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5 PROTEOMICS ANALYSIS IN FROZEN HORSE
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ABSTRACT

The effect of high-pressure processing (HPP) (150, 300 and 450 MPa for 0, 2.5 and 5 min) on total sodium dodecyl sulphate (SDS)-soluble and sarcoplasmic proteins in frozen (−10°C for 3 months) horse mackerel (*Trachurus trachurus*) was evaluated. Proteomics tools based on image analysis of SDS-PAGE protein gels and protein identification by tandem mass spectrometry (MS/MS) were applied. Although total SDS-soluble fraction indicated no important changes induced by HPP, this processing modified the 1-D SDS-PAGE sarcoplasmic patterns in a direct-dependent manner and exerted a selective effect on particular proteins depending on processing conditions. Thus, application of the highest pressure (450 MPa) provoked a significant degradation of phosphoglycerate mutase 2, glycogen phosphorylase muscle form, pyruvate kinase muscle isozyme, beta-enolase and triosephosphate isomerase and phosphoglucomutase-1. Conversely, protein bands assigned to tropomyosin alpha-1 chain, fast myotomal muscle troponin T and parvalbumin beta 2 increased their intensity after applying a 450-MPa processing.

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

Running title: Proteomics and pressurised frozen horse mackerel.

1. INTRODUCTION

Freezing technology is often used to retain the sensory and nutritional properties of fish products for direct consumption or as raw materials for other technological processes. However, proteins of fish muscle have shown to undergo different chemical and physical changes during frozen storage which, under certain conditions (namely, long periods of storage, poor freezing practices, temperature fluctuations, initial low quality, etc.), may result in quality loss, reflected mainly by an unacceptable texture as well as an undesirable flavour, odour and colour (Sikorski, & Kolakowska, 1994; Sotelo, Piñeiro, & Pérez-Martín, 1995).

In agreement with the actual increasing consumer's demand for high quality marine food, a wide range of advanced technologies have been developed that are able to present attractive, nutritional and safe products. Among such technologies, high pressure processing (HPP) has shown to maintain sensory and nutritional properties while inactivating microbial development (Ashie, & Simpson, 1996; Gómez-Estaca, Montero, Giménez, & Gómez-Guillén, 2007; Yagiz, Kristinsson, Balaban, & Welt, 2009). Concerning the protein fraction, previous research has focused on the ability of HPP for inhibiting the endogenous enzyme activity; such effect would be based on its denaturation and modification capacity towards the protein fraction, being the intensity and reversibility of this effect strongly dependent on the strength of the HPP conditions (Hendrickx, Ludikhuyze, van den Broek, & Weemaes, 1998; Campus, 2010). Thus, HPP has shown to affect non-covalent bonds (hydrogen, ionic, and hydrophobic bonds) substantially. As a result, marked changes are likely to be produced in quaternary (namely, dissociation of oligomers), tertiary (namely, modification of hydrophobic and ionic interactions) and secondary (namely, modification of hydrogen bonds) protein structures.

Over the last decade, proteomics has successfully been applied to evaluate quality in different kinds of food systems (D'Alessandro, & Zolla, 2012; Gallardo, Carrera, & Ortea, 2013). In particular, proteomics analysis based on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and tandem mass spectrometry (MS/MS) has been found a powerful technology to identify global changes in protein constituents of tissues and subcellular compartments (Han, & Wang, 2008; Xu, Liu, Tian, Dong, Shen, & Qu, 2015). Despite this outstanding potential of proteomics, the application of proteomics tools to high-pressure treated fish is still scarce, although different topics having potential perspectives have been described such as fish authentication, allergen detection, microorganism contamination, aquaculture production and quality changes during storage or processing (Rodrigues, Silva, Dias, & Jessen, 2012; Carrera, Cañas, & Gallardo, 2013).

Thus, a marked content decrease in 94-, 50- and 43-kDa bands was noticed in mackerel (*Scomber japonicus*) muscle when treated at 200 MPa or higher, although their protein identity was not characterised (Ohshima, Nakagawa, & Koizumi, 1992). Chevalier, Le Bal, Choutor and Chantreau (1999) also found losses in a non-identified 48-kDa protein band in turbot (*Scophthalmus maximus*) muscle when treated at a pressure higher than 150 MPa. Further, sodium dodecyl sulphate (SDS)-PAGE analysis of Coho salmon (*Oncorhynchus kisutch*) sarcoplasmic fraction showed a partial loss of a band corresponding to 29 kDa that was identified by MS/MS analysis as phosphoglycerate mutase (Ortea, Rodríguez, Tabilo-Munizaga, Pérez-Won, & Aubourg, 2010). More recently, image analysis of SDS-PAGE protein gels and protein identification by tandem MS/MS showed that myofibrillar proteins were more stable under pressurisation treatment (150-450 MPa for 0-5 min) than sarcoplasmic ones when

a fatty fish species (mackerel, *Scomber scombrus*) was studied (Pazos, Méndez, Gallardo, & Aubourg, 2014).

Atlantic horse mackerel (*Trachurus trachurus*) is a medium-fat abundant underutilised species. It has shown an important endogenous enzyme activity and significant quality losses during frozen storage (Simeonidou, Govaris, & Vareltzis, 1997; Aubourg, Piñeiro, & González, 2004), so that different preservative strategies have been applied to inhibit its quality loss (Vareltzis, Koufidis, Graviilidou, Papavergou, & Vasiliadou, 1997; Lugasi, Losada, Hóvári, Lebovics, Jakóczy, & Aubourg, 2007). The effect of different HPP conditions on some physical and chemical properties of horse mackerel was studied by Erkan, Üretener, Alpas, Selçuk, Özden, & Buzrul (2011); however, changes throughout a further storage were not analysed in such study. Previous research carried out by our group has shown a lipid damage inhibition (Torres, Vázquez, Saraiva, Gallardo, & Aubourg, 2013) and sensory acceptance enhancement (Torres, Saraiva, Guerra-Rodríguez, Aubourg, & Vázquez, 2014) of frozen horse mackerel when a previous HPP (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 HPP. For this purpose, protein solubility measurement and a proteomics approach consisting of image analysis of one-dimensional (1-D) 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 horse mackerel previously submitted to HPP.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents employed

Dithiothreitol (DTT), Tris-HCl, bicinchoninic acid (BCA), sodium dodecyl sulphate (SDS) and the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate (APS) and bromophenol blue 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).

2.2. Raw fish, HPP and frozen storage

Atlantic horse mackerel (65 kg) caught near the Bask coast in Northern Spain was obtained at the Ondarroa harbour (Bizkaia, Spain) and transported in ice to the AZTI Tecnalia (Derio, Spain) pilot plant for HPP within 6 h after catch. Whole horse mackerel individuals (25-30 cm and 200-250 g range) were placed in flexible polyethylene bags (three individuals per bag) and vacuum sealed at 400 mbar.

HPP was carried out at 150, 300 and 450 MPa for 0, 2.5 and 5 min in a 55-L high pressure unit (WAVE 6000/55HT; NC Hyperbaric, Burgos, Spain). Water applied as the pressurising medium at 3 MPa/s yielded 50, 100 and 150 s as the come up times for the 150, 300 and 450 MPa treatments, respectively, while decompression time was less than 3s. Cold pressurising water was used to maintain temperature conditions during HPP at room temperature (20°C).

After HPP, horse mackerel individuals were kept at -20°C for 48 hours and then

stored at -10°C with samples analysed after 0, 1 and 3 months of storage. Fish without HPP (namely, at 0.1-MPa condition) and subjected to the same freezing and frozen storage conditions was used as control. Three batches or replicates ($n=3$) for each treatment were analysed independently. Each analysis was based on the whole white muscle of fish pooled from two individual fish.

A frozen storage temperature (-10°C) higher than commercial practice (-18°C) was chosen as an accelerated test condition to estimate the extent of the HPP on the protein damage development of the actual medium-fat frozen fish species. In addition, the response to HPP of marine species has been reported to vary with species, chemical composition and size (Yagiz et al., 2009; Campus, 2010). Consequently, a preliminary study was undertaken to confirm the above mentioned pressure conditions as reported in previous research (Torres et al., 2013, 2014). Accordingly, 300 MPa was chosen as the mid pressure point to be studied in the present research which included also a lower and a higher pressure value of 150 and 450 MPa, respectively.

2.3. Extraction of total SDS-soluble and sarcoplasmic protein fractions

Previously to proteomics and protein solubility analysis, proteins were separated into two fractions, i.e. total SDS-soluble and sarcoplasmic proteins.

Extraction of total SDS-soluble and sarcoplasmic proteins was performed by following a published protocol (Pazos et al., 2014). Briefly, 0.5 g of raw mince was homogenised in eight volumes of Tris buffer (10 mM Tris-HCl, pH 7.2) supplemented with 2% of the denaturing agent SDS and 5 mM of the protease inhibitor PMSF. Homogenates were boiled, re-homogenised by Ultra-Turrax, and centrifuged at 40000g (12 min at 4°C). Supernatants were separated and labelled as total SDS-soluble protein fraction.

Sarcoplasmic protein fractions were obtained by homogenising 0.5 g of minced muscle in eight volumes of a non-denaturing Tris buffer solution (10 mM Tris-HCl, pH 7.2) and supplemented with 5 mM of PMSF in an Ultra-Turrax blender for 2 min. Supernatants were separated after centrifugation of the homogenates at 40000g (12 min at 4°C), and labelled as sarcoplasmic protein fraction.

Both protein fractions were maintained at –80°C until the electrophoretic analysis was carried out.

2.4. Protein solubility

Protein concentrations were determined in the sarcoplasmic and total SDS-soluble fractions by employing the BCA (Smith et al., 1985) and Bradford (1976) assays, respectively. Concentration curves were built using bovine serum albumin as standard reference. Protein solubility was expressed as g protein kg⁻¹ flesh.

2.5. SDS-Polyacrylamide gel electrophoresis

1-D laboratory-made 10% (v/v) polyacrylamide gels (acrylamide:N,N'-ethylene-bis-acrylamide, 200:1) with stacking gel (4% polyacrylamide) were loaded with 20 or 30 µg of protein per well, and run in a Mini-PROTEAN 3 cell (Bio-Rad, Hercules, CA, USA) (Laemmly, 1970). The running buffer consisted of 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, Uppsala, Sweden). Scanned coomassie-stained 1-DE gels were analysed by means of the 1-DE gel analysis software LabImage 1D (Kapelan Bio-Imaging Solutions, Halle, Germany).

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

Protein bands of interest were manually excised from 1-D gels. Proteins were in-gel reduced, alkylated and digested with trypsin as previously described (Jensen, Wilm, Shevchenko, & Mann, 1998). Briefly, protein spots were three times washed with water and dehydrated with acetonitrile. Gel plugs were then dehydrated in a vacuum centrifuge and finally rehydrated with a 0.5 μ M solution of sequencing grade bovine trypsin in 50 mM ammonium bicarbonate buffer, pH 8.0, for at least 40 min on ice. After the rehydration step, samples were digested overnight at 37°C. The peptide mixture was dried in vacuum centrifugation, and dissolved again in 20 μ l of 5% acetic acid prior to mass analysis.

Trypsin-digested proteins were analysed by a binary LC system model Agilent 1260 Series (Agilent, Palo Alto, CA, USA), coupled to a linear ion trap (LIT) mass spectrometer LTQ Velos Pro with an electrospray ionisation (ESI) interface (Thermo Scientific, San Jose, CA, USA). 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 μ m. Mobile phases A and B were respectively 0.5% acetic acid in water and in 100% acetonitrile. A 90 min linear gradient from 5 to 35% B, at a flow rate of 1.5-1.7 μ L/min, was used. Peptide detection was performed by using survey scans from 350 to 1600 Da (2 μ scans), followed by MS/MS scans (2 μ scans) of the six more intense peaks using an isolation width of 1 Da and a normalised collision energy of 35%. Singly charged ions were excluded from MS/MS fragmentation and a dynamic exclusion enabled, with repeat count set to 2 and exclusion duration of 30 seconds. Protein identification was performed using the database searching function of the PEAKS 7 software (Bioinformatics Solutions Inc, Waterloo, Ontario, Canada), to compare experimental MS/MS spectra against reference MS/MS spectra from the UniProtKB/Swiss-Prot

database, which also included their respective decoy sequences. The following limitations were used for the searches: tryptic cleavage, up to 2 missed cleavage sites, and tolerances ± 1.2 Da for precursor ions and ± 0.8 Da for MS/MS fragments ions. The FDR was kept below 1%.

2.7. 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 SDS-PAGE gels. Data were reported as mean $\pm$ 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 Fisher least squares difference (LSD) test. 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.

3. RESULTS AND DISCUSSION

3.1. Protein solubility and proteomics analysis of total SDS-soluble protein fraction

The solubility of total SDS-soluble proteins was not found dependent on pressure intensity (Table 1), but was found affected by the duration of the frozen storage. Thus, SDS-soluble protein solubility in month-0 and month-1 frozen horse mackerel previously either pressurised (150-450 MPa for 0 min) or non-pressurised were not significantly different, ranging values from 102 to 118 g kg⁻¹ muscle (Table 1). However, protein solubility severely dropped to values of 19-24 g kg⁻¹ muscle in month-3 frozen horse mackerel; similarly to the results obtained at earlier frozen storage

(months 0 and 1), solubility was not found to be dependent on HPP at the end of the experiment.

In a related research, turbot (*Scophthalmus maximus*) fillets were frozen either by pressure release (i.e. pressure shift freezing, 140 MPa, -14°C) or by air-blast freezing (-20°C) and were then stored at -20°C for 75 d (Chevalier, Sequeira-Muñoz, La Bail, Simpson, & Ghoul, 2000); contrary to the present research, a marked extractability decrease of salt-soluble proteins was observed 2 d after freezing for pressure-shift freezing samples as compared with non-frozen samples, which was correlated with a decrease in the apparent viscosity of the salt-soluble protein extract; meantime, air-blast freezing induced lower changes in protein extractability. Later on (Tironi, de Lamballerie, & Le Bail, 2010), sea bass (*Dicentrarchus labrax*) muscle was stored at -15 and -25°C for 1, 3 and 5 months after a pressure-shift freezing process (200 MPa; -18°C) and then thawed by pressure assisted process (200 MPa for 20 min); as a result, parameters related to protein denaturation, extractability and water-holding capacity did not present a significant effect of HPP when compared with samples frozen and thawed using conventional methods; however, non-pressurised samples experienced changes during frozen storage, such as protein denaturation and water-holding capacity modifications. In agreement with the present results, total SDS-soluble mackerel (*Scomber scombrus*) proteins showed to be stable under pressurisation treatment (150-450 MPa for 0-5 min) in terms of solubility; however, a marked decrease could be outlined in all kinds of samples after 3 months at -10°C (Pazos et al., 2014).

The analysis of total SDS-soluble proteins on 1-D SDS-PAGE gels indicated no important changes induced by HPP in terms of fragmentation or cross-linking (Figure 1). A significant appearance or disappearance of protein bands was not observed in month-0 frozen horse mackerel previously subjected HPP (Figure 1A). However, frozen

storage showed an important effect on 1-D gel profiles. Thus, two of the most important protein bands found in month-0 (Figure 1A) and month-1 (Figure 1B) frozen horse mackerel, named as 1T and 2T, respectively, were completely lost after a 3-month frozen storage either in HPP-treated or -untreated fish muscle (Figure 1C). Protein bands 1T and 2T were, respectively, identified as the skeletal muscle fast myosin heavy chain (MHC) and the skeletal muscle alpha-actin (Table 2), being both myofibrillar proteins. In contrast, the protein band 3T, identified as tropomyosin alpha-1 chain, was found to be stable after a 3-month frozen storage (Figure 1).

Pressure holding times from 0 to 5 min did not significantly affect total SDS-soluble proteins when considering fish samples treated at 150 MPa (Figure 2). Thus, previously mentioned bands (1T, 2T and 3T; Table 2) showed a similar pattern in fish samples hold at 150 MPa for 0 to 5 min.

Some previous information is in agreement with the low extent of fish changes produced in the total SDS-soluble proteins as a result of HPP. Thus, the SDS-PAGE and immunoblot analyses in sea bream (*Sparus aurata*) subjected to HPP (200-400 MPa for 10 min) and subsequently stored under refrigerated (3°C up to 13 d) conditions did not reveal degradation of main structural proteins as a result of HPP (Campus et al., 2010); however, authors noted the capacity of HPP applied at 400 MPa to prevent a refrigeration time-dependent degradation of desmin, an important protein in the muscle cell architecture. Recently (Pazos et al., 2014), total SDS-soluble mackerel (*Scomber scombrus*) proteins showed to be stable under pressurisation treatment (150-450 MPa for 0-5 min) and throughout frozen storage (3 months at -10°C) in terms of electrophoretic profiles.

Contrary, other previous studies reported significant effects on myofibrillar proteins in fish species under HPP conditions. Thus, turbot (*Scophthalmus maximus*)

fillets were frozen either by pressure release (i.e. pressure shift freezing, 140 MPa, –14°C) or by air-blast freezing (–20°C) and were then stored at –20°C for 75 d (Chevalier et al., 2000); as a result, the electrophoresis analysis led to a significant decrease in the intensity of myosin heavy chains. Effects of pressure-shift freezing and/or pressure assisted thawing on the quality of sea bass (*Dicentrarchus labrax*) muscle proteins were evaluated and compared with conventional (air-blast) frozen and thawed samples (Tironi, Le Bail, & de Lamballerie, 2007); according to SDS-PAGE results, HPP (200 MPa for 20 min) produced a partial denaturation with aggregation and insolubilisation of the myosin as well as a water-holding capacity decrease.

3.2. Protein solubility and proteomics analysis of sarcoplasmic protein fraction

The solubility of sarcoplasmic proteins did not change either by pressure intensity or frozen storage (Table 1). Thus, solubility of sarcoplasmic proteins ranged from 20.9 to 26.5 g kg⁻¹ muscle in HPP-fish and in non-processed fish muscle during the whole frozen storage period.

According to the present results, extraction of water-soluble proteins was barely affected by HPP (50-300 MPa for 1-12 hours) when applied to tilapia (*Oreochromis niloticus*) fillets (Ko, Jao, Hwang, & Hsu, 2006). Contrary to the actual research, Ohshima et al. (1992) proved that a marked sarcoplasmic content decrease was produced on cod (*Gadus macrocephalus*) and mackerel (*Scomber japonicus*) muscle after HPP (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 HPP (135, 170 and 200 MPa for 30 s) (Ortea et al., 2010); this decrease was maintained throughout a 20-day chilled storage. Similarly, a marked sarcoplasmic

protein content decrease was implied in mackerel (*Scomber scombrus*) muscle by pressure increase during HPP (150-450 MPa for 0-5 min) (Pazos et al., 2014); this effect was maintained throughout a 3-month frozen storage at -10°C .

Figure 3 shows the effect of HPP on the 1-D SDS-PAGE profiles of horse mackerel sarcoplasmic proteins. Protein profiling on 1-D gels showed significant HPP-induced changes under 450 MPa for 0 min condition at month 0 (Figure 3A). Thus, horse mackerel subjected to this processing showed an important reduction of the protein band 1S (identified as phosphoglycerate mutase 2; Table 2) when compared with fish submitted to lower pressure intensities (150 and 300 MPa). Moreover, several protein bands appeared/disappeared in the range of 35-40 KDa in fish muscle previously treated with 450 MPa for 0 min and corresponding to month 0. A similar behaviour was concluded for month-3 frozen fish (Figure 3B).

To further deepen the understanding of how HPP can affect the chemical stability of sarcoplasmic proteins, pressurised horse mackerel with holding times ranging from 0 to 5 min was also analysed by 1-D gel electrophoresis (Figure 4) and MS/MS identification (Table 2). Results indicated no significant changes of protein profiles by holding 150 MPa from 0 to 5 min (Figure 4A). However, there were significant modifications of 1-D profiles in horse mackerel treated at 300 MPa for 2.5 and 5 min. Thus, holding 300 MPa up to 2.5 min (Figure 4B) reduced the presence of protein bands identified as phosphoglycerate mutase 2 (band 1S), beta-enolase (band 6S) and phosphoglucomutase-1 (band 8S). Conversely, protein bands assigned to tropomyosin alpha-1 chain (band 2S) and parvalbumin beta 2 (band 9S) increased their presence intensity after maintaining a pressure value of 300 MPa for 2.5-5 min (Figure 4B and Table 2). Interestingly, the parvalbumin beta 2 is considered one of the major fish allergens (Carrera, Cañas, & Gallardo, 2012).

As noted above, 450 MPa was the intensity that provoked the most substantial changes in the electrophoretic pattern of sarcoplasmic proteins. Moreover, modifications were significantly more important by holding 450 MPa up to the midpoint of 2.5 min, and ever more with a holding time of 5 min (Figure 4C). Thus, in addition to a dramatic reduction of phosphoglycerate mutase 2 (band 1S) at 450 MPa/0 min condition, maintaining 450 MPa for 2.5 min provoked a significant degradation of several sarcoplasmic proteins such as glycogen phosphorylase muscle form (band 4S), pyruvate kinase muscle isozyme (band 5S), beta-enolase (band 6S), triosephosphate isomerase (band 7S) and phosphoglucomutase-1 (band 8S). In contrast, this pressure-time combination significantly increased the presence of protein bands assigned to tropomyosin alpha-1 chain (band 2S), fast myotomal muscle troponin T (band 3S) and to a lesser extent, parvalbumin beta 2 (band 9S). Further, when a 450-MPa pressure was maintained for 5 min, phosphoglycerate mutase 2 (band 1S), pyruvate kinase muscle isozyme (band 5S), beta-enolase (band 6S) and triosephosphate isomerase (band 7S) reduced drastically their band intensity. Tropomyosin alpha-1 chain (band 2S) and fast myotomal muscle troponin T (band 3S) also increased their presence intensity after maintaining a 450-MPa pressure value for 5 min (Figure 4C and Table 2).

Earlier investigations have proved that denaturation of sarcoplasmic proteins has an important impact on meat and fish quality parameters such as colour and water holding capacity (Sotelo et al., 1995; Sayd et al., 2006). Additionally, stability of specific sarcoplasmic proteins such as creatine kinase, glycogen phosphorylase and triosephosphate isomerase B was found to be correlated with firmness and texture parameters of rainbow trout (*Oncorhynchus mykiss*) (Godiksen, Morzel, Hyldig, & Jessen, 2009). The present investigation emphasises the higher susceptibility of certain horse mackerel sarcoplasmic proteins to be disrupted by HPP compared to myofibrillar

proteins, being this susceptibility for particular sarcoplasmic protein dependent on pressure conditions. These changes induced on sarcoplasmic proteins, mainly involved in energy homeostasis, glycolysis and carbohydrate metabolism, as well as on the contractile tropomyosin protein seem to be behind the decline of textural properties previously observed in horse mackerel treated at 300-450 MPa (Torres et al., 2014); conversely, no protein alterations at 150 MPa were observed. In such study, pressure-treated fish showed a higher quality retention than its counterpart frozen fish without previous HPP; additionally, lower water holding ability and lower sensory acceptability values were obtained in fish treated under 300- and 450-MPa conditions when compared with 150-MPa treated fish.

An important effect of HPP on the electrophoresis profile of sarcoplasmic proteins has already been reported in previous research, although MS identification of protein bands modified has been very scarce. Thus, Ohshima et al. (1992) analysed the electrophoresis profiles changes produced on cod (*Gadus macrocephalus*) and mackerel (*Scomber japonicus*) muscle after HPP (200, 400 and 600 MPa for 30 min); it could be observed that the SDS-PAGE analysis showed that most of the sarcoplasmic components in cod samples were not detected in the pressurised samples when 400 and 600 MPa conditions were applied, while a marked content decrease could be observed for 94, 50 and 43 kDa components in mackerel muscle when pressurised at 200 MPa. Later on, HPP showed to provoke dissociation and inactivation of pyruvate kinase proteins (De Felice, Soares, & Ferreira, 1999); this activity was found to be irreversibly inhibited at 350 MPa.

Pressure-assisted thawing was applied to frozen whiting (*Gadus merlangus*) fillets (Chevalier et al., 1999). The influence of different factors such as pressure applied (0-200 MPa), freezing rate, pressurization rate, and pressure holding time (15-

60 min) was studied in comparison with conventional thawing process; the electrophoresis analysis of sarcoplasmic protein fraction showed a modification of the 48 kDa band, whose intensity decreased when applying a pressure higher than 150 MPa. Electrophoresis profiles of sea bass (*Dicentrarchus labrax*) muscle that was HPP-treated (100-300 MPa for 5 min) showed an increase of some sarcoplasmic protein (21.5, 51 and 97 kDa molecular weight) presence with the pressure treatment (Chéret, Hernández-Andrés, Delbarre-Ladrat, de Lamballerie, & Vérrez-Bagnis, 2006). Other study in sea bass fillets suggested that HPP causes lysosomes disruption and also denaturation, fragmentation and aggregation of sarcoplasmic proteins, together with a decrease in enzyme activity (Teixeira et al., 2013). Recently, Ortea et al. (2010) analysed the sarcoplasmic proteins modifications of Coho salmon (*Oncorhynchus kisutch*) subjected to HPP (135, 170 and 200 MPa for 30 s); as a result, the SDS-PAGE analysis of such protein fraction showed a partial loss of a band corresponding to 29 kDa, which after excision, digestion and mass spectrometry analysis was identified as phosphoglycerate mutase.

In a recent and closely related research focused on a fatty fish species (mackerel, *Scomber scombrus*; Pazos et al., 2014), pressurisation (150-450 MPa for 0-5 min) modified the 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 creatin kinase, fructose-bisphosphate aldolase A, glycogen phosphorylase, and beta-enolase were degraded at 300-450 MPa. Additionally, the stability of triosephosphate isomerase B, phosphoglucomutase, and phosphoglycerate kinase-1 was found to be HPP-reduced when submitted at 450 MPa. HPP (300-450 MPa) was also found to induce the formation of a cross-linking product of pyruvate kinase and two compounds derived from tropomyosin at 450 MPa.

4. CONCLUSIONS

The solubility of total SDS-soluble proteins was not found dependent on pressure conditions, but was found affected by the duration of the frozen storage; thus, a marked drop after a 3-month storage was observed. Meantime, the solubility of sarcoplasmic proteins did not change either by pressure intensity or frozen storage.

The analysis of total SDS-soluble proteins on 1-D SDS-PAGE gels indicated no important changes induced by HPP in terms of fragmentation or cross-linking. However, a 3-month storage led in all kinds of samples to the loss of two bands corresponding to two myofibrillar proteins (band 1T, skeletal muscle fast myosin heavy chain; band 2T, skeletal muscle alpha-actin).

The study of electrophoretic profiles on 1-D SDS-PAGE showed substantial modifications in specific sarcoplasmic proteins, so that a targeted effect was found to be critically dependent on pressurization conditions. After MS/MS analysis of sarcoplasmic fraction, it could be concluded that no significant changes of protein profiles by holding 150 MPa from 0 to 5 min was present. However, application of a higher pressure (300 and 450 MPa) reduced the presence of protein bands identified as phosphoglycerate mutase 2 (band 1S), beta-enolase (band 6S) and phosphoglucomutase-1 (band 8S); further, application of the highest pressure (450 MPa) provoked a significant degradation of phosphoglycerate mutase 2 (band 1S), glycogen phosphorylase muscle form (band 4S), pyruvate kinase muscle isozyme (band 5S), beta-enolase (band 6S), triosephosphate isomerase (band 7S) and phosphoglucomutase-1 (band 8S). Conversely, protein bands assigned to tropomyosin alpha-1 chain (band 2S), fast myotomal muscle troponin T (band 3S) and parvalbumin beta 2 (band 9S) increased their intensity after applying a 300-MPa and/or 450-MPa processing.

This selective behaviour of HPP conditions on sarcoplasmic proteins agree with previous parallel research where sensory and physical properties of frozen horse mackerel were studied. In it, pressure-treated fish showed a higher quality retention than its counterpart frozen fish without previous HPP; meantime, lower water holding ability and lower sensory acceptability values were obtained in fish treated under 300- and 450-MPa conditions when compared with 150-MPa treated fish. Additionally, the presence increase of the allergen parvalbumin beta 2 (band 9S) can impair the food security of frozen horse mackerel if previously treated under 300- and 450-MPa conditions.

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Conflict of interest

The authors have declared no conflict of interest.

FIGURE CAPTIONS

Figure 1. Effect of high-pressure processing (HPP) intensity on the 1-D SDS-PAGE profiles of total SDS-soluble proteins of horse mackerel muscle that was subsequently stored at -10°C for 0 (Panel A), 1 (Panel B) and 3 (Panel C) months. Bands marked as 1T, 2T and 3T denote highly abundant proteins affected or unaffected by HPP and/or frozen storage. Protein identifications as shown in Table 2.

Figure 2. Effect of the pressure holding time (0, 2.5 and 5 min) on the 1-D SDS-PAGE profiles of total SDS-soluble proteins of horse mackerel muscle subsequently stored under frozen conditions (-10°C) for 1 month. Bands labelled denote proteins affected by high-pressure processing and/or frozen storage. Protein identifications as shown in Table 2.

Figure 3. Effect of pressure conditions (150, 300 and 450 MPa for 0 min) on the 1-D SDS-PAGE profiles of sarcoplasmic proteins of horse mackerel muscle subsequently stored under frozen conditions (-10°C) for 0 (Panel A) and 3 (Panel B) months. Bands labelled denote proteins affected by HPP and/or frozen storage. Protein identifications as shown in Table 2.

Figure 4. Effect of the pressure holding time on the 1-D SDS-PAGE profiles of sarcoplasmic proteins of horse mackerel muscle that was pressurised at 150 (Panel A), 300 (Panel B) or 450 (Panel C) MPa for 0, 2.5 and 5 min and subsequently stored under frozen conditions (-10°C) for 0 months. Bands labelled from 1S to 9S denote proteins affected by pressure conditions. Protein identifications as shown in Table 2.

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TABLE 1

Effect of high-pressure processing (HPP) on the solubility (g kg^{-1} muscle)* of total SDS-soluble and sarcoplasmic proteins in frozen horse mackerel**

Protein fraction	HPP condition	Frozen storage time (months)		
		0	1	3
Total SDS-soluble proteins	Control	102.2 $\pm$ 7.2 b	118.2 $\pm$ 12.1 b	23.0 $\pm$ 3.0 a
	150 MPa-0 min	109.0 $\pm$ 17.0 b	116.8 $\pm$ 6.6 b	23.7 $\pm$ 8.5 a
	300 MPa-0 min	107.2 $\pm$ 19.0 b	106.6 $\pm$ 10.7 b	22.8 $\pm$ 3.5 a
	450 MPa-0 min	103.5 $\pm$ 0.1 b	115.1 $\pm$ 7.8 b	19.2 $\pm$ 1.3 a
Sarcoplasmic proteins	Control	26.5 $\pm$ 3.3	25.1 $\pm$ 4.1	23.8 $\pm$ 1.4
	150 MPa-0 min	21.3 $\pm$ 3.4	22.4 $\pm$ 2.7	20.9 $\pm$ 2.4
	300 MPa-0 min	25.9 $\pm$ 4.0	24.3 $\pm$ 1.8	22.4 $\pm$ 1.1
	450 MPa-0 min	21.3 $\pm$ 3.4	22.3 $\pm$ 1.2	20.9 $\pm$ 2.4

* Mean values of three ($n = 3$) replicates $\pm$ standard deviations. Control samples correspond to frozen fish without previous HPP (namely, 0.1 MPa).

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

TABLE 2: Identification of proteins from the total SDS-soluble (T) fraction and from the sarcoplasmic (S) fraction of horse mackerel affected by high-pressure processing (HPP) and/or frozen storage. Band Id refers to the numbered band in 1-D gels shown in Figures 1-4. For each protein band, different parameters are indicated: protein identification, gene name, UniProtKB/Swiss-Prot Code, protein mass, score, significant peptides (FDR < 1%), unique peptides and % sequence coverage

Band Id	Identification	Gene name	UniProtKB/Swiss-Prot Code	Theoretical MW (kDa)	-10lgP Score	#Peptides	#Unique Peptides	Sequence Coverage (%)
1T	Myosin heavy chain, fast skeletal muscle	-	Q90339	221.6	213.37	32	16	17
2T	Actin, alpha skeletal muscle	Acta1	Q98972	42.0	229.62	19	2	53
3T	Tropomyosin alpha-1 chain	Tpm1	P13104	32.7	136.89	6	3	25
1S	Phosphoglycerate mutase 2	Pgam2	P16290	28.7	113.83	9	6	19
2S	Tropomyosin alpha-1 chain	Tpm1	P84335	32.7	163.36	35	13	69
3S	Troponin T, fast skeletal muscle	Tnnt3	P09739	30.7	94.71	7	7	14
4S	Glycogen phosphorylase muscle form	Pygm	P11217	97.0	197.05	35	2	29
5S	Pyruvate kinase muscle isozyme	Pkm	Q92122	57.5	153.65	20	8	24
6S	Beta-enolase	Eno3	Q3ZC09	47.1	124.63	26	9	37
7S	Triosephosphate isomerase	Tpi1	Q7ZWN5	26.8	122.95	27	13	31
8S	Phosphoglucomutase-1	Pgm1	Q08DP0	61.6	145.18	15	4	28
9S	Parvalbumin beta 2	-	P86765	11.3	100.89	4	1	29

Figure 1

Figure 2

Figure 3

Figure 4