LIPID CHARACTERIZATION AND PROPERTIES OF PROTEIN HYDROLYSATES OBTAINED FROM DISCARDED MEDITERRANEAN FISH SPECIES

Lipids and protein hydrolysates from Mediterranean discarded species

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ABSTRACT

BACKGROUND: Discards are an important fraction of the by-products produced by the fishing industry. As consequence of their low commercial acceptance, it is required to provide an added value to these underutilized materials. In this study, it was carried out the characterization of the lipid fraction of three fish discarded species in the west Mediterranean Sea, sardine (*Sardine pilchardus*), mackerel (*Scomber colias*) and horse mackerel (*Trachurus trachurus*), as well as the evaluation of both angiotensin I-converting enzyme (ACE)-inhibitory and antioxidative activities of their protein hydrolysates.

RESULTS: The processing of these biomaterials led to oils with a high content of Omega-3 polyunsaturated fatty acids (PUFAs), ranging from 220.5 g kg^{-1} for horse mackerel to 306.0 g kg^{-1} for sardine. Regarding the protein fraction, most of the hydrolysates presented ACE inhibition values higher than 60%, which corresponds to IC_{50} values varying from 345 μg protein mL^{-1} for mackerel to 400 μg protein mL^{-1} for sardine. Moreover, most of the hydrolysates exhibited an acceptable antioxidative activity, 35-45% of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) inhibition.

CONCLUSION: This study suggests that the three discarded species evaluated are valuable raw material for the production of bioactive ingredients such as Omega-3 PUFAs and protein hydrolysates exhibiting antihypertensive and antioxidant activities.

KEYWORDS: Fish oil, fish protein hydrolysates, Omega-3, antihypertensive activity, antioxidative activity.

INTRODUCTION

Discards are an important fraction of the by-products produced by the fishing industry. They represent an underutilization of the marine resources and they also produce a significant ecological impact on the marine organism's food chain because most of discards are dumped at sea dead or dying.[1] To this regard, the EU Commission has lately considered several regulations avoiding progressively discard practices in the EU fishing fleet with the aim of adopting a policy of zero-discard in EU fisheries as a part of a reformed Common Fisheries Policy in 2013.[2]

Spanish fishing vessels in the west Mediterranean Sea (FAO fishing area 37.1.1.) employ trawling and purse seine as main fishing gears, which generate average discard rates of 23% and 5-10%, respectively.[3] Discards in west Mediterranean Sea comprise non-target species such as mackerel, which appears as by-catch species in trawling fisheries. Other species, such as sardine and horse mackerel, are mostly discarded due to quota restriction or minimum landing sizes (11 cm for sardine and 12 cm for horse mackerel). Horse mackerel is the commercial species presenting the largest discard rate (5-10%) for the Spanish Mediterranean fisheries employing purse seine.[3] In the case of sardine, although the average discard rate is estimated at only 0.1 – 0.2% of the total catches in Mediterranean Spanish fisheries, the total mass of the sardine discarded yearly is significant.[4]

Given the low commercial acceptance of discards, it is necessary to provide an added value to these underutilized materials. In this sense, the development of new extraction technologies and research has permitted the identification and isolation of an increasing number of bioactive compounds from remaining fish muscle proteins, collagen and gelatine, fish oil, fish bone, internal organs and shellfish and crustacean shells.[5] These

bioactive compounds have recently received much attention in the biotechnological, nutraceutical and pharmaceutical fields.

Considering protein fraction, many scientific studies have demonstrated that some peptides present in fish muscle tissue exhibit a number of biological activities such as antihypertensive, antithrombotic, immunomodulatory and antioxidative activities, among others.[6,7]

Antihypertensive activity of peptides lies on their capacity to inhibit the action of the angiotensin converting enzyme (ACE, EC 3.4.15.1), a dipeptidil carboxipeptidase able to convert the plasma peptide angiotensin I into angiotensin II, which possesses a strong vasoconstrictor activity. Furthermore, this enzyme is responsible for the degradation of bradykinin, an endogenous vasodilator nonapeptide. Consequently, the administration of ACE inhibitor peptides can reduce blood pressure, which is one of the major risk factors for cardiovascular diseases. To this regard, hydrolysates from marine origin have been widely reported to exhibit ACE-inhibitory activity,[8] some of them belonging to target species in Mediterranean fisheries such as sardinelle[9] or cuttlefish.[10]

Another biological activity of interest is the antioxidative capacity. Oxidative damage is caused by natural occurring radicals, such as superoxide anion ($O_2 \cdot^-$), hydroxyl radical ($OH\cdot$) or hydrogen peroxide (H_2O_2), which are unavoidably generated by cell respiration. These radicals, known as reactive oxygen substances (ROS) can act as initiators or propagators of chain reactions, which result in the damage of lipid membranes, structural proteins, enzymes and DNA structure. Many studies have related the accumulative oxidative damage with the occurrence of several chronic diseases such as cancer, diabetes, inflammatory and neurodegenerative diseases.[11] In the field of food processing, lipid oxidation reactions are responsible for the appearance of undesirable

odors and flavors due to the formation of secondary oxidation products.[12] Antioxidant compounds can delay or interrupt chain oxidation reactions by scavenging free radicals or acting as chain terminators. The current regulations restraining the use of synthetic antioxidants as food additives, due to their potential hazardous effects, has paved the way to the research of new natural antioxidants. To this regard, antioxidant potency has been reported in protein hydrolysates from several marine species,[13] some of them present in the Mediterranean Sea such as sardinelle.[14]

Besides the protein content, fish oil is another important fraction which can be extracted from fish materials of high availability such as non-commercial species, fish by-products or wastes from fish processing. Its incorporation into feedstuffs for both animal and human consumption is justified by its unique content in polyunsaturated fatty acids belonging to the n-3 family, such as eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA). Both have been reported to promote several benefits on human health, such as prevention of atherosclerosis, reduction of blood pressure and protection against arrhythmias;[15] improvement of the anti-inflammatory response;[16] and development of brain and eye retina in infants.[17]

Therefore, the aim of this research work was: i) to study the characterization of fish oil, and ii) to evaluate the antihypertensive and antioxidative activity of protein hydrolysates, both produced from three discarded fish species in the west Mediterranean Sea, sardine (*Sardina pilchardus*), mackerel (*Scomber colias*) and horse mackerel (*Trachurus trachurus*).

MATERIALS AND METHODS

Raw material

Sardine (*Sardina pilchadus*), mackerel (*Scomber colias*) and horse mackerel (*Trachurus trachurus*) were chosen for this study as species discarded by the Spanish fleet in the Mediterranean Sea. The raw material was provided by the fishing harbour of Motril (Spain), kept in ice during the transportation and pressed in the same day.

Proximate chemical composition

The moisture and ash content were estimated gravimetrically by heating the samples until attaining constant weight at 103ºC and 550ºC, respectively. Total nitrogen was determined by the Kjeldahl method, and the content of crude protein was then obtained by employing a nitrogen-to-protein conversion factor of 6.25. Total lipid content was determined according to the Soxhlet semi-continuous extraction method.[18]

Separation of protein and oily fractions

The whole fish, included viscera and gonads, was preheated at 40ºC for 30 minutes by means of a water bath model Digiterm 100 (Selecta, Spain). Then, it was fed into an electric press model ESP-K (Sanahuja, Spain) where it was submitted to three consecutive pressing steps until attaining a final pressure of 150 bar. The press liquor released during the operation was collected and then centrifuged at 20,000×g in order to recover the oily phase. The cakes resulting from the pressing operation were frozen at -20ºC prior to their use as substrate for protein hydrolysis. It should be noted that the pressing stage also allowed a reduction in the moisture content and in the volume of the raw material which produce a better preservation of these materials prior to their up-grading and reduce the handling and isolation costs.

Fatty acid profile of the oils

The oils obtained were methylated by direct transesterification following the method of Lepage and Roy[19] modified by Rodríguez-Ruiz et al.[20] These methyl esters were then analysed according to the method described by Camacho-Paez et al.[21] with an Agilent 7890A gas chromatograph (Agilent Technologies, S.A.) connected to a capillary column of fused silica Omegawax (0.25 mm × 30 m, 0.25 μm standard film; Supelco, Bellefonte, PA), and a flame-ionisation detector. Matreya (Pleasant Gap, PA) n-3 PUFAs standard (catalogue number 1177) was used for the qualitative fatty acid determination. Nonadecanoic acid (19:0) (Sigma-Aldrich) was used as internal standard for quantitative determination of fatty acids. Each sample was analysed in triplicate.

Composition of the lipid fraction

The composition of the lipid fraction was determined by thin layer chromatography (TLC) according to Hita et al.[22] Plates of silica-gel (Precoated TLC plates, SIL G-25; Macherey-Nagel, Sigma–Aldrich) were activated by heating at 105 ºC for 30 min. The samples were spotted directly on the plate by adding 0.2 mL of reaction product mixture. The plates were developed in chloroform/acetone/methanol. Spots of each lipid were visualized by spraying the plate with iodine vapour in a nitrogen stream. Fractions corresponding to each lipid type were scraped from the plates and methylated and analysed as described above. Nonadecanoic acid (19:0) (Sigma-Aldrich) was used as internal standard for quantitative determination of fatty acids.

Enzymes and hydrolysis procedure

The press cakes were processed in order to obtain hydrolysates displaying ACE inhibitory and antioxidative activities. To this end, the cakes were hydrolysed employing two serine endoprotease enzymes, one of bacterial origin (subtilisin, EC

3.4.21.62) and other from an animal source (trypsin, EC 3.4.21.4), both provided by Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S, respectively. All the experiments were conducted at pH 8 and 50ºC, while enzyme-substrate ratio was set at 30 g kg^{-1} for both enzymes, being substrate the protein.

For each experiment, the press cake was grinded by means of cutter SK-3 (Sammic, Spain). A given mass of grinded press cake was then homogenised with demineralised water until reaching a final volume of 200 mL. This suspension was then transferred into a jacketed reactor of volume capacity 250 mL. Three reaction patterns were studied: (a) 2-hour hydrolysis with subtilisin followed by addition of trypsin until completing 4 h of reaction; (b) 2-hour reaction with trypsin followed by subtilisin and; (c) 4-hour hydrolysis with simultaneous addition of both enzymes. The degree of hydrolysis, defined as the percentage ratio of the number of peptide bonds cleaved to the total number of peptide bonds in the substrate studied, was calculated as a function of the base consumption throughout the reaction,[23] employing an automatic titrator 718 Stat Titrino (Metrohm, Switzerland). According to this method, the degree of hydrolysis (DH) can be related to the amount of base consumed to keep the pH constant during the reaction, as follows:

$$DH = B \cdot N_b / (\alpha \cdot m_P \cdot h_{TOT}) \times 100 \qquad (1)$$

where B (mL) is the amount of base consumed, N_b (eq L^{-1}) is the normality of the base, α is the 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^{-1}) 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 milliequivalents of peptide bonds per gram of protein, as reported in literature.[24]

A set of 150 hydrolysates, originated from the three species and three enzymatic treatments studied and drawn at different times of reaction, were evaluated in this research work. To this end, the samples were heated at 100ºC for 15 min to deactivate the enzyme and were then centrifuged in order to remove the remained solids. They were kept under refrigerated conditions until performing the analysis.

Determination of the ACE inhibitory activity

The ACE inhibitory activity of the hydrolysates was determined *in vitro* by means of the assay described by Shalaby et al.[25] This method is based in the hydrolysis of the tripeptide N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG, Sigma F7131) with the Angiotensin converting enzyme (ACE) from rabbit lung (Sigma A6778). The assay was carried out in 96-well microplate at 37ºC. Each well contained 10 μL of enzyme solution (0.25 U mL^{-1}), 10 μL of sample, and 150 μL of 0.88mM of FAPGG in buffer Tris-HCl 50 mM, pH 7.5 and 0.3 M of NaCl. The wavelength was set at 340 nm and the absorbance was monitored during 30 minutes by means of a Multiskan FC microplate photometer (Thermo Scientific, Finland). Each sample was analysed in triplicate.

The absorbance decreases linearly with time as ACE hydrolyses the substrate FAPGG. The slope of this descent is commonly used as a measurement of the enzyme activity. Indeed, the numerical value of inhibitory activity of each hydrolysate can be calculated by Eq. 2:

$$\text{ACE inhibition (\%)} = \left(1 - \frac{\rho_i}{\rho_0}\right) \times 100 \qquad (2)$$

where ρ_i was the slope in the presence of inhibitor (hydrolysate) and ρ_o the slope obtained in the absence of inhibitor (pure water). These slopes were calculated from the values obtained within the interval of 10 to 25 minutes, where a better linearity was observed.

The ACE inhibitory activity of a given hydrolysate is widely reported in literature by the IC_{50} value. This value is defined as the concentration of hydrolysate (μg protein mL^{-1}) needed to inhibit ACE activity by half. In this work, the IC_{50} values were calculated for the samples presenting the highest values of ACE inhibitory activity. Each IC_{50} value was reported to the content of soluble protein in the hydrolysates, which was determined using the Micro-Lowry assay (Sigma Aldrich, USA).

Determination of antioxidative activity

The antioxidative activity of the hydrolysates was estimated by determination of their DPPH radical scavenging activity according to Brand-Williams et al.[26] 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-picryl-hydrazyl (DPPH) at 0.1 mM concentration in methanol. The mixture was then kept at room temperature in the dark for 30 minutes, and the reduction of DPPH radical was measured at 515 nm. The DPPH radical scavenging activity (%) was calculated as:

$$\text{DPPH inhibition (\%)} = \left(\frac{A_0 - A_i}{A_0} \right) \times 100 \qquad (3)$$

where A_0 was the absorbance of the control and A_i the absorbance obtained in the presence of hydrolysate. Each sample was analysed in duplicate.

206 Then, the antioxidative capacity of the hydrolysates presenting the highest activity were
207 expressed as Trolox Equivalent Antioxidant Capacity value (TEAC), which is
208 determined by interpolating the DPPH inhibition on a calibrating curve using different
209 Trolox (Sigma-Aldrich, USA) concentrations.[27]

210 ### Statistical analysis

211 The Statgraphics software (version 5.1) was used to generate the statistical analysis.
212 Differences were considered significant at $p < 0.05$.

213 ## RESULTS AND DISCUSSION

214 ### Proximate composition of fish species

215 The proximate composition of the fish discarded species studied is shown in Table 1.
216 The three species presented similar protein content, ranging from 169 g kg^{-1} for horse
217 mackerel to 184 g kg^{-1} for mackerel. On the other hand, the lipid content varied much
218 more from one species to another. The horse mackerel exhibited the highest lipid
219 content (78 g kg^{-1}) and mackerel the lowest (25 g kg^{-1}). Regarding moisture content, it
220 was found to correlate inversely with the lipid content. The ash content ranged from 28
221 g kg^{-1} for mackerel to 40 g kg^{-1} for horse mackerel. Nevertheless, it should be taken into
222 account that, although proximate composition of fish is specific for each species, it
223 varies according to the stage of maturity, the sex, the spawning cycle, the environment,
224 the season, and the nutritional condition of the animal.[28]

225 Moisture was found to correlate inversely with the lipid content for the five species

226 ### Lipid fraction

227 Table 2 shows the fatty acid profile of sardine, mackerel and horse mackerel oils. The
228 major fatty acids identified for the three oils were: (i) saturated fatty acids (SFAs),
229 palmitic (C16:0), myristic (C14:0) and stearic (C18:0); (ii) monounsaturated fatty acids

(MUFAs), oleic (C18:1n-9), palmitoleic (C16:1n-7), erucic (C22:1n-9), vaccenic (C18:1n-7) and gadoleic (C20:1n-9); and (iii) polyunsaturated fatty acids (PUFAs), docosahexaenoic (C22:6n-3), eicosapentaenoic (C20:5n-3), stearidonic (C18:4n-3), docosapentaenoic (C22:5n-3), linoleic (C18:2n-6) and linolenic (C18:3n-3).

The SFAs composition of the oils ranged from 259.0 g kg^{-1} for mackerel to 296.3 g kg^{-1} for sardine, being the palmitic acid the primary SFA for the three species and contributing to 164.7-197.8 g kg^{-1} of the total content of lipids. The MUFAs content was significantly different ($p<0.05$) for the three species and it varied from 231.7 g kg^{-1} for sardine to 365.3 g kg^{-1} for horse mackerel. As a result, the MUFAs composition of the oils was found to be higher than the SFAs for mackerel and horse mackerel oils but not for sardine. Oleic acid was the most represented of the MUFAs for the three species, accounting for 290-400 g kg^{-1} of the total MUFA. Similarly, significant differences in the PUFAs composition were found among the three species, ranging from 330.6 g kg^{-1} for horse mackerel to 439.7 g kg^{-1} for sardine. The PUFAs were the predominant in the oils except for horse mackerel where MUFA were the major lipids. These results are in line with previous studies on the fatty acids profile of the same species.[29,30]

The ratio of n-3/n-6 ranged from 135.5 for mackerel to 140.8 for sardine. This fact strongly supports that the three discarded fish species studied are important sources of Omega-3 PUFAs. Especially relevant is the content of EPA and DHA, which together account for 306.0 g kg^{-1} of the total fatty acids for sardine, followed by mackerel (279.2 g kg^{-1}) and horse mackerel (220.5 g kg^{-1}). Among the three species, sardine presented the highest content of both EPA (129.6 g kg^{-1}) and DHA (176.3 g kg^{-1}).

Table 3 shows the composition of the lipid fraction for each species. It is observed that the three oils were mostly composed by triacylglycerols (TAG), ranging from 973 g kg^{-1}

for mackerel to 998 g kg^{-1} for sardine. On the other hand, the free fatty acids content (FFAs) was not significant for the three species when compared to the triacylglycerols content, varying from 2 g kg^{-1} for sardine to 27 g kg^{-1} for mackerel.

As a consequence of these results, the oils obtained from the three discarded fish species exhibited a high quality due to the fact that they presented most of the Omega-3 PUFAs as TAGs form which are more resistant to oxidation[31] and which are also more bioavailable for the human digestive system.[32]

Protein fraction

Hydrolysis curves

In figure 1, the hydrolysis curves are shown for the three fish species studied. All the curves presented a high rate of hydrolysis after the addition of the corresponding enzyme. This rate decreased throughout the reaction until reaching a steady-state phase when no apparent hydrolysis took place.

The DH reached after 4 hours of reaction was different depending on what species and treatment was used. In general, the lowest values of DH were obtained for mackerel, while horse mackerel protein presented the best degradability to the enzymes used. Regarding the enzymatic treatments, the sequential enzymatic treatment subtilisin plus trypsin led to the highest degree of hydrolysis (DH) for sardine (18%), as shown in Figure 1a. In the case of mackerel, Figure 1b, both the two-step reaction subtilisin plus trypsin and the simultaneous addition of both enzymes led to the maximum DH (14%), while the maximal extent of the hydrolysis (21%) was obtained for horse mackerel after the simultaneous addition of subtilisin plus trypsin (Fig. 1c). In the three cases, the increase of DH after the addition of trypsin was less marked than that obtained when subtilisin was added in the second step. This fact was attributed to the specificity of

trypsin, which only cleaves peptidic bonds involving Arginine and Lysine, while subtilisin is an endoprotease of broad spectrum.[24]

Antihypertensive activity

For all the combinations between species and enzymatic treatments the ACE-inhibitory activity increased sharply within the first 20 – 30 min of hydrolysis, attaining a maximum value of ACE inhibition at DH between 5 – 10%. This period corresponds to the higher reaction rates observed in the hydrolysis curves. Above this point, ACE inhibition values remained constant or presented a slight decreasing trend. As an example, Fig. 2a shows the ACE inhibition of sardine hydrolysates versus the time of hydrolysis for each enzymatic treatment, and Fig. 2b depicts the ACE-inhibitory activity of the hydrolysates produced with the simultaneous addition of subtilisin plus trypsin for the three species studied.

A similar relationship between DH and ACE inhibitory activity was reported in previous works.[9,33] This phenomenon may be explained due to the fact that the hydrolysate was constituted by a mixture of proteins and peptides where antihypertensive peptides would be continuously destroyed by proteolytic action while new active sequences might be released to the medium. Moreover, it was observed that the addition of the second enzyme had not significant effect in the ACE-inhibitory activity of the hydrolysate. Therefore, it was concluded that the degree of hydrolysis had no significant effect on the antihypertensive potency of the hydrolysates.

Although most of the active peptides identified to date are di- and tripeptides, active peptides larger than 20 amino acid residues have been reported.[34] The ACE inhibition potency of a given peptide is thought to be linked to its structure and amino acid sequence, specially the tripeptide sequence at the C-terminal end. Indeed, it has been

reported that the presence of hydrophobic amino acid residues in this region seems to favor the ACE inhibitory activity of the peptides, while hydrophilic peptides display weak or no affinity for the ACE active sites.[35,36]

Considering sardine hydrolysates, the enzymatic treatments assayed which produced the highest ACE-inhibitory activity were the simultaneous addition of subtilisin plus trypsin and the sequential enzymatic reaction with subtilisin (2h) followed by trypsin (2h), Fig 2a. ACE inhibition values up to 70% were obtained when reaching DH values of 13% (Table 4). An estimation of the average peptide chain length (PCL) of the hydrolysates can be made from the percentage degree of hydrolysis (DH), according to the expression:[24]

$$PCL = 100/DH \qquad (4)$$

This means that the hydrolysate at DH 13% presented a peptide length distribution around an average value of 8 amino acid residues. Some of these peptides are responsible for the ACE inhibitory activity (70%). The IC_{50} values for these hydrolysates were 430 and 400 μg protein mL^{-1}, respectively (Table 4), which was of the same order of magnitude than that reported by Matsui et al.[37] for the crude hydrolysates (i.e. without further fractionation or purification) of sardine muscle, IC_{50} = 260 μg protein mL^{-1}. These authors employed an alkaline protease from *Bacillus licheniformis*, at pH 9, 50ºC and 17 hours of hydrolysis. This value is slightly better than that obtained in our work, expense of larger times of hydrolysis (17 h).

ACE inhibition values up to 65% were obtained for mackerel hydrolysates when employing the sequential treatment subtilisin plus trypsin and the simultaneous addition of subtilisin and trypsin (Table 4). Degree of hydrolysis around 14%, corresponding to

325 PCL of 7 amino acids, maximized the ACE-inhibition activity. IC_{50} values of 345 and
326 360 μg protein mL^{-1} were obtained for these hydrolysates (Table 4).

327 Regarding horse mackerel hydrolysates, Table 4, it was noticed that there is no
328 difference on the ACE-inhibitory activity when employing the sequential reaction
329 subtilisin plus trypsin or the simultaneous treatment with subtilisin and trypsin. ACE
330 inhibition values up to 66.5% were obtained for those enzymatic patterns when DH
331 values of 16% were reached, corresponding to PCL of 6 amino acids. IC_{50} values of 395
332 and 364 μg protein mL^{-1} were obtained for these hydrolysates, respectively.

333 To the authors' knowledge, there is no previous reference on the IC_{50} of crude
334 hydrolysates from mackerel and horse mackerel. It can be concluded that the enzymatic
335 treatment performed in this work favored the release of ACE inhibitory peptides.
336 Nevertheless, further fractionation and purification of the hydrolysates is required, in
337 order to obtain peptidic fractions with enhanced antihypertensive potency or even
338 identify the peptide or peptides responsible for the ACE inhibition.

339 **Antioxidative activity**

340 The antioxidative activity of fish protein hydrolysates could be related to the degree of
341 hydrolysis and the enzyme specificity. Besides, the amino acid composition and
342 sequence of the different peptides released during the hydrolysis reaction play a
343 significant role in the antioxidant capacity of the final hydrolysate.[38] In this sense, it is
344 believed that the presence of cationic and hydrophobic peptides enhance the capacity of
345 the hydrolysate to prevent lipid oxidation.[39]

346 In this work, it was generally observed that, for the different enzymatic patterns used to
347 hydrolyse the three cakes obtained, the DPPH inhibition increased with the degree of
348 hydrolysis, reaching the following maxima values after 90 minutes of reaction: 40% for

349 sardine, 35% for mackerel and 45% for horse mackerel. These values of DPPH
350 inhibition remained practically constant until completion of the reaction. For instance,
351 Fig. 3a depicts the DPPH inhibition of horse mackerel hydrolysates versus the time of
352 hydrolysis for each enzymatic treatment, and Fig. 3b shows the DPPH scavenging
353 activity of the hydrolysates produced with the simultaneous addition of subtilisin plus
354 trypsin for the three species evaluated. This result is in agreement with previous
355 works[40,41] which stated that the DPPH scavenging capacity of fish hydrolysates
356 improves as the degree of hydrolysis increases.

357 Regarding sardine hydrolysates (Table 5), it was observed that the two-step reaction
358 subtilisin plus trypsin at 90 minutes of reaction, and the simultaneous addition of both
359 enzymes at 150 minutes, produced the hydrolysates presenting the highest antioxidative
360 activity, being 40% inhibition of DPPH. It corresponded to a DH of 15% and a PCL of
361 7 amino acids with TEAC values of 0.033 and 0.030 μmol Trolox mg^{-1} protein,
362 respectively. On the contrary, hydrolysates presenting a lower DH and, as consequence,
363 a longer peptide chain length exhibited lower DPPH inhibition capacity (Fig. 3). These
364 results are in accordance with the study of Bougatef et al.[14] where sardinelle
365 hydrolysates presenting a DPPH radical-scavenging activity ranging from 15-55% were
366 obtained. In addition, these authors revealed that peptides presenting shorter chain
367 length exhibited higher DPPH inhibition, which it is in line with the results obtained in
368 this research work.

369 Considering the mackerel hydrolysates, the three enzymatic patterns assayed produced
370 considerable differences in the DPPH radical-scavenging activity. It is shown in Table 5
371 that the sequential addition of subtilisin plus trypsin was the most appropriate treatment,
372 obtaining hydrolysates with a PCL of 7 amino acids, which presented the highest DPPH

inhibition (35%). It corresponded to a TEAC value of 0.025 μmol Trolox mg^{-1} protein. In this line, Wu et al.[40] produced mackerel hydrolysates employing Protease N with a DPPH radical-scavenging activity up to 80% after 10 hours of hydrolysis. This fact suggests that, for mackerel, longer hydrolysis and different enzymatic treatments may result in hydrolysates presenting a higher antioxidative activity.

Regarding horse mackerel hydrolysates, it was observed that the different enzymatic treatments produced significant differences in the DPPH inhibition (Fig. 3a). It was revealed that the most suitable combination of enzymes was subtilisin and trypsin simultaneously, producing hydrolysates with a PCL of 5 amino acids, which exhibited a DPPH radical-scavenging activity up to 45%. This corresponds to a TEAC value of 0.030 μmol Trolox mg^{-1} protein.

The results commented above stated that the produced hydrolysates of sardine, mackerel and horse mackerel were significant DPPH radical scavengers. Nevertheless, further fractionation and characterization of the hydrolysates is required in order to obtain peptidic fractions with enhanced antioxidative activity.

CONCLUSIONS

The three discarded species considered, sardine, mackerel and horse mackerel, were found to have a potential as raw material for the production of bioactive compounds. The oils recovered from their lipid fraction exhibited a high content of Omega-3 PUFAs, presenting the sardine oil the highest content in EPA (129.6 g kg^{-1}) and DHA (176.3 g kg^{-1}). Moreover, the three oils were mostly composed by triacylglicerols (973 – 998 g kg^{-1}) which means higher quality in terms of oxidation stability and bio-availability. Regarding the protein fraction, most of the hydrolysates produced, exhibited ACE inhibition values higher than 60%, mackerel presented the lowest IC_{50}

397 value (345 μg protein mL^{-1}) and sardine the highest one (400 μg protein mL^{-1}). In
398 addition, most of the hydrolysates presented an acceptable antioxidative activity with
399 DPPH inhibition values ranging from 35 to 45%, when the highest TEAC value (0.033
400 μmol Trolox mg^{-1} protein) was obtained for sardine hydrolysates, employing the
401 enzymatic treatments subtilisin (2h) plus trypsin (2h).

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Tables

Table 1. Proximate composition (g kg^{-1}) of sardine, mackerel and horse mackerel

Composition	Sardine	Mackerel	Horse mackerel
Moisture	718	740	704
Protein	176	184	169
Lipid	44	25	78
Ash	38	28	40

Table 2. Fatty acid profile (g kg^{-1}) of sardine, mackerel and horse mackerel oils

Fatty acids	**Sardine**	**Mackerel**	**Horse mackerel**
14:0	58.8a	43.7b	67.9c
16:0	197.8a	175.3b	164.7c
18:0	39.7a	40.6b	31.5a
Σ SFA	296.3a	259.0b	264.1b
16:1n-7	64.2a	50.7b	53.6c
18:1n-9	86.9a	132.8b	106.4c
18:1n-7	29.0a	29.4b	14.6b
20:1n-9	27.1a	48.2b	72.5c
22:1n-9	24.5a	70.7b	118.1c
Σ MUFA	231.7a	331.0b	365.3c
16:2n-4	12.9a	11.4b	12.2ab
16:3n-4	5.9a	6.5b	5.8a
16:4n-1	5.1a	0.9b	6.7a
18:2n-6	17.2a	16.6a	12.7b
18:3n-3	18.2a	12.1b	11.6c
18:4n-3	34.2a	26.2b	27.6c
20:4n-6	10.4a	9.0b	8.0c
20:4n-3	10.4 a	9.7a	8.6b
20:5n-3 (EPA)	129.6a	103.4b	107.8c
22:5n-3	19.4a	18.9a	16.9b
22:6n-3 (DHA)	176.3a	169.7b	112.7c
Σ PUFA	439.7a	390.2b	330.6c
Others	32.3a	19.1a	40.0a
Σ n-6	27.6a	25.5ab	20.7b
Σ n-3	388.2a	345.8b	285.2c
n-3/n-6	140.8a	135.5a	137.9a
EPA + DHA	306.0a	279.2b	220.5c

Data are means of triplicate determinations. SD< 2 %. Mean values within a row followed by different letter mean significant differences ($p<0.05$).

Table 3. Composition of the lipid fractions (g kg^{-1}) of sardine, mackerel and horse mackerel

	Sardine	**Mackerel**	**Horse mackerel**
TAG	998	973	988
FFA	2	27	12

Table 4. ACE inhibitory activity for sardine, mackerel and horse mackerel hydrolysates

Species	Maximum ACE inhibition (%)	Enzymatic treatment	Reaction time (min)	DH (%)	PCL	IC_{50} ($\mu g\ mL^{-1}$)
Sardine	70.0	Subtilisin + Trypsin (simultaneous)	30	13.0	≈ 8	430
		Subtilisin + Trypsin	60			400
Mackerel	65.0	Subtilisin + Trypsin (simultaneous)	60	14.0	≈ 7	345
		Subtilisin + Trypsin	150			360
Horse mackerel	66.5	Subtilisin + Trypsin (simultaneous)	30	16.0	≈ 6	364
		Subtilisin + Trypsin	150			395

ACE Inhibition data are means of triplicate determinations.

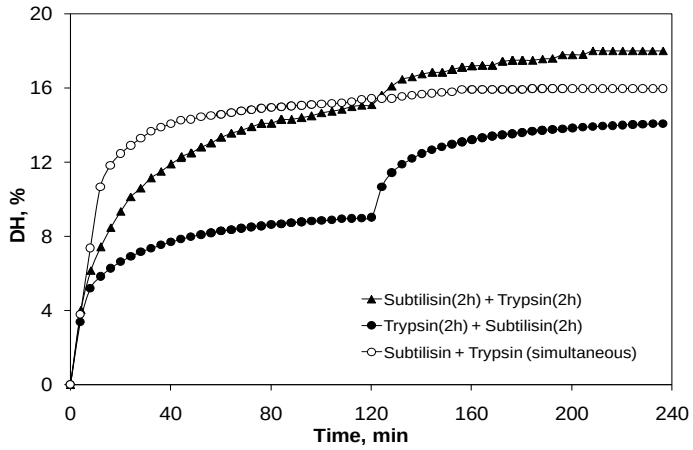
Table 5. Antioxidative activity for sardine, mackerel and horse mackerel hydrolysates.

Species	Maximum DPPH inhibition (%)	Enzymatic treatment	Reaction time (min)	DH (%)	PCL
Sardine	40	Subtilisin + Trypsin (simultaneous)	150	15.0	≈ 7
		Subtilisin + Trypsin	90		
Mackerel	35	Subtilisin + Trypsin	150	14.0	≈ 7
Horse mackerel	45	Subtilisin + Trypsin (simultaneous)	150	20.0	≈ 5

DPPH Inhibition data are means of duplicate determinations.

Figures

(a)



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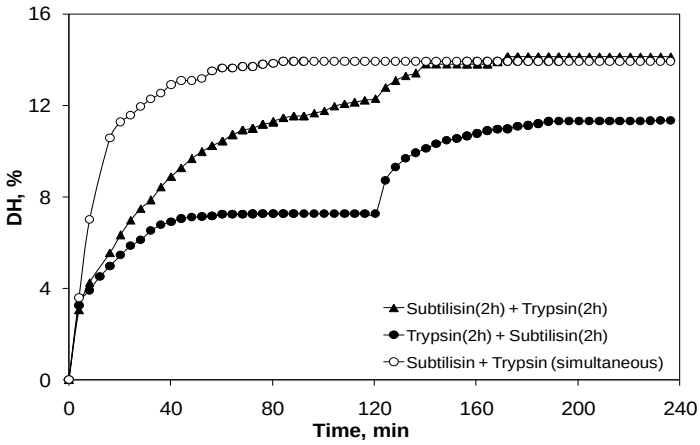

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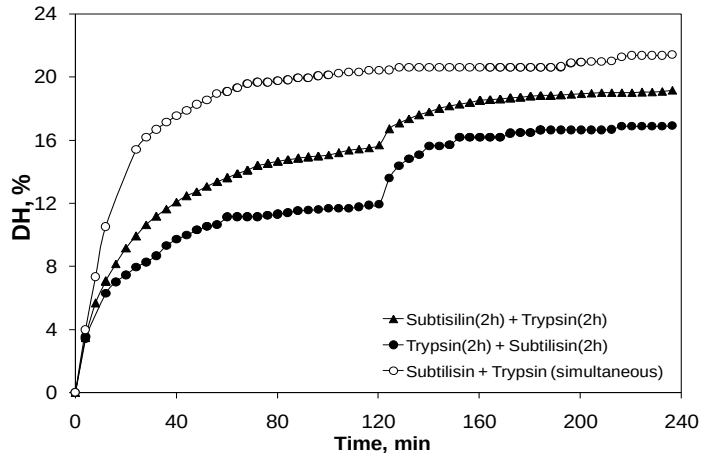
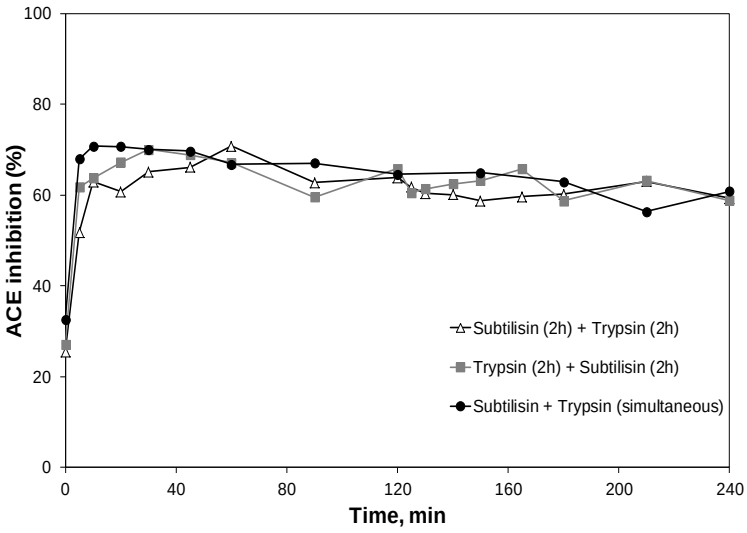


Fig. 1. Hydrolysis curves for sardine (a), mackerel (b) and horse mackerel protein (c).

(a)



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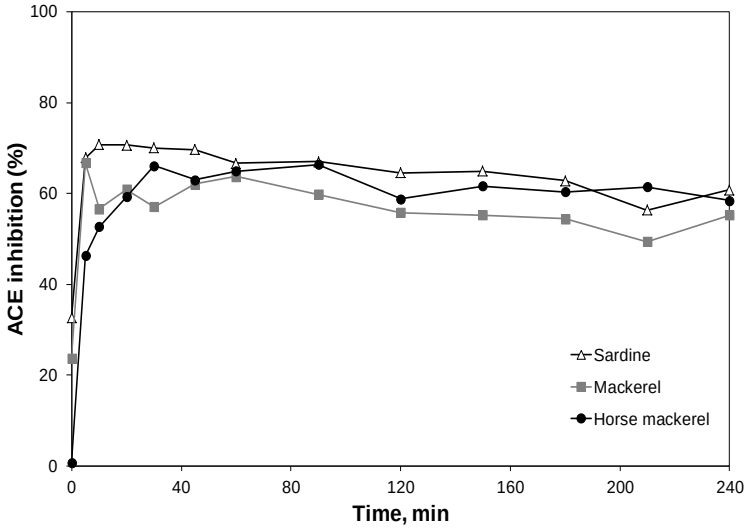
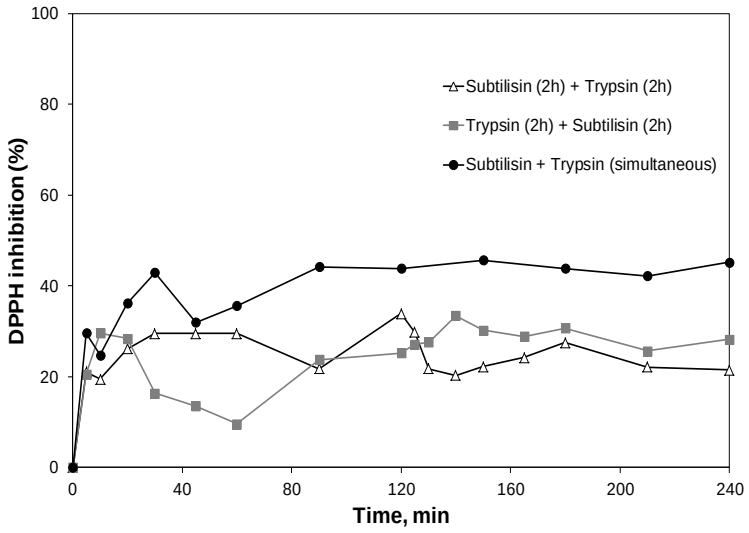


Fig. 2. ACE inhibition of sardine hydrolysates at different times of hydrolysis (a), ACE inhibition of the hydrolysates obtained with the simultaneous addition of subtilisin plus trypsin (b).

(a)



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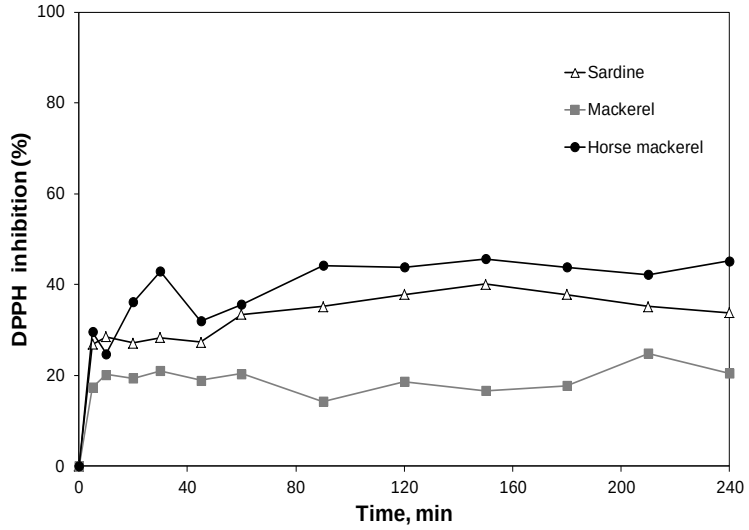

Fig. 3. DPPH inhibition of horse mackerel hydrolysates at different times of hydrolysis (a), DPPH inhibition of the hydrolysates obtained with the simultaneous addition of subtilisin plus trypsin (b).