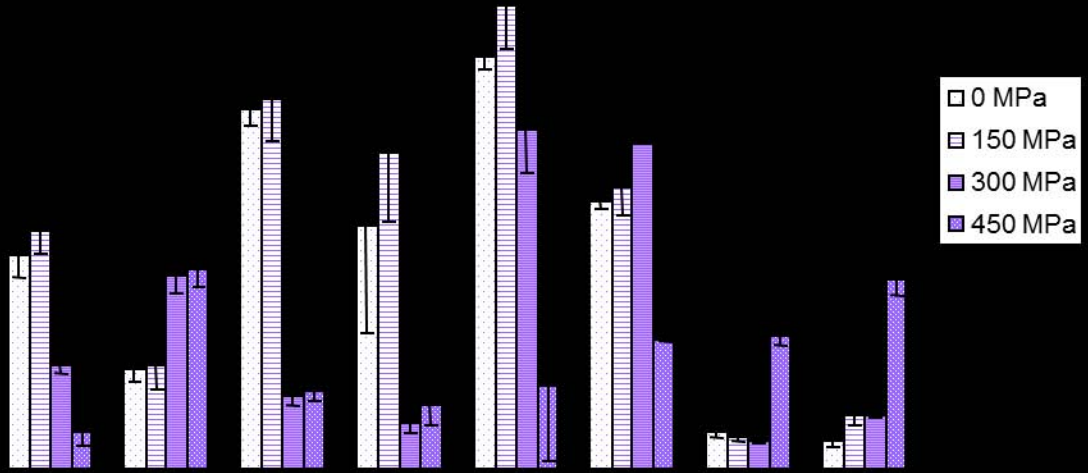

0 MPa
150 MPa
300 MPa
450 MPa

Figure 4

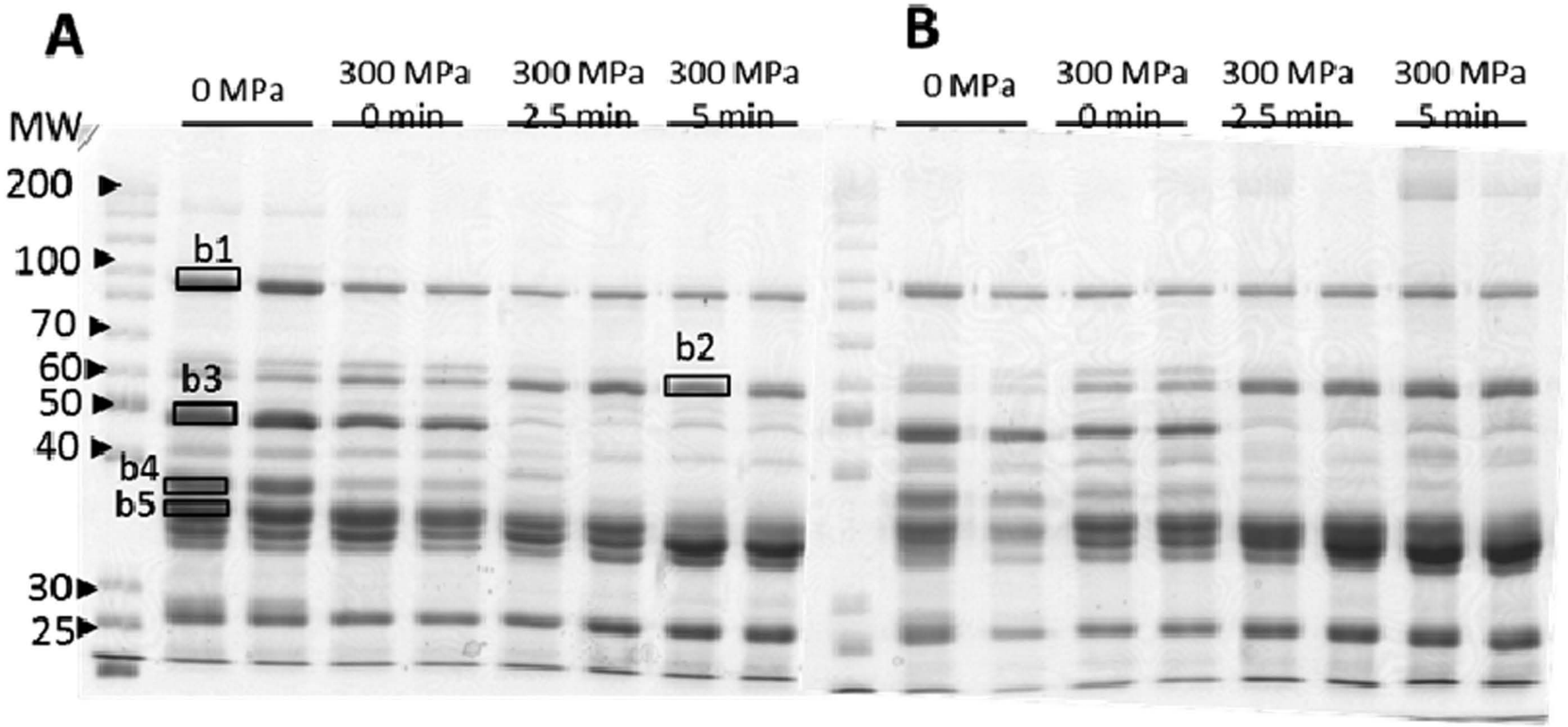
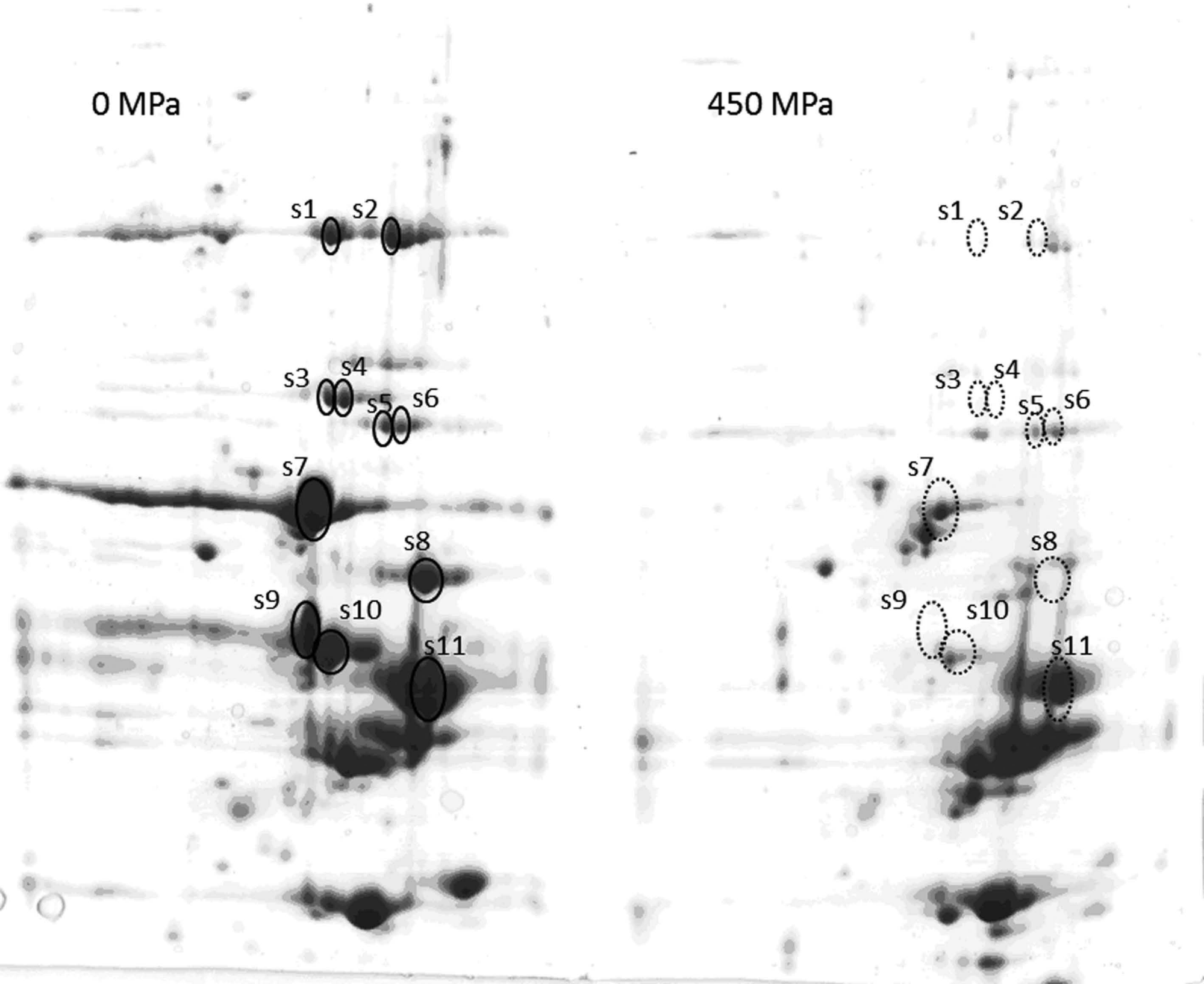

A
0 MPa
300 MPa 0 min
300 MPa 2.5 min
300 MPa 5 min
MW
200
100
70
60
50
40
30
25
b1
b2
b3
b4
b5
B
0 MPa
300 MPa 0 min
300 MPa 2.5 min
300 MPa 5 min

Figure 5


0 MPa
s1
s2
s3
s4
s5
s6
s7
s8
s9
s10
s11
450 MPa
s1
s2
s3
s4
s5
s6
s7
s8
s9
s10
s11