ANTIOXIDANT ACTIVITY OF PROTEIN HYDROLYSATES OBTAINED FROM DISCARDED MEDITERRANEAN FISH SPECIES

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ABSTRACT

In this study, five discarded species in the Mediterranean Sea, namely sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark, were evaluated as raw material for obtaining fish protein hydrolysates exhibiting antioxidant activity. The DH of the hydrolysates ranged from 13.2 to 21.0 %, with a protein content varying from 60.7 to 89.5 %. The peptide profile of all hydrolysates was very similar, except for the hydrolysate of small-spotted catshark. Their lipid content was found to be between 4.6 and 25.3 %. The highest DPPH scavenging activity was found for the hydrolysates of sardine and horse mackerel with EC_{50} values varying from 0.91 to 1.78 mg protein/mL. Sardine and small-spotted catshark hydrolysates exhibited the highest ferrous chelating activity with an EC_{50} value of 0.32 mg protein/mL. Moreover, sardine and bogue hydrolysates presented the highest reducing power. Finally, a total of six antioxidant peptides were theoretically identified within the structure of myosin and actin proteins from sardine and small-spotted catshark. The potential antioxidant activity exhibited by the hydrolysates suggests that it is feasible to obtain added-value products such as natural antioxidants from these discarded species.

Keywords: discards, fish protein hydrolysates, antioxidant activity, DPPH, Fe^{2+} chelating activity, reducing power

1. INTRODUCTION

Marine discards are that portion of total fish catch which is not retained for sale and returned to the sea. It comprises non-target species with low commercial value, fish below minimum commercial size, fish caught in excess of individual quota and damaged fish which is not worthy for fishermen to keep on board (Kelleher, 2005). In the Alboran Sea, the portion of the West Mediterranean Sea lying between the Spanish southern coast and the north of Morocco, discards are mainly composed of commercial species such as sardine (*Sardina pilchardus),*

horse mackerel (*Trachurus mediterraneus*) and axillary seabream (*Pagellus acarne*) which are dumped at the sea due to high-grading practices, quota restriction and minimal commercial-size requirements. Other species such as bogue (*Boops boops*) and small-spotted catshark (*Scyliorhinus canicula*) are discarded due to their reduced commercial value (García-Moreno, Pérez-Gálvez, Morales-Medina, Guadix A, & Guadix EM, 2013a).

These practices represent an important underutilization of marine resources. Since discards are generally dead or dying when returned to the sea, they also cause significant environmental problems such as alterations on marine trophic chains (Bozzano & Sardà, 2002). In order to ensure the sustainability of EU fisheries, the EU Commission is in the process of implementing a reformed Common Fisheries Policy which aims to gradually implement a practice of zero-discards (EU, 2011). Nevertheless, technical measures should also be applied in order to successfully meet discards bans, since discards can be reduced (i.e. by improving the selectivity of the fishing gears) but cannot be completely eliminated. In this sense, it seems to be of special importance the development of up-grading processes which permit to obtain added-value products from this underutilized raw material.

In this context, discarded species in the Alboran Sea are good sources of protein, with protein contents ranging from 17 to 23 % depending on the species (García-Moreno et al., 2013a). Thus, enzymatic hydrolysis of their protein fraction is a convenient method for the production of bioactive compounds that could be utilised in the nutraceutical and pharmaceutical fields. In this regard, several fish protein hydrolysates have shown numerous bioactivities such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, antimicrobial, among others (Je, Lee, Lee, & Ahn, 2009; Kim & Wijesekara, 2010).

Due to the increasing interest in finding antioxidants from natural sources which may have less potential hazard than synthetic ones, research on fish protein hydrolysates exerting

antioxidant activity has gained an increased interest. Antioxidants are generally employed to prevent lipid oxidation in foods in order to avoid the formation of toxic compounds and undesirable odours and flavours (Lin & Liang, 2002). Furthermore, oxidative stress has also been involved in the occurrence of several diseases such as hypertension, cancer, diabetes, Alzheimer's and aging (Hajieva & Behl, 2006).

In the last decade, several authors have reported a strong antioxidant activity for fish protein hydrolysates obtained from different species such as black scabbardfish (*Aphanopus carbo)* (Batista, Ramos, Coutinho, Bandarra, & Nunes, 2010), sardinelle (*Sardinella aurita*) (Bougatef et al., 2010), saithe (*Pollachius virens*) (Chabeaud, Dutournié, Guérard, Vandanjon, & Bourseau, 2009), yellowfin sole (*Limanda aspera*) (Jun, Park, Jung, & Kim 2004), mackerel (*Scomber austriasicus*) (Wu, Chen, & Shiau, 2003), and herring (*Clupea harengus*) (Sathivel et al., 2003). However, there is little information about the production of fish protein hydrolysates with antioxidant activity from discarded species in the Alboran Sea.

Another important aspect to consider is the choice of the enzyme since it has a great impact on the release of antioxidant peptides by hydrolysis of fish protein (Laroque, Chabeaud, & Guérard, 2008). The endoproteases subtilisin (EC 3.4.21.62) and pancreatic trypsin (EC 3.4.21.4) have previously shown good results in the production of fish protein hydrolysates exhibiting antioxidant activity (Amarowicz & Shahidi, 1997; Rajapakse, Mendis, Byun, & Kim, 2005; Thiansilakul, Benjakul, & Shahidi., 2007). Nevertheless, only a few studies have addressed the effect of a combination of these enzymes (García-Moreno et al., 2013b).

Therefore, the objective of this study was to investigate the potential of five discarded species in the Alboran Sea (*Sardina pilchardus, Trachurus mediterraneus, Pagellus acarne, Boops boops and Scyliorhinus canicula*) as raw material for the production of fish protein

hydrolysates exhibiting antioxidant activity. For that purpose, the combined effect of subtilisin and trypsin as enzymatic treatment was evaluated.

2. MATERIALS AND METHODS

2.1 Raw material

Raw sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), bogue (*Boops boops)*, axillary seabream (*Pagellus acarne*) and small-spotted catshark (*Scyliorhinus canicula*), was provided by the fishing harbour of Motril (Spain) in September 2011. All fish were kept in ice during the transportation and pressed in the same day.

2.2 Separation of protein fraction

The whole fish, included viscera and gonads, was preheated at 40ºC for 30 min (Digiterm 100, Selecta, Barcelona, Spain). Then, it was fed into an electric press (ESP-K, Sanahuja, Castellón, Spain) where it was subjected to three consecutive pressing steps until attaining a final pressure of 150 bar. The pressing stage permitted to reduce the moisture content and the volume of the protein rich material, which also implies a diminution of the handling and insulation costs. The cakes obtained from the pressing operation were grinded in a cutter (SK-3, Sammic, Sevilla, Spain) and then frozen at -20ºC prior to their use as substrate for protein hydrolysis. For small-spotted catshark, the grinding and homogenization of the press cake was not possible due to the high resistance of its skin. Thus, muscle of this species was employed as substrate for protein hydrolysis. It was obtained by de-heading, de-gutting and removing the skin from the whole fish.

2.3 Hydrolysis procedure

For the enzymatic hydrolysis, two serine endoprotease enzymes were employed; one of bacterial origin (subtilisin, EC 3.4.21.62) and other from an animal source (pancreatic trypsin, EC 3.4.21.4), both provided by Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S,

107 respectively. The following hydrolysis conditions were studied: (a) 2 h hydrolysis with
108 subtilisin followed by 2 h hydrolysis with trypsin; (b) 2 h reaction with trypsin followed by 2
109 h incubation with subtilisin and; (c) 4h hydrolysis with simultaneous addition of both
110 enzymes. The first enzyme utilized in treatments a) and b) was not inactivated prior to
111 addition of the second enzyme.

112 The protein content of the raw material for the hydrolysis was determined by using the
113 Kjeldahl method (AOAC, 2006), with a nitrogen-to-protein conversion factor of 6.25. The
114 results, expressed as % wet base, were as follows: sardine 19.2 %, horse mackerel 20.2 %,
115 axillary seabream 23.4 %, bogue 21.9 % and small-spotted catshark 27.4 %.

116 Then, a given mass of grinded press cake was homogenised with demineralised water until
117 reaching a final volume of 200 mL. This suspension, having a protein concentration of 25 g/L,
118 was then transferred into a jacketed reactor of volume capacity 250 mL. The experiments
119 were conducted at pH 8 and 50ºC, while enzyme-protein ratio was set at 3 % (w/w) for both
120 enzymes. Protein was considered as substrate.

121 The degree of hydrolysis, defined as the percentage of the number of peptide bonds cleaved
122 compared to the total number of peptide bonds in the substrate studied, was calculated as a
123 function of the base consumption throughout the reaction employing an automatic titrator
124 (718 Stat Titrino, Metrohm, Herisau, Switzerland) (Camacho, González-Tello, Páez-Dueñas,
125 Guadix EM, & Guadix A, 2001). According to this method, the degree of hydrolysis (DH)
126 can be related to the amount of base (NaOH, 1 N) consumed to keep the pH constant during
127 the reaction, as follows (Eq. 1):

128 $$DH = B \cdot N_b / \left(\alpha \cdot m_p \cdot h_{TOT} \right) \times 100 \quad (1)$$

129 where B (mL) is the amount of base consumed, N_b (eq/L) is the normality of the base, α is the
130 average degree of dissociation of the α-NH_2 amino groups released during the hydrolysis,

which is dependent on the temperature and the pH, m_P (g) is the mass of protein in the substrate and h_{TOT} (meq/g) is the number of equivalents of peptide bonds per gram of protein. At pH 8 and temperature of 50ºC, the 88.5% of the amino groups are dissociated, while h_{TOT} was assumed to be 8.6 meq/g of protein, as reported in literature (Adler-Nissen, 1986).

A set of 250 hydrolysates, originated from the five species and three enzymatic treatments studied and drawn at different times of reaction (0, 5, 10, 20, 30, 45, 60, 90, 120, 125, 130, 140, 150, 165, 180, 210 and 240 min), were evaluated in order to determine the influence of DH on the DPPH scavenging activity. The samples were heated in a boiling water bath for 15 min to inactivate the enzyme and were filtered in order to remove the remained solids. They were kept at -20 ºC until performing the analyses.

After completion of the hydrolysis, the final hydrolysates were also heated in a boiling water bath for 15 min and filtered. Then, they were lyophilized and stored at -20 ºC until analyses were performed.

2.4 Characterization of the hydrolysates

2.4.1 Protein content

The protein content of the lyophilized hydrolysates was determined using a FP-528 LECO nitrogen analyser (LECO, St Joseph, MI, USA) calibrated with ethylenediaminetetraacetic acid according to the Dumas method (Saint-Denis & Goupy, 2004).

2.4.2 Lipid content and lipid classes

The lipid content of the lyophilized hydrolysates was determined according to the method described by Folch et al. (1956). Lipid classes were determined by thin-layer chromatography (TLC) using hexane/diethyl ether/acetic acid (65:35:1, v/v/v) as the developing solvent system. The developed plates were sprayed with 10% phosphomolybdic acid in ethanol and heated at 120 ºC for 5 min. The identification of the different classes was done by comparison

with the standards from Sigma. For quantification purposes, the TLC plates were scanned (GS-800 densitometer, Bio-Rad, Alcobendas, Spain) and analyzed with Quantity One analysis software (Bio-Rad, Alcobendas, Spain).

2.4.3 Molecular mass distribution of hydrolysates

The molecular mass distribution of the fish protein hydrolysates was estimated by gel filtration chromatography with a FPLC ÄKTA (Amersham Biosciences, Uppsala, Sweden) using a Superdex Peptide 10/300 GL column with a UV detector at 254 nm. The eluent was 30% acetonitrile with 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. A molecular mass calibration curve was prepared using the following standards: ribonuclease A (13,700 Da), aprotinin (6500 Da), angiotensin I (1296 Da), bradykinin (1060 Da) and triglycine (189 Da).

2.5 Determination of antioxidant activity

2.5.1 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity of the hydrolysates was determined by two different methods depending on the objective pursued.

In order to evaluate the evolution of the DPPH scavenging activity of the hydrolysates with the degree of hydrolysis, the method reported by Brand-Williams et al. (1995) which requires a low amount of sample was employed. Briefly, an aliquot of each sample (50 μL) was mixed with 100 μL of Tris buffer solution (50 mM, pH 7.4) and with 850 μL of a daily-prepared solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) at 0.1 mM in methanol. The mixture was then kept at room temperature in the dark for 30 min, and the reduction of DPPH radical was measured at 515 nm. A blank was run in the same way by using distilled water instead of sample, and sample control was also made for each sample by adding methanol instead of DPPH solution. Then, DPPH radical scavenging activity was calculated according to Eq. 2:

$$\text{DPPH inhibition (\%)} = \left(1 - \frac{(A_{sample} - A_{sample\_control})}{A_{blank}}\right) \times 100 \quad (2)$$

For the determination of the EC_{50} value of the final hydrolysates, which is defined as the concentration of hydrolysate (mg protein/mL) needed to inhibit DPPH activity by 50%, the method described by Picot et al. (2010) was used. This method is in the line of most of the methods used to measure the DPPH scavenging activity because they employ the same amount of sample as volume of DPPH solution. It was used for the final hydrolysates since enough volume of these samples was available. In short, a volume of 1 mL of each protein hydrolysate having different protein concentrations (0.2–5 mg/mL) was added to 1 mL of 0.1 mM DPPH in methanol. The mixture was shaken for 1 h at 25 ºC in the dark. Then, the absorbance of the reaction mixture was measured at 517 nm. A blank was run in the same way by using distilled water instead of sample, and sample control was also made for each sample by adding methanol instead of DPPH solution. Triplicate measurements were carried out for each sample and DPPH scavenging activity was also calculated by Eq. 2.

2.5.2 Reducing power

The reducing power of fish protein hydrolysates samples was determined according to the method of Oyaizu (1992). Two mL of each hydrolysate at different protein concentrations (3–20 mg/mL) were added to 2 mL of 0.2 mM phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. The reaction mixture was incubated at 50 ºC for 20 min and then 2 mL of 10% TCA were added. The mixture was centrifuged at 1500×g for 10 min. A 2 ml aliquot of the supernatant was mixed with 2 mL distilled water and 0.4 mL of 0.1% ferric chloride. The absorbance of the resulting solution was recorded at 700 nm after 10 min. An equivalent volume of distilled water instead of sample was used as control. Analyses were carried out in triplicate.

2.5.3 Iron (Fe^{2+}) chelating activity

The iron chelating activity of the fish protein hydrolysates was estimated by the method described by Decker and Welch (1990). Distilled water (3.7 mL) was added to 1 mL of each fish protein hydrolysate solution at different concentrations (0.2–1 mg/mL). Then, 100 μL of ferrous chloride 2 mM were added and after 3 min the reaction was inhibited by the addition of 200 μL of ferrozine solution 5 mM. The mixture was shaken vigorously and left at room temperature for 10 min, and the absorbance was measured at 562 nm. A blank was run in the same way by using distilled water instead of sample. Sample control was made for each sample without adding ferrozine. Triplicate measurements were carried out. The chelating capacity was calculated as follows (Eq. 3):

$$\text{Iron chelating activity (\%)} = \left(1 - \frac{(A_{sample} - A_{sample\_control})}{A_{blank}}\right) \times 100 \quad (3)$$

The EC_{50} value was calculated for each hydrolysate produced. This value is defined as the concentration of hydrolysate (mg protein/mL) needed to have a chelating activity of 50 %.

2.6. Theoretical identification of antioxidant peptides

The presence of previously reported antioxidant sequences within the structure of the fish proteins would provide an idea of the peptides that could be responsible for the antioxidant activity of the hydrolysates produced in this study. The main proteins of fish (65-75%) are structural proteins such as actin and myosin. Another important group is the sarcoplasmic proteins (myoalbumin, globulin and enzymes) which represent 20-35% of the total protein (Torres, Chen, Rodrigo-García & Jaczynski, 2006).

Swissprot database was employed to obtain the sequence of proteins contained in the species studied in this work: Myosin heavy chain (H6UPM0) and Beta actin (H6UPL9) for *Sardine*

pilchardus and Beta-actin (Q8QFS7) for *Scyliorhinus canicula*. Then, an exhaustive search was carried out in order to find sequences of previously reported active peptides within the structure of these proteins. For that purpose, Microsoft Excel 2010 was employed.

2.7 Statistical analysis

The Statgraphics software (version 5.1) was used to carry out a one-way analysis of variance (ANOVA) on the data. Means values were compared by using the Tukey's test. Differences between means were considered significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Hydrolysis of protein fraction

Hydrolysis curves with similar shape were obtained for the five protein species evaluated. High rates of hydrolysis were observed after the addition of each protease or mixture of proteases, which decreased with reaction time until reaching a plateau. Subtilisin, which cleaves a broad spectrum of peptide bonds (Adler-Nissen, 1986), resulted in higher DH when compared with the addition of trypsin. Besides, an increase in the DH took place immediately after the addition of the second enzyme in the processes where the enzymes were added in sequence. In the same line, Guerard et al. (2002) also observed an increase in the hydrolysis rate as a result of intermediate addition of a commercial neutral protease for the hydrolysis of tuna waste.

For instance, Fig. 1 shows the hydrolysis curves for the press cake of horse mackerel with the three enzymatic treatments assayed. It was observed that the simultaneous addition of subtilisin and trypsin produced the highest DH followed by the sequential treatment of subtilisin plus trypsin. However, different results were found for the other species, where the sequential treatment with subtilisin plus trypsin resulted in the highest DH (data not shown).

247 This is explained by the fact that trypsin produced a worse degradability of the protein of
248 these species compared to the protein of horse mackerel.

249 Table 1 shows the DH values obtained for the 15 hydrolysates produced after 4 h of
250 hydrolysis. It was noticed that the hydrolysates of horse mackerel presented the highest DH,
251 ranging from 18.2 to 21.0 %. They were even higher than the final DH obtained from the
252 muscle of small-spotted catshark, 17.3 – 19.2 %. DH varying from 16.0 to 17.2 were obtained
253 for the hydrolysates of axillary seabream. Similar results were observed for the hydrolysates
254 of bogue, 15.3 – 17.6 %, while the lowest DH were found for sardine hydrolysates, ranging
255 from 13.2 to 14.9 %. These DH were in the range of values mentioned by other authors
256 (Batista, Ramos, Mendonca, & Nunes, 2009; Pires, Clemente, & Batista 2013).

257 **3.2 Influence of DH on DPPH scavenging activity**

258 It has been reported that apart from the properties of the substrate hydrolysed, the choice of
259 the enzymatic treatment and the processing conditions (temperature, pH and enzyme/substrate
260 ratio), the extent of the hydrolysis reaction also has an important significance on the release of
261 antioxidant peptides (Laroque, Chabeaud, & Guérard, 2008).

262 In this sense, the DPPH method was chosen for the evaluation of the antioxidant activity of
263 the hydrolysates with different DH. This method is commonly employed for the
264 determination of the scavenging potency of protein hydrolysates due to its reliability and
265 reproducibility (Laroque, Chabeaud, & Guérard, 2008). Nevertheless, it needs to be said that
266 some peptides released in the course on the enzymatic treatment may also exhibit antioxidant
267 activity by other mechanisms such as chelating of transition metals and participating in the
268 redox reactions involved in the oxidation process (Frankel & Meyer, 2000).

269 Fig. 2 shows the evolution of the DPPH scavenging activity of the hydrolysates with DH.
270 Comparing the results from the three enzymatic treatments assayed, differences were found

among species. For instance, for horse mackerel it was observed that the sequential treatment subtilisin plus trypsin and the enzymatic treatment of trypsin plus subtilisin produced hydrolysates with a higher DPPH scavenging activity, around 45%, than the one resulted from the simultaneous addition of both enzymes, 35 % (Fig. 2a). On the contrary, the sequential treatment subtilisin plus trypsin led to the hydrolysate with the lowest DPPH inhibition for bogue. In the case of sardine and small-spotted catshark, the simultaneous addition of subtilisin and trypsin resulted in the hydrolysates exhibiting the lowest scavenging of DPPH radical; whereas for axillary seabream no differences were obtained by the three enzymatic patterns employed (data not shown). These findings reveal that, for all the species apart from bogue and axillary seabream, the simultaneous addition of subtilisin and trypsin did not enhance the release of peptides with hydrophobic amino acid residues at the C-terminal.

Fig. 2b depicts the evolution of DPPH inhibition with the time of hydrolysis for all species. It shows that when employing the enzymatic pattern subtilisin plus trypsin , the hydrolysates obtained from horse mackerel and sardine exhibited higher DPPH inhibition (40-45 %) than the hydrolysates produced from the other species, around 15 %. This trend was also observed for the other two enzymatic treatments assayed (data not shown).

It was also noted that the DH had a different effect on the DPPH scavenging activity of the hydrolysates, which depended on the raw material hydrolysed and on the enzymatic treatment employed. In this sense, a maximum of DPPH inhibition was reached in the first hydrolysis period with subtilisin for sardine (at 30 min), bogue (at 30 min) and small-spotted catshark (at 10 min) (Fig. 2b). It may be due to the fact that peptides exhibiting DPPH scavenging activity were released until the maximum and then they were hydrolysed to inactive sequences. On the other hand, for horse mackerel and axillary seabream a gradual increase of DPPH scavenging activity was obtained with DH in this period. This phenomenon may be explained

by the fact that peptides exhibiting DPPH scavenging activity were continuously produced by proteolytic action.

The addition of trypsin also led to an increase in the radical scavenging activity of the hydrolysates, which was more remarkable in the case of sardine hydrolysate (Fig. 2b). A maximum of activity was also found in this period particularly for sardine (at 130 min), axillary seabream (at 165 min) and small-spotted catshark (at 180 min). Likewise, maximum values of DPPH inhibition were obtained for most of the hydrolysates when adding trypsin as first enzyme. As an example, Fig. 2a shows a maximum for horse mackerel in the period with trypsin at 60 min, and in the period with subtilisin at 180 min. In this line, Jao and Ko (2002) also reported a maximum of DPPH inhibition at 2.5 h for hydrolysates obtained from tuna cooking juice. Then, the scavenging activity of the hydrolysates decreased until completion of the reaction. In contrast, different results were reported by Wu et al. (2003) which found that the DPPH inhibition of mackerel hydrolysates increased with DH and then tended to plateau after 5 h of reaction.

As a consequence of the results discussed above, the hydrolysates obtained after 4 h hydrolysis, which presented an important DPPH inhibition and the highest DH, were chosen as the best candidates to exhibit antioxidant activity. Hydrolysates with the highest DH were preferred in order to ensure the presence of small peptides which have been reported to show potent antioxidant activities (Jeon, Byun, & Kim, 2000; Kim et al., 2001). Moreover, peptides with low molecular mass have less chance to be modified during digestion and, thus can be absorbed intact from the gastrointestinal tract and produce specific biological actions (Roberts, Burney, Black, & Zaloga, 1999).

3.3 Characterization of final lyophilized hydrolysates

The protein and lipid contents of the final lyophilized hydrolysates are presented in Table 1. The highest protein content was found for the hydrolysates produced from the muscle of small-spotted catshark, ranging from 87.0 to 89.5 %. Axillary seabream and bogue hydrolysates presented lower protein content, varying from 73.0 to 76.8 %, whereas the lowest protein content was found in sardine and horse mackerel hydrolysates, 60.7-67.8 %. These results are in line with previous studies which reported fish protein hydrolysates with a protein content varying from 60 to 92 % (Šližyte, Daukšas, Falch, Storrø, & Rustad, 2005; Ovissipour et al., 2013). These results also confirmed the study carried out by Šližyte et al (2005) which stated that the raw material to be hydrolysed containing the highest amount of lipids such as pelagic species gave the hydrolysates with the lowest content of protein.

Regarding the lipid content, it was found to correlate inversely with the protein content (Table 1). It was observed that the lyophilized hydrolysates of sardine and horse mackerel presented the highest content, 17.4-25.3 %, axillary seabream and bogue hydrolysates had lower values, 8.0-8.8 %, whereas small-spotted catshark hydrolysates presented the lowest lipid amount, 4.6-6.5 %. These results are explained due to the lipid content reported for these species in autumn, which was higher for sardine (11.3 %) and horse mackerel (5.0 %), followed by axillary seabream (3.7 %), small-spotted catshark (2.5 %) and bogue (0.8 %) (García-Moreno et al., 2013a). Chalamaiah et al. (2013) obtained similar fat content for rohu egg protein hydrolysates which was attributed to fat globules present in the supernatant after removing undigested material by filtration. However, it should be mentioned that hydrolysates with low lipid content are preferred in order to obtain final products with improved oxidative stability (Raghavan, Kristinsson, & Leeuwenburgh 2008; Khantaphant, Benjakul, & Ghomi, 2011).

Moreover, different lipid content was found for the hydrolysates obtained from the same raw material but by different enzymatic treatment (Table 1). Daukšas et al. (2005) attributed this

fact to the formation of more or less peptides with accessible hydrophobic regions which are more lipid-binding than those with the hydrophobic regions embedded in the interior.

In order to better characterize the hydrolysates, the lipid class composition was determined for the lyophilized samples obtained with the sequential treatment subtilisin plus trypsin . For the hydrolysates produced from press cakes, it was found that triacylglycerols represented the major lipid class. They were around 75.0 % for sardine and horse mackerel and 45.0 % for axillary seabream and bogue. In the hydrolysates from these species free fatty acids were the second most important lipids. Its content was species dependent, but it was considerably higher in axillary seabream and bogue hydrolysates, approximately 35.0 %. This high content of free fatty acids is detrimental for the oxidative stability of the hydrolysates, since free fatty acids are even more prone to oxidation than esterified fatty acids (Aidos, Van der Padt, Boom & Luten, 2001). Considering cholesterol content, it ranged from 3.9 % for sardine to 14.7 % for bogue. Phospholipids content of these hydrolysates was practically constant and it was around 9.0 %. On the other hand, phospholipids were the most abundant lipids for small-spotted catshark muscle hydrolysate, 54.4 %, followed by cholesterol, 35.2 %. This finding may be due to the fact than in lean fish, phospholipids make up most of the lipids of the cell (Liang & Hultin, 2005). Although antioxidant activity has been previously described for phospholipids, a recent study reported that it was insignificant when compared to other antioxidants such as BHT, EDTA and ascorbic acid (García-Moreno, Horn & Jacobsen, 2014). Conversely, triacylglycerols and free fatty acids contents of this hydrolysate were practically negligible, 5.0 and 5.4 % respectively. Similarly, Daukšas et al. (2005) reported that triacylglycerols content of cod hydrolysates depended on the raw material and enzyme employed, varying from 37 to 88 %. These authors also reported phospholipid content for cod hydrolysates of 59 %, which is in the range of the content found in small-spotted catshark hydrolysate.

The molecular mass size distribution of the hydrolysates obtained from the press cakes by the three enzymatic treatments was very similar (Fig. 3). In general, the gel filtration profile of the hydrolysate samples indicated hydrolysis of the fish proteins into small molecular mass peptides (<1000 Da) and free amino acids. With regard to the small-spotted catshark hydrolysate, it showed a rather different peptide profile from the hydrolysates prepared from the press cake of the bony fish. Although it also had most of the peptides with a molecular mass below 1000 Da, it presented two narrow peaks at short (7-8.5 mL) and long (23.5-25.5 mL) elution volume which were not found in the other hydrolysates (dotted squares in Fig. 3).

3.4 Antioxidant activity of final hydrolysates

It is widely known that antioxidants can act by different mechanisms. Therefore, the use of various methods for the evaluation of the antioxidant activity is recommended (Frankel & Meyer, 2000).

The DPPH radical scavenging and the Fe^{2+} chelating activities of the 15 final hydrolysates were measured as a function of the protein concentration in order to determine their EC_{50} values (Table 3). It was observed that sardine and horse mackerel hydrolysates presented the highest DPPH scavenging activity with EC_{50} values ranging from 0.91 to 1.78 mg protein/mL. They were followed by axillary seabream and bogue hydrolysates with EC_{50} values varying from 1.94 to 2.91 mg protein/mL, whereas small-spotted catshark hydrolysates presented the lowest DPPH scavenging-activity with EC_{50} values in the range of 3.82 - 4.45 mg protein/mL. These results are in line with the EC_{50} value reported for rohu egg protein hydrolysates, 1.5 mg hydrolysate/mL, (Chalamaiah et al., 2013), but are considerable lower than the EC_{50} value found for protein hydrolysates from toothed ponyfish muscle, 25 mg hydrolysate/mL, (Klomklao, Benjakul, & Kishimura, 2013) and for hake protein hydrolysates when concentrations up to 30 mg hydrolysate/mL did not reach a DPPH inhibition of 50 % (Pires, Clemente, & Batista 2013).

In most of the cases, significant differences were found in the DPPH scavenging activity of the hydrolysates when employing different enzymatic treatments (Table 3). In general, the sequential addition of trypsin plus subtilisin resulted in the hydrolysates with the lowest EC_{50} values. It may be due to the fact that adding subtilisin as second enzyme may favor the cleavage at the C-terminal of hydrophobic residues which can contribute to the DPPH inhibitory activity (Je, Lee, Lee, & Ahn, 2009).

The capacity to bind transition metals is also a useful indication of antioxidant activity. It is due to the fact that transition metal ions, Fe^{2+} and Cu^{2+}, catalyze the generation of reactive oxygen species such as hydroxyl radical (OH·) which initiates lipid peroxidation (Stohs & Bagchi, 1995). Sardine and small-spotted catshark hydrolysates exhibited the highest ferrous-chelating activity with EC_{50} values of 0.32 mg protein/mL (Table 3). The hydrolysates prepared from horse mackerel, axillary seabream and bogue presented slightly higher EC_{50} values ranging from 0.42 to 0.63 mg protein/mL. They showed higher ferrous binding capacity than those of silver carp hydrolysates produced with Flavourzyme, which exhibited only 60 % at a concentration of 5 mg hydrolysate/mL (Dong et al., 2008). Nonetheless, they were in the line of EC_{50} values obtained by Ktari et al. (2012) for zebra blenny protein hydrolysates, which ranged from 0.15 to 0.25 mg hydrolysate/mL depending on the enzymatic treatment used.

The metal binding capacity of protein hydrolysates is generally attributed to their content in effective sites capable of chelating metal ions (Ovissipour et al., 2013). In this sense, histidine containing peptides have been reported to exhibit metal chelating activity through their imidazole ring (Bougatef et al., 2009). Considering the different enzymatic treatments assayed, it was revealed that for axillary seabream, bogue and small-spotted catshark, the simultaneous addition of subtilisin and trypsin led to the hydrolysates with the lowest metal chelating activity (Table 3). This fact may be caused by differences in the structure of the

417 peptides in these hydrolysates (Thiansilakul, Benjakul, & Shahidi., 2007). However, further
418 investigations are required in order to obtain more information about the amino acid
419 sequences of the active peptides which can confirm it.

420 The reducing power assay is another common test employed to determine the antioxidant
421 activity of fish protein hydrolysates. Particularly, this method evaluates the capacity of fish
422 protein hydrolysates to act as a reducing agent (Batista, Ramos, Coutinho, Bandarra, &
423 Nunes, 2010). As observed in Fig. 4, the reducing power of all the hydrolysates increased
424 with the concentration of protein. A similar trend was reported in previous studies (Ktari et
425 al., 2012; Pires, Clemente, & Batista 2013). From Fig. 4, it was observed that sardine and
426 bogue hydrolysates exhibited the highest reducing power independently of the enzymatic
427 treatment, whereas small-spotted catshark hydrolysates presented the lowest. This fact may be
428 probably due to the differences observed in the molecular mass profile of these hydrolysates
429 (Fig. 3), as well as the amino acid composition of their peptides (Theodore, Raghavan, &
430 Kristinsson, 2008). It also should be mentioned that the simultaneous addition of subtilisin
431 plus trypsin was the least appropriate enzymatic treatment for the production of fish protein
432 hydrolysates with a high reducing power (Fig. 4). The results obtained in this work were
433 similar to that reported for black scabbardfish (Batista, Ramos, Coutinho, Bandarra, & Nunes,
434 2010), higher than those found for hake by products (Pires, Clemente, & Batista 2013), but
435 considerably lower than those obtained for zebra blenny (Ktari et al., 2012) and sardinelle
436 (Bougatef et al., 2010).

437 **3.5 Theoretical identification of antioxidant peptides**

438 A total of six sequences of previously reported antioxidant peptides, namely YA, PR, HH,
439 EL, VKV and KD, were identified within the proteins studied. The dipeptides YA and PR
440 previously identified by Tang et al. (2010) and Wang et al. (2008), respectively, showed good
441 DPPH scavenging properties. Both peptides had been found in the primary structure of

sardine myosin, which point toward both peptides could contribute to the good values of DPPH radical scavenging activity found for sardine hydrolysate. Other dipeptides with good radical scavenging properties previously found in the sardine proteins studied were HH encrypted also in myosin (Chen, Muramoto, Yamauchi, Fujimoto & Nokihara, 1998) and EL which was found in both myosin and beta-actin (Suetsuna, Ukeda, & Ochi, 2000). Apart from these peptides with scavenging properties, two more sequences, VKV and KD, are encrypted in myosin and beta-actin of the sardine. These peptides were identified as antioxidant peptides (Suetsuna & Ukeda 1999) using the methyl linoleate model. With respect to small-spotted catshark, only four of the previous sequences (KD, YA, HH and EL) where identified within the Beta-actin sequence. Almost all the peptides identified in sardine and small-spotted catshark proteins, presented a size 300-200 Da.

Although these myosin and actin-derived peptides may be present within the sardine and small spotted catshark hydrolysates, further studies (e.g. in silico studies) are required in order to confirm that these peptides are the responsible of the observed antioxidant activity.4. CONCLUSIONS

This work denoted that it is feasible to produce fish protein hydrolysates exhibiting strong *in vitro* antioxidant activity when employing press cakes of discarded species as raw material. These hydrolysates, with less potential hazard than synthetic antioxidants, are suitable products to be used by the food industry in order to prevent lipid oxidation. They presented a varying protein (60.7-89.5 wt%) and lipid (4.6-25.3 wt%) content depending on the species. The molecular mass profiles indicated that the hydrolysates were mainly constituted of small peptides, below 1000 Da, which have been reported to contribute to their antioxidant activity. The highest DPPH scavenging activity was found for the hydrolysate of sardine produced by the enzymatic treatment trypsin plus subtilisin , with an EC_{50} value of 0.91 mg protein/mL.

Sardine and small-spotted catshark hydrolysates exhibited the highest ferrous chelating activity, with EC_{50} value of 0.32 mg protein/mL, except for the small-spotted catshark hydrolysate obtained by the simultaneous addition of subtilisin and trypsin. In terms of reducing power, sardine and bogue hydrolysates presented the highest electron donating capacity, with an absorbance higher than 0.8 at a concentration of 20 mg protein/mL. Therefore, it can be concluded that, among the five species evaluated, sardine is the discarded fish species with the highest potential for the production of fish protein hydrolysates with antioxidant activity. Besides, six sequences of antioxidant peptides previously described in literature (YA, PR, HH, EL, VKV and KD) were also found to be encrypted in the structure of myosin and actin proteins from sardine.

5. ACKNOWLEDGEMENTS

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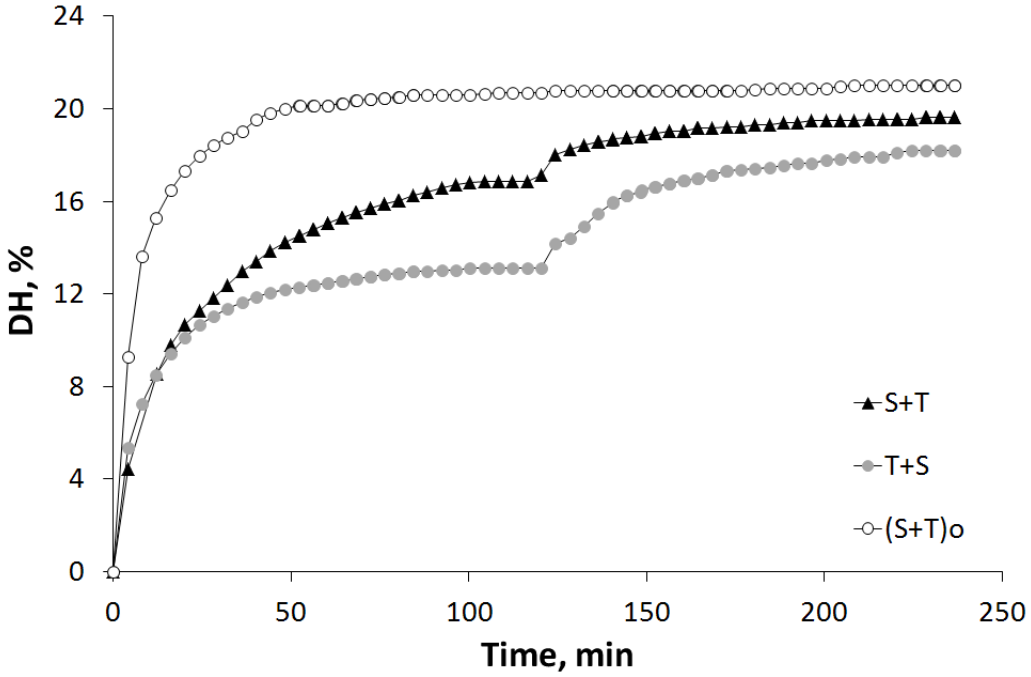
650

FIGURE AND TABLE CAPTIONS

- Figure 1. Hydrolysis curves for the press cake of horse mackerel
- Figure 2. (a) DPPH inhibition of horse mackerel hydrolysates at different times of hydrolysis, (b) DPPH inhibition of the hydrolysates obtained with the enzymatic pattern subtilisin + trypsinfor the five species studied.
- Figure 3. Gel filtration chromatograms showing the molecular mass distribution of the final hydrolysates obtained by the enzymatic treatment subtilisin + trypsin.
- Figure 4. Reducing power of the final hydrolysates produced by: (a) subtilisin + trypsin, (b) trypsin + subtilisin and (c) subtilisin+trypsin (simultaneous). Results are average of triplicate determination ± standard deviation.
- Table 1. Degree of hydrolysis and protein and lipid content of the final hydrolysates
- Table 2. Lipid classes presented in the final hydrolysates produced by the enzymatic treatment subtilisin + trypsin
- Table 3. EC_{50} values of the final hydrolysates



666

667 S+T: subtilisin plus trypsin; T+S: trypsin plus subtilisin; (S+T)o: simultaneous addition of subtilisin and trypsin.

668 Fig. 1. Hydrolysis curves for the press cake of horse mackerel

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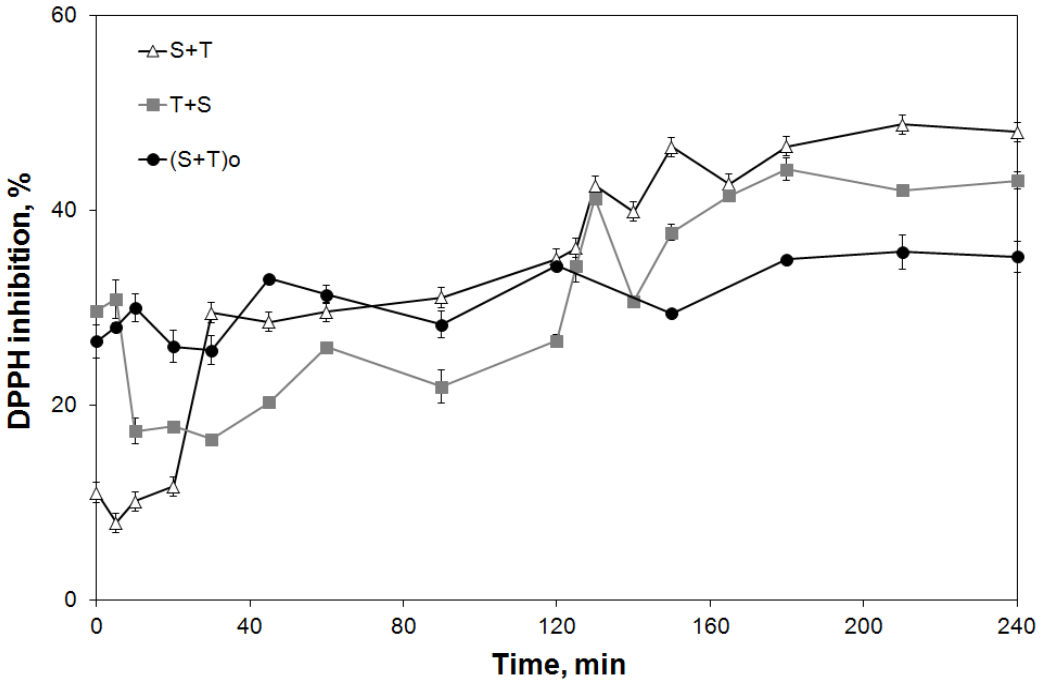
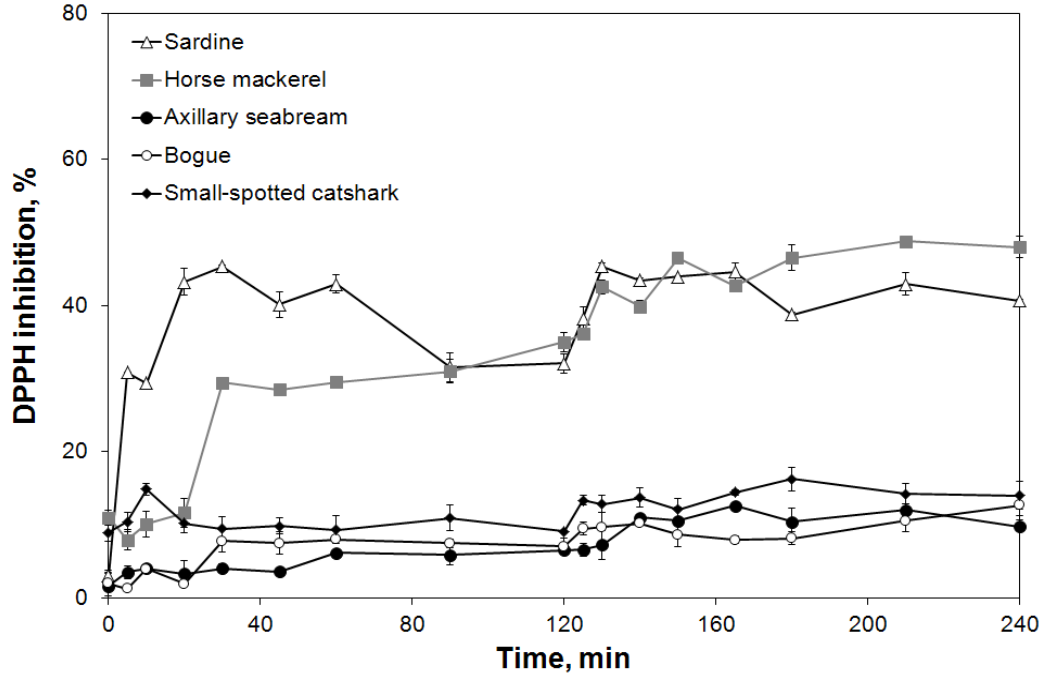
(a)

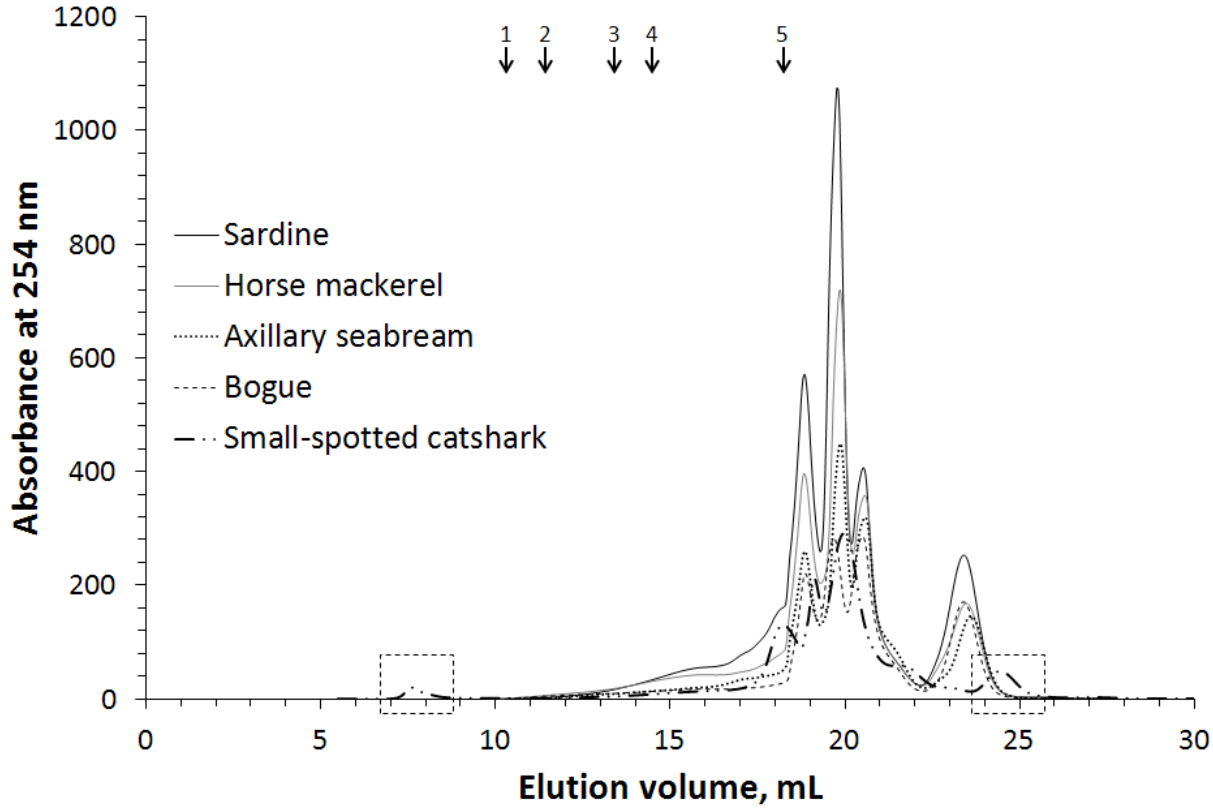


(b)



671 S+T: subtilisin plus trypsin; T+S: trypsin plus subtilisin; (S+T)o: simultaneous addition of subtilisin and trypsin.

672 Fig. 2. (a) DPPH inhibition of horse mackerel hydrolysates at different times of hydrolysis,
673 (b) DPPH inhibition of the hydrolysates obtained with the enzymatic pattern subtilisin +
674 trypsin for the five species studied.



675

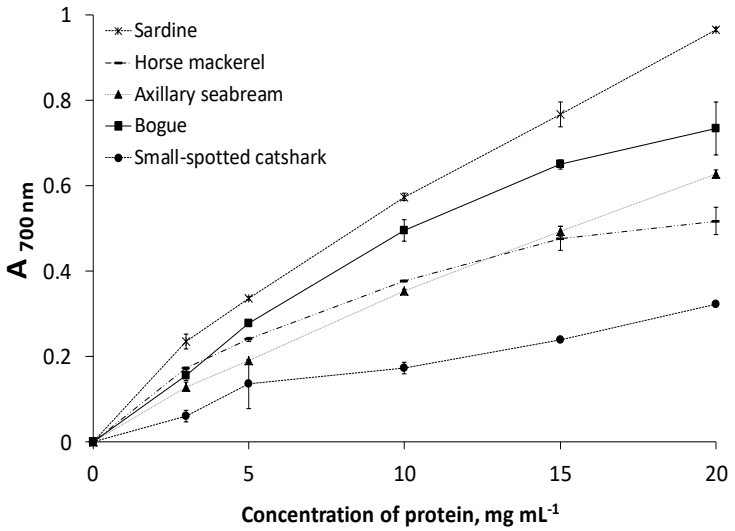
676 1, ribonuclease A (13,700 Da); 2, aprotinin (6500 Da); 3, angiotensin I (1296 Da); 4,bradykinin (1060 Da); 5,
677 triglycine (189 Da).

678 Fig. 3. Gel filtration chromatograms showing the molecular mass distribution of the final
679 hydrolysates obtained by the enzymatic treatment subtilisin + trypsin.

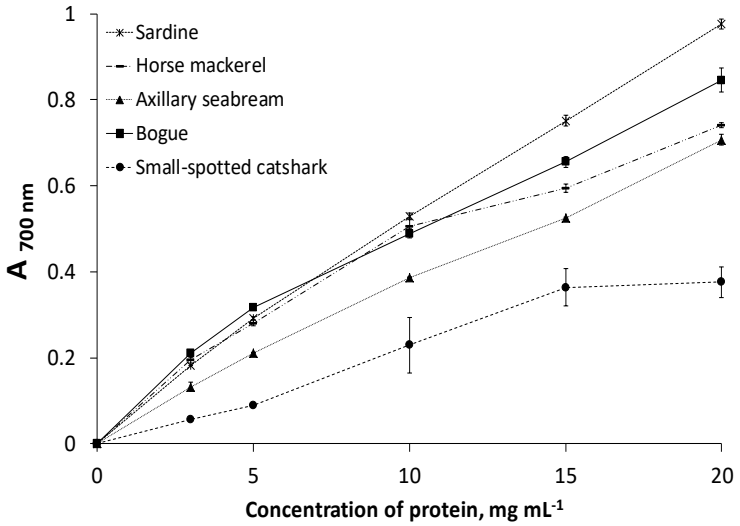
680

681

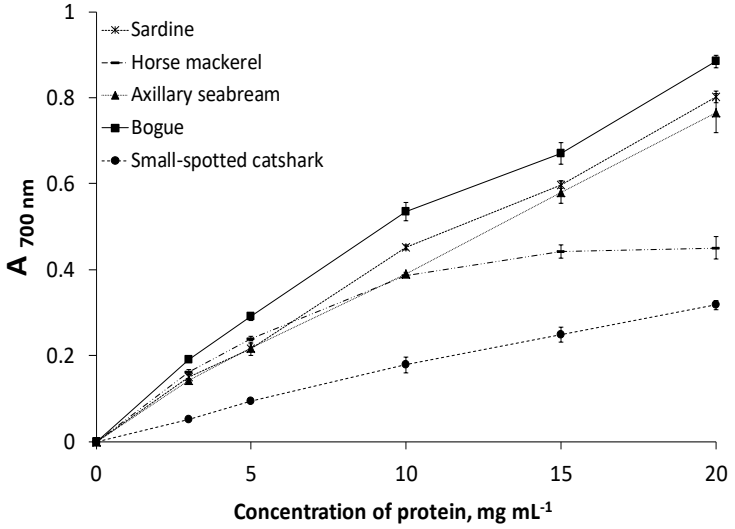
(a)


Sardine
Horse mackerel
Axillary seabream
Bogue
Small-spotted catshark
A 700 nm
Concentration of protein, mg mL-1

(b)


Sardine
Horse mackerel
Axillary seabream
Bogue
Small-spotted catshark
A 700 nm
Concentration of protein, mg mL-1

(c)


Sardine
Horse mackerel
Axillary seabream
Bogue
Small-spotted catshark
A 700 nm
Concentration of protein, mg mL-1

682 Fig. 4. Reducing power of the final hydrolysates produced by: (a) subtilisin + trypsin, (b)
683 trypsin + subtilisin and (c) subtilisin + trypsin (simultaneous). Results are average of triplicate
684 determination ± standard deviation.

685

686 Table 1. Degree of hydrolysis and protein and lipid content of the final hydrolysates

Hydrolysate		DH, %	Protein, %	Lipid, %
Sardine	S+T	14.9	61.5 ± 0.4^{a}	22.3 ± 0.2^{a}
	T+S	13.2	60.7 ± 0.4^{b}	25.3 ± 0.0^{a}
	(S+T)o	13.7	66.4 ± 0.4^{c}	19.7 ± 0.9^{b}
Horse mackerel	S+T	19.7	67.8 ± 0.2^{d}	17.4 ± 0.3^{c}
	T+S	18.2	67.1 ± 0.2^{e}	17.6 ± 0.3^{c}
	(S+T)o	21.0	62.5 ± 0.2^{f}	21.0 ± 0.1^{ab}
Axillary seabream	S+T	17.2	73.0 ± 0.3^{g}	8.8 ± 0.9^{d}
	T+S	16.0	73.5 ± 0.3^{g}	8.0 ± 0.6^{d}
	(S+T)o	16.3	74.9 ± 0.2^{h}	8.4 ± 0.9^{d}
Bogue	S+T	17.6	75.7 ± 0.7^{i}	8.3 ± 0.1^{d}
	T+S	17.0	76.8 ± 0.2^{j}	8.1 ± 0.0^{d}
	(S+T)o	15.3	76.4 ± 0.2^{j}	8.4 ± 0.2^{d}
Small-spotted catshark	S+T	19.2	87.0 ± 0.2^{k}	6.5 ± 1.9^{e}
	T+S	18.3	89.5 ± 0.2^{l}	4.6 ± 1.3^{f}
	(S+T)o	17.3	88.7 ± 0.5^{m}	4.8 ± 1.8^{f}

687 DH: degree of hydrolysis; S+T: subtilisin plus trypsin; T+S: trypsin plus subtilisin; (S+T)o: simultaneous
688 addition of subtilisin and trypsin.

689 Protein and lipid data are means of triplicate determinations ± standard deviation. Mean values within a column
690 followed by different letter mean significant differences ($p<0.05$).

691

692 Table 2. Lipid classes presented in the final hydrolysates produced by the enzymatic treatment
693 subtilisin+trypsin

Lipid class, %	**Sardine**	**Horse mackerel**	**Axillary seabream**	**Bogue**	**Small-spotted catshark**
Triacylglycerols	74.9 ± 6.6^{a}	73.9 ± 3.7^{a}	45.0 ± 0.5^{b}	43.8 ± 3.5^{b}	5.0 ± 1.1^{c}
Free fatty acids	12.2 ± 2.3^{a}	6.5 ± 0.9^{b}	36.2 ± 0.4^{c}	33.8 ± 3.7^{c}	5.4 ± 1.0^{b}
Cholesterol	3.9 ± 2.3^{a}	9.7 ± 1.0^{b}	10.2 ± 0.1^{b}	14.7 ± 0.1^{c}	35.2 ± 1.7^{d}
Phospholipids	9.0 ± 2.0^{a}	9.9 ± 1.9^{a}	8.6 ± 0.2^{a}	7.7 ± 0.3^{a}	54.4 ± 1.9^{b}

694 Data are means of triplicate determinations ± standard deviation. Mean values within a row followed by different
695 letter mean significant differences ($p<0.05$).

696

697 Table 3. EC_{50} values of the final hydrolysates

Hydrolysate		EC_{50} (mg protein/mL)	
		DPPH radical scavenging	Fe^{2+} Chelating activity
Sardine	S+T	1.30 ± 0.12[a]	0.32 ± 0.01[a]
	T+S	0.91 ± 0.02[b]	0.32 ± 0.01[a]
	(S+T)o	1.75 ± 0.05[c]	0.32 ± 0.01[a]
Horse mackerel	S+T	1.63 ± 0.03[d]	0.42 ± 0.03[b]
	T+S	1.47 ± 0.01[e]	0.49 ± 0.01[c]
	(S+T)o	1.78 ± 0.08[c]	0.46 ± 0.01[d]
Axillary seabream	S+T	2.56 ± 0.02[f]	0.45 ± 0.01[d]
	T+S	2.34 ± 0.05[g]	0.5 ± 0.03[c]
	(S+T)o	2.44 ± 0.03[g]	0.51 ± 0.01[c]
Bogue	S+T	2.91 ± 0.06[h]	0.51 ± 0.01[c]
	T+S	2.84 ± 0.05[h]	0.50 ± 0.01[c]
	(S+T)o	1.94 ± 0.02[i]	0.63 ± 0.03[e]
Small-spotted catshark	S+T	4.45 ± 0.06[j]	0.32 ± 0.02[a]
	T+S	3.82 ± 0.06[k]	0.32 ± 0.02[a]
	(S+T)o	4.35 ± 0.11[l]	0.51 ± 0.01[c]

698 S+T: subtilisin plus trypsin; T+S: trypsin plus subtilisin; (S+T)o: simultaneous addition of subtilisin and trypsin.
699 Data are means of triplicate determinations ± standard deviation. Mean values within a column followed by
700 different letter mean significant differences ($p<0.05$).

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