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Hydrolysates of fish skin collagen: an opportunity for valorizing fish industry byproducts

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Abstract: During fish processing operations, such as skinning and filleting, the removal of collagen-containing materials could account up to 30% of the total fish byproducts. Collagen is the main structural protein in skin, representing up to 70% on a dry weigh depending on the species, age and season, having a wide range of applications including cosmetic, pharmaceutical, food industry and medical. In the present work, collagen was obtained by pepsin extraction of skin from two species of teleost and two species of chondrychtyes with yields varying between 14.16-61.17%. Storage conditions of skins appear to influence these collagen extractions yields. Pepsin soluble collagen (PSC) was enzymatically hydrolyzed and resultant hydrolysates were ultrafiltrated and characterized. Electrophoretic patterns showed the typical composition of type I collagen, with denaturation temperatures ranged between 23ºC and 33ºC. In terms of antioxidant capacity, results revealed significant intraspecific differences between hydrolysates, retentate and permeate fractions when using β-Carotene and DPPH methods and also showed interspecies differences between those fractions when using DPPH and ABTS methods. Under controlled conditions, PSC hydrolysates from *Prionace glauca*, *Scyliorhinus canicula*, *Xiphias gladius* and *Thunnus albacares* provide a valuable source of peptides with antioxidant capacities constituting a feasible way to efficiently upgrade fish skin biomass

Keywords: collagen; enzymatic hydrolysis; antioxidant activity; β-carotene; DPPH; ABTS.

1. Introduction

As the human population is growing and their consumption behavior changing, the worldwide demand for fishery products is also increasing and so is doing the demand for ready to cook meals in the form of loins or steaks. These kinds of processed products generate a large amount of by-products in the form of skin, bones, viscera, heads, scales, etc. Those organic materials are considered postharvest fish losses (by-products) and are a main concern for current fishery management policies because they represent a significant source of valuable compounds as proteins, fat, minerals, etc. Although part of these by-products are already being used, either for fish meal or oil production (35% of world fishmeal production was obtained from fish residues in 2012) [1], it is considered that this kind of utilization produces very little added-value and that, with present technological development, a more valuable and profitable use is possible [2].

Fishing activity in Galicia (North-West Spain) constitutes a key sector for the economy of the region, with a high concentration of small, medium and big businesses dedicated to fish processing activities rendering a wide variety of by-products susceptible of valorisation. During fish processing operations the removal of collagen-containing materials (mainly skin, bones and scales) could

account for as much as 30% of the total by-products generated after filleting (75% of the total catch weight) [2;3;4].

Although collagen is the main protein component of fish skin and its particular heterotrimeric structure [α_1(I)]$_2$ α_2(I)] has been previously described, there have been only a few publications describing the properties of fish skin collagen hydrolysates [5;6;7], and even less research has been conducted on the characterization of hydrolysates obtained from pepsin soluble collagen of marine origin [7]. As acid solubilisation of collagen has been shown to render low yields, enzymatic proteolysis has been studied as an alternative to enhance the yield and at the same time obtaining hydrolysates with good nutritional composition, increased solubility and better emulsifying, foaming and gelating properties as well as with biologically active peptides [8;9;10]

Two sharks, blue shark (*Prionace glauca;* PGLA) and small-spotted catshark (*Scyliorhinus canicula;* SCAN), and two bonny fishes, yellowfin tuna (*Thunnus albacares;* TALB) and swordfish (*Xiphias gladius;* XGLA) were selected since a significant amount of those are industrially processed generating significant amounts of skin [11, 12, 13]. The objective of this study was to evaluate the potential use of skins which are obtained as a by-product of the fish processing industry to obtain fish skin collagen hydrolysates and to test the influence of some biochemical properties, as the amino acid content or molecular weight, on antioxidant capacity of hydrolysates. This is the first time, as far as we know that the extraction, characterization and comparison of collagen hydrolysates from these species, is described.

2. Results and Discussion

Fish skin can be an important by-product for some fishery industries, for example some companies produce piece of skinned and deboned fish which render important amounts of skins and bones as by-products. One of the problems associated with these by-products is the heterogeneity of them: they are originated from different species, previous frozen storage conditions can be different (frozen storage in brine), they can be mixed with bones or other by-products…etc. Appropriate management of these by-products should take into account these problems, and one important and initial step is to estimate the value associated with each type of product. Therefore, the initial chemical characterization and the estimations of collagen content are important data to evaluate the potential value of these by-products. Low yield of collagen extraction can be expected in industrial conditions because of the previous treatment and storage history of the raw materials. Hydrolysis would help to overcome some of the problems associated to this previous treatments, increasing the yield of a valuable product, collagen hydrolysates, with many interesting properties, such as antioxidant activity [14;15].

2.1. Chemical composition of skin by-products

2.1.1. Proximate composition

Table 1. Chemical composition of fish skins from the four species used for the study. Values, expressed in a wet basis, are means of 3 determinations ± standard deviation (Protein=N x 5.4).

Species	Composition (%)			
	Moisture	Protein	Lipids	Ash
PGLA	76.03 ± 0.83	20.14 ± 0.97	0.24 ± 0.03	4.24 ± 0.24
SCAN	61.5 ± 0.79	22.09 ± 0.96	0.36 ± 0.01	14.01 ± 0.5
XGLA	42.87 ± 0.54	16.28 ± 2.21	30.53 ± 1.99	2.49 ± 0.21
TALB	62.57 ± 2.4	26.96 ± 2.04	3.22 ± 0.72	0.67 ± 0.14

Table 1 shows the chemical composition of the skins of the four analysed species, these were similar to other fish species skins. Skin of the two elasmobranch contained similar amounts of protein, while swordfish skin presented the lowest protein content of all species while those from tuna were the highest. In the case of swordfish, it is remarkable the highest lipid content (30.53%) which may

also be the target of valorisation for this type of by-product. The higher ash content in the skin of small-spotted catshark is remarkable and it could be attributed to its particular skin structure; a thinner skin with a higher proportion of scales compared to the skin of blue shark. The skin of blue shark is thicker and present two different layers with scales being present only in the upper one.

2.1.2. Hydroxyproline (HPro) content

Hydroxyproline has been used as a method to quantify the amount of collagen in a particular tissue [16]. This analytical approach was used to estimate the collagen content in the skin of all the species analysed, assuming that all HPro content of skin is due to collagen and taking into account that the ratio of HPro in collagen is 12.5 g of HPro/100 g of collagen [17]. Table 2 shows that the collagen content was higher in the skin of TALB, followed by the two species of elasmobranch which showed similar values (SCAN and PGLA), and finally the lowest value corresponded to the skin of XGLA, these results are in coherent with the protein content found in the skin of these species (table 1). Collagen content reported previously for other fish species was similar with slight variations depending on the species [18].

Sotelo et al. [19] have reported a low collagen content in the skin of SCAN (11.6% in a wet basis), which may be explained by differences in the previous treatment of skins for this species (used fresh in this study).

Table 2. Hydroxyproline content in skin (g OHPro/100 g skin), collagen content calculated from the hydroxiproline values, and yield of PSC_1 (g collagen/100 g skin), and PSC_2 (g collagen/100 g collagen of the skin). The average values (± SD) expressed in a wet weight basis are means of three replicates.

	Hydroxyproline content in skin (%)	Collagen content (%)	PSC_1 yield (%)	PSC_2 yield (%)
PGLA	1.23 ± 0.11	9.84 ± 0.88	5.87 ± 0.49	61.17 ± 5.15
SCAN	1.85 ± 0.14	14.8 ± 1.14	4.89 ± 0.85	33.00 ± 5.25
XGLA	1.08 ± 0.16	8.64 ± 1.28	2.59 ± 0.22	31.33 ± 5.55
TALB	2.69 ± 0.26	21.53 ± 2.09	2.97 ± 0.98	14.16 ± 6.14

2.2. Extraction of collagen

2.2.1. Yield of PSC

Previous reports have shown that pepsin enhances the extraction efficiency in collagen because it is able to cleave specifically telopeptide regions of collagen [20, 21]. Besides, by hydrolysing the non-triple helice domain, non-collagen proteins are more easily removed, and thus collagen becomes readily to be solubilized in acid solution and the antigenicity caused by telopeptides is reduced, obtaining a collagen with higher purity with the possibility of using it in different applications [22;23;24].

Table 2 shows PSC yields obtained for PGLA, SCAN, TALB and XGLA. Extraction yields obtained for PGLA and SCAN were similar to other PSC extracted from different fish species, such as bigeye snapper skin [25], brownstripe red snapper skin [26] or largefin longbarbel catfish [27]. However, the yields obtained for TALB and XGLA are lower than those values. While TALB showed the highest collagen content values (determined by means of hydroxyproline analysis in skin), it also showed (together with XGLA skins) lower extraction yields (PSC_1 and PSC_2). These results could be attributed to several factors such as differences in the structure of the collagen fibers or the storage conditions; processing of tuna usually involves freezing and frozen storage, most of the times in brine. This treatment may cause protein denaturation, higher degree of crosslinking and therefore lower collagen solubility and extraction yield [27;28;29;30].

125 2.2.2. Characterization of PSC

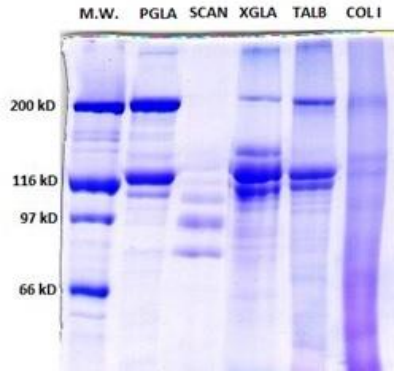
126 Polyacrylamide gel electrophoresis (SDS-PAGE).

127 Figure 1 shows the PSC electrophoretic patterns of the analysed species. PSC SDS-PAGE pattern
128 from PGLA and TALB were more similar to the type I collagen pattern were two identical α_1-chains
129 (120 kDa), one α_2-chain (110 kDa) and one β dimer band of about 200 kDa can be observed [16;31].
130 The molecular weight data obtained for α and β chains of PSC from TALB are similar to those
131 previously published for the same species [23;32]. The cross-linking rate of collagen has been reported
132 to be low; which might explain why highly cross-linked components (γ-component) in PGLA, TALB
133 and XGLA are shown only as a faint bands in figure 1 [33;34]. This result indicates that pepsin was
134 able to hydrolyse the cross-links in the telopeptide region without damaging the integrity of the
135 triple-helix.

136 PSC from SCAN was characterised by a high susceptibility to pepsin hydrolysis, as revealed that
137 neither dimer nor trimer could be observed in SDS-PAGE, and also by the presence of several weak
138 α subunits nor lower than 110 kDa bands, which could be products of enzymatic hydrolysis of
139 collagen components (figure 1). In fact, previous publications have shown that β and γ-components
140 were present in acid soluble collagen from SCAN skin [19].

141 In the electrophoretic pattern of XGLA, one intermediate band was observed between β and α
142 component with an approximate molecular weight of about 150 kDa. The presence of similar
143 components have also been reported for PSC from different species, suggesting either an incomplete
144 hydrolysis of β dimers, or the presence of a mixture of different collagens [35;36].

145 **Figure 1.** 7% SDS-PAGE showing PSC from PGLA, SCAN, TALB and XGLA. M.W: Molecular Weight
146 Standards. Col I: standard collagen type I from mammal.



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148 Amino acid content.

149 Table 3 shows the amino acid composition of PSC of the four studied species and also that from
150 calf skin (data obtained from Zhang et al., [21]). To our knowledge, amino acid composition has never
151 previously been reported for PSC collagen of these species except for TALB [32]. Although Glycine
152 was the most abundant amino acid in all the species studied, yet did not represent one third of total
153 amino acid residues as expected [19;20]. Similar results have been previously reported in PSC
154 obtained from yellowfin tuna skin [32] and squid skin collagen [7]. This result might be explained
155 due to the presence of telopeptide fractions in which the repetitive occurrence of glycine every three
156 amino acid is absent [30].

157 The lower imino acid content found in SCAN PSC, contributes to the low stability of the triple
158 helix structure [35], a result which is in agreement with the SDS profiles showed above, indicating
159 the higher susceptibility of this species to the action of pepsin.

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Table 3. Amino acid composition of PSC of PGLA, SCAN, TALB and XGLA(residues/1000). Data from calf skin collagen is also included [21]. Imino acids includes proline and hydroxyproline.

	PSC				*CALF
Amino acid	PGLA	SCAN	TALB	XGLA	
Hydroxyproline	84.62 ±0.98	88.28 ±0.62	87.38 ±0.60	76.55 ±0.87	94
Aspartic acid	46.58 ±0.42	52.16 ±0.43	55.40 ±0.54	61.32 ±0.46	45
Serine	35.98 ±0.42	54.02 ±0.14	35.53 ±0.25	39.89 ±0.74	33
Gultamic acid	92.02 ±1.00	92.10 ±0.47	97.89 ±0.43	94.64 ±0.96	75
Glycine	214.80 ±2.92	234.69 ±1.36	217.22 ±1.32	210.20 ±3.22	330
Histidine	15.80 ±0.20	17.35 ±0.10	12.70 ±0.05	15.67 ±0.34	5
Arginine	111.50 ±1.09	91.26 ±1.08	92.16 ±2.97	89.54 ±2.26	50
Threonine	33.59 ±0.16	33.41 ±0.44	40.00 ±1.81	42.89 ±1.60	18
Alanine	108.57 ±0.87	89.79 ±0.97	111.78 ±2.58	105.20 ±2.39	119
Proline	107.68 ±0.76	95.22 ±0.29	114.86 ±0.45	121.89 ±1.30	121
Cystine	0.88 ±0.01	0.31 ±0.00	0.07 ±0.00	0.61 ±0.01	0
Tyrosine	3.39 ±0.05	1.36 ±0.00	4.42 ±0.07	6.45 ±0.15	3
Valine	27.77 ±0.39	34.13 ±0.12	25.64 ±.015	26.95 ±0.40	21
Methionine	13.51 ±0.33	14.06 ±0.20	6.29 ±0.13	3.53 ±0.15	6
Lysine	33.48 ±0.36	37.78 ±0.13	35.37 ±0.23	31.52 ±0.43	26
Isoleucine	24.62 ±0.30	18.29 ±0.02	14.26 ±0.15	20.47 ±0.38	11
Leucine	25.97 ±0.36	27.30 ±0.07	28.28 ±0.21	31.19 ±0.68	23
Phenylalanine	19.25 ±0.22	18.49 ±0.01	20.75 ±0.15	21.50 ±0.47	3
Iminoacids	192.3	183.5	202.24	198.44	215
% hydroxylation of proline	44.00	48.10	43.20	38.57	44

1.

Determination of denaturation temperature.

DSC analyses of lyophilized PSC were performed. Calf skin type I collagen was used for comparison purposes. Denaturation temperatures for PGLA, SCAN, TALB and XGLA PSCs were 33ºC, 23.6 ºC, 30.6ºC and 31.4ºC respectively, which are similar to those found in literature for other PSC in different marine organisms: paper nautilus [37], striped catfish [38], bighead carp [35] or blueshark [38]. Denaturation temperatures of PSC were in all species lower than that of collagen type I of calf skin (T_d=40ºC). Among the four species studied, the lower denaturation temperature was found in SCAN PSC. These results agree with the lower iminoacid content (hydroxyproline and proline) found in the collagen obtained from this species. Thermal stability of collagen is related to the restriction of the secondary structure imposed by the pyrrolidine rings of proline and hydroxyproline, contributing to the strength of the triple helix [20;40]. Sotelo et al. [19] have found higher denaturation temperature for ASC obtained from small-spotted catshark skin, suggesting the influence of pepsin cross-link cleavage on lower thermal stability found in PSC. Similar results were obtained for ASC and PSC from the skin of brownbanded bamboo shark [32].

2.3. Enzymatic Hydrolysis of PSC

2.3.1. Degree of hydrolysis

Hydrolysis curves were similar to others previously reported for different marine skin proteins [41;42]. The hydrolysis degree (DH) (average values ± SD) calculated using the pH-STAT method were 16.52 ± 3.74, 15.80 ± 0.99, 11.49 ± 1.5 and 12.56 ± 1.79 % for PGLA, SCAN, TALB and XGLA,

respectively. Enzymatic proteolysis and the resulting degree of hydrolysis are key parameters influencing peptide length and other related characteristics such as solubility and nutritional, functional or sensory properties [7;9].

2.3.2. Antioxidant activities in hydrolysates

Table 4 shows data of antioxidant analysis in collagen unfractionated hydrolysates (H) and 3kDa ultrafiltration fractions: retentates (R) and permeates (P). The antioxidant capacities were evaluated using 3 methods, including two based on free radical scavenging capacity, that is, DPPH and ABTS, and one based on the inhibition of lipid peroxidation, determined by the β-carotene assay. The highest antioxidant capacity measured by DPPH and ABTS were obtained with XGLA hydrolysate, while the highest value showed by the β-carotene assay was obtained in SCAN hydrolysate. Regarding ultrafiltrated fractions (retentates and permeates) the highest values determined with DPPH and ABTS were found in SCAN and XGLA respectively. PGLA showed the lowest value of antioxidant activity in H, R and P determined both with DPPH and ABTS assays. This result might be in relation to the highest hydrolysis degree found for this species (16.52%). The hydrolysis degree dependence of radical-scavenging activity has been also reported for other fish protein hydrolysates [14;15]. Regarding β-carotene assay, it showed yet again differences compared to DPPH and ABTS methods as the highest values found in ultrafiltrated fractions where observed in PGLA followed by TALB, SCAN and XGLA. The precise mechanism explaining the antioxidant activity of peptides has not been entirely elucidated, however several authors suggested the influence of hydrolysis degree and also the presence of some amino acids such as cysteine in the hydrolysate as potential factors [14;43;44], which may interact with free radicals by their SH group [45]. The higher values found in XGLA hydrolysate for DPPH and ABTS analysis together with the higher values found in XGLA ultrafiltrated fractions for ABTS analysis might be in relation to the higher content of cysteine in XGLA hydrolysates (56.03/1000 residues)(table 5). Contrary, PGLA showed the lowest cysteine content (8.96 residues/1000 residues) which might be the reason for the lowest antioxidant activity found in this species both in hydrolysate and ultrafiltrated fractions regarding with DPPH and ABTS analysis.

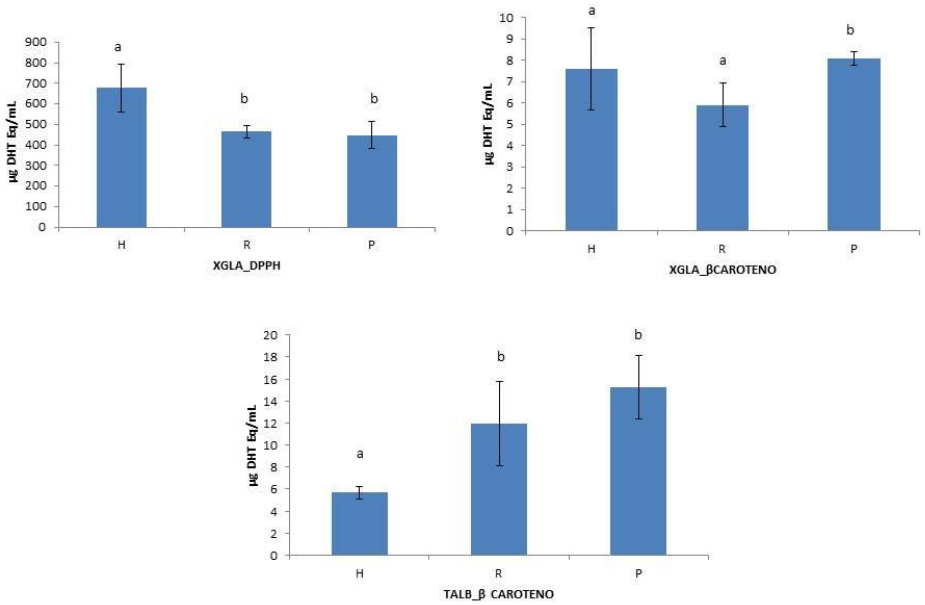
Table 4. Antioxidant activities (Mean ± SD) of collagen unfractionated hydrolysates (H), retentates (R) and permeates (P) quantified by means of three methods (DPPH, ABTS and β-carotene) and calculated as equivalents (in μg) of BHT per mL of hydrolysate.

Species	Fraction	DPPH (mg BHT Eq/mL)	ABTS (mg BHT q/mL)	β-carotene (mg BHT Eq/mL)
XGLA	H	677,2 ±114.42	253,8 ±1.85	7,6 ±1.93
TALB	H	578,9 ±57.81	199,6 ±37.54	5,7 ±0.61
SCAN	H	494,2 ±210.3	159,2 ±30.78	20,9 ±3.53
PGLA	H	405,3 ±9.89	151,2 ±43.49	15,3 ±5.02
XGLA	R	465,6 ±30.47	247,3 ±10.70	5,9 ±1.04
TALB	R	436,0 ±85.54	174,1 ±70.05	11,9 ±3.86
SCAN	R	603,4 ±30.88	143,6 ±29.80	7,4 ±11.69
PGLA	R	423,0 ±41.32	124,9 ±35.76	19,2 ±1.92
XGLA	P	448,0 ±66.45	264,9 ±18.86	8,1 ±0.33
TALB	P	457,7 ±95.61	192,8 ±56.66	15,3 ±2.91
SCAN	P	601,7 ±175.33	209,7 ±53.71	12,4 ±9.14
PGLA	P	416,0 ±18.88	134,9 ±26.76	17,0 ±2.64

On the other hand, the β-carotene method showed highest antioxidant capacity with those hydrolysates with the highest DH (SCAN and PGLA), while those with the lowest DH showed also the lowest antioxidant capacity.

Several studies have shown the positive influence of molecular size reduction of peptides on the functional properties of collagen hydrolysates [10;14;46]. The antioxidant capacity of retentates (R) and permeates (P) were statistically analyzed to test the influence of peptide molecular weight. One-way ANOVA analysis of data revealed some significant intraspecific differences between H, R and P when using β-Carotene and DPPH methods (figure 2) and also showed interspecies differences between H, R and P when using DPPH and ABTS methods (figure 3). The unfractionated hydrolysate (H) of XGLA showed significant higher value ($p \leq 0.05$) of antioxidant activity determined with DPPH compared to retentate or permeate fractions (figure 2). However, when the antioxidant activity was determined with β-Carotene method, XGLA showed a higher value in permeates than in unfractionated hydrolysate or retentates. Significant differences were also observed in TALB, when data from β-Carotene method were analyzed, between unfractionated hydrolysate and the other two fractions (R and P). Interspecies significant differences of hydrolysates, retentates and permeates are presented in figure 3 ($p \leq 0.05$). Figure 3A shows the differences found for unfractionated hydrolysates with ABTS; XGLA showed the highest antioxidant activity whereas SCAN and PGLA were the lowest. However, unfractionated hydrolysates did not show significant differences between species when the antioxidant activity was determined with DPPH or β-Carotene method (data not shown). In figure 3B it can be also observed that retentate fraction of SCAN presented the highest activity compared to other three species when DPPH was used, while ABTS data (figure 3C) showed significant differences in retentate fractions only between XGLA and PGLA (lowest). Regarding permeate fractions (figure 3D) significant differences were observed only between XGLA and PGLA when ABTS data were analyzed.

Figure 2. Intraspecific differences between Hydrolysate (H), retentate (R) and permeate (P) fractions for different antioxidant methods (ABTS, DPPH). No significant differences were observed between those fractions in the other two antioxidant methods analyzed. Different letters indicate significant differences among means ($p \leq 0.05$).



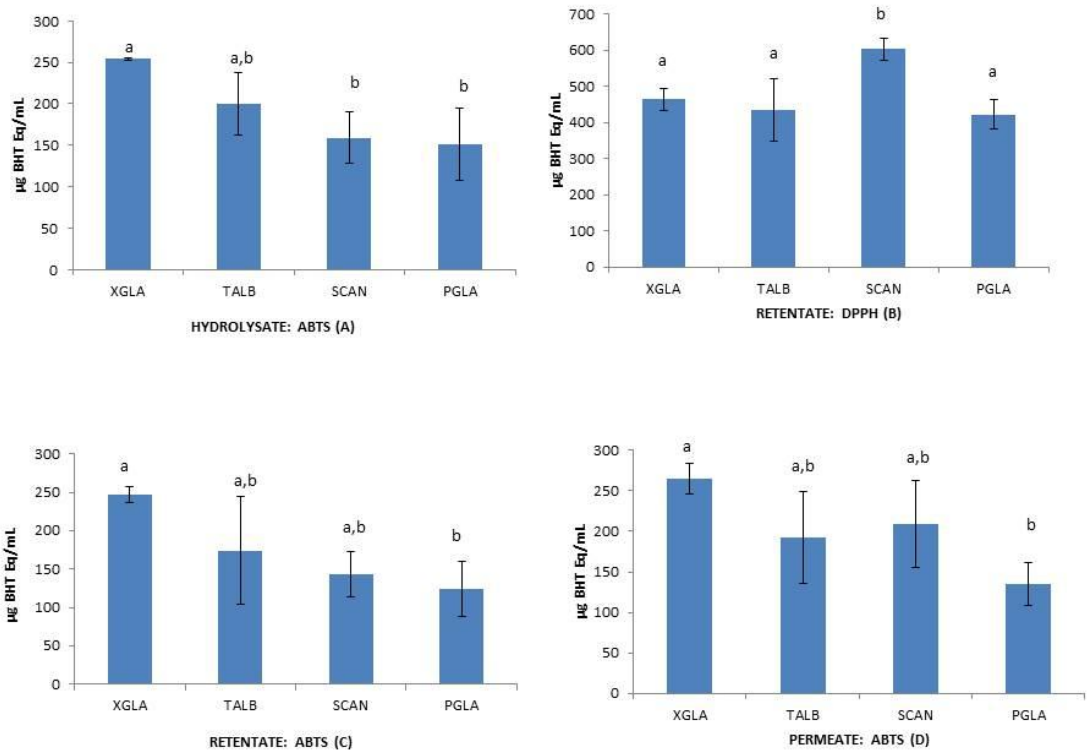


Figure 3. Interspecies differences in Hydrolysate (H), retentate (R) and permeate (P) fractions for XGLA and TALB using different antioxidant methods (DPPH, β-Caroteno). Different letters indicate significant differences among means ($p \leq 0.05$).

Besides, significant differences ($p \leq 0.05$) were also observed with unfractionated hydrolysates antioxidant capacity of teleost (XGLA and TALB) and chondrychtyes (PGLA and SCAN) with the β-carotene assay. Thus, the two teleost species XGLA and TALB showed lower antioxidant capacity than chondrychtyes, result that might be in relation with the higher content of hydrophilic amino acids (Asp, Ser, Gly, His, Arg, Thr and Cys) in chondrychtyes hydrolysates compared to teleost (table 5).These results agree with other studies suggesting differences on the antioxidant defense system between elasmobranchs and teleosts, due to different evolutionary rates and also due to different physical activity, nutrient intake and environment in which each species develops [47].

In summary, antioxidant capacity results suggest that there is not a unique factor responsible for this antioxidant capacity of hydrolysates, which seems to be influenced by the species which is being studied, the type and length of the peptides present in the sample and the methodology employed to determine the antioxidant activity.

2.3.3. Amino acid content

Table 5 shows the amino acid content of unfractionated collagen hydrolysates. Besides the influence of amino acid composition and other factors on antioxidant activity (discussed above), it is also of importance to highlight the increase in Cystine content in hydrolysates, in comparison to non-hydrolyzed collagen (PSC). These variations might be explained because the alkaline pH achieved during hydrolysis promotes reoxidation of cysteine residues to generate the original disulfide bond [48]. The higher Cystine content found in TALB and XGLA hydrolysates is therefore related to the low collagen yield obtained for those skins (section 2.2.1). As it was previously reported, the positive correlation between high disulfide bond content and low extraction yields is because of a higher stabilization of supramolecular assemblies [49]. The higher content of methionine in SCAN hydrolysates compared to the other species is also noteworthy.

Table 5. Amino acid composition of collagen hydrolysates of four species (residues/1000). Imino acids includes proline and hydroxyproline.

Amino acid	HYDROLYSATES			
	PGLA	SCAN	TALB	XGLA
Hydroxyproline	84.65 ±0.80	87.50 ±1.22	86.97 ±0.54	75.15 ±0.36
Aspartic acid	48.56 ±0.45	53.33 ±0.77	53.08 ±0.24	59.39 ±0.34
Serine	36.39 ±0.34	52.45 ±0.65	34.81 ±0.20	38.83 ±0.19
Gultamic acid	92.49 ±0.89	90.97 ±1.27	90.69 ±0.42	92.02 ±0.43
Glycine	230.71 ±2.10	227.17 ±2.96	215.82 ±0.66	211.01 ±1.06
Histidine	16.53 ±0.13	16.49 ±0.18	11. 18 ±0.12	14.91 ±0.03
Arginine	93.64 ±0.98	93.00 ±1.08	90.92 ±0.65	76.46 ±0.16
Threonine	27.99 ±0.32	36.62 ±0.59	40.00 ±.035	39.00 ±0.26
Alanine	105.81 ±1.11	93.50 ±1.27	108.72 ±0.74	97.97 ±0.62
Proline	106.47 ±1.14	89.31 ±1.26	100.22 ±0.77	99.87 ±0.61
Cystine	8.93 ±0.16	8.29 ±0.33	31.91 ±0.33	53.03 ±0.16
Tyrosine	2.17 ±0.01	1.68 ±0.02	1.84 ±0.02	2.24 ±0.00
Valine	27.84 ±0.28	34.12 ±0.42	26.17 ±0.17	27.61 ±0.12
Methionine	13.68 ±0.15	17.06 ±0.26	15.19 ±0.24	12.39 ±0.09
Lysine	34.16 ±0.32	37.55 ±0.48	33.88 ±0.14	32.70 ±0.17
Isoleucine	24.65 ±0.26	17.45 ±0.20	13.05 ±0.10	19.15 ±0.09
Leucine	26.11 ±0.25	25.95 ±0.27	26.20 ±0.13	28.58 ±0.07
Phenylalanine	19.23 ±0.19	17.56 ±0.17	19.34 ±0.10	19.67 ±0.04
Iminoacids	191.12	176.81	187.19	175.02
% hydroxylation of prol	44.29	49.48	46.45	42.93

3. Experimental Section

3.1 Raw material

Fresh skin of small-spotted catshark was obtained by local fishing fleet, while frozen skin of blue shark, swordfish and yellowfin tuna was provided by a Lumar S.L industry (Galicia, Spain) and stored at -20ºC until used. Fins, fat and muscle residues were removed from skins, then skin was cut into small pieces (0.5 x 0.5 cm^2) and mixed thoroughly. These skin pieces of each species were divided into three batches which were kept frozen at -20 ºC until collagen extraction.

Identification of fish species was performed by DNA analysis, following the methodology of Blanco et al.[50].

3.1.1. Proximate composition

Skin was analyzed for crude protein content by Kjeldhal method [51] in a DigiPREP HT digestor (SCP Science) and a TitroLine easy tritation unit (SCHOTT). Lipid content was determined by Bligh and Dyer [52]. Moisture was determined after heating the sample overnight at 105ºC and ash content was determined after heating the sample overnight at 600ºC. The conversion factor used for calculating the protein content from Kjeldahl nitrogen data was 5.4 as collagen, the main protein present in skin, contains approximately 18.7% nitrogen [53;54].

3.1.2. Hydroxyproline content

30 mg of dried grinded skin was introduced in hydrolysis microwaves tubes and 4 mL of 6 M HCl were added. Hydrolysis was performed in a microwave (Berghof speed wave MWS-2 microwave) at a 150ºC for 90 min at 70% power. Once the hydrolysis step finished, samples were allowed to cool down to room temperature and were made up to a known volume with 6 M HCl. 400 µL of this solution were transferred to glass vials and let dry in a vacuum desiccator at 60ºC in presence of solid NaOH, after drawing the air for 3 days. The resulting dry matter was suspended in 8 mL of buffer (0.13 M citric acid, 0.75% glacial acetic acid, 0.6 M sodium acetate, 0.15 M sodium hydroxide and 20.13 % n-propanol, pH was adjusted to 6.5 with 0.2 M NaOH and volume was brought to 660 mL with distilled water).

Hydroxyproline primary standard was prepared by dissolving 50 mg of hydroxyproline (Sigma) in 100 mL of buffer. From this primary standard a calibration curve of hydroxyproline, ranging from 0.5 µg/mL up to 10 µg/mL, was prepared. Chloramine-T reagent was freshly prepared just before using it (0.05 M Chloramine in distilled water). 3 ml of either samples or standards were placed in a tube and 1.5 ml of Chloramine-T reagent was added, the mixture was allowed to react for 25 min. Upon completion of that time, chromogenic reagent (15 g of p-dimethyl-amino-benzaldehyde, 60 mL of n-propanol, 26 mL of 70% perchloric acid were made up to a volume of 100 mL with distilled water) was added and tubes introduced in a water bath at 60ºC for 15 min. Samples were left to cool to room temperature and after, absorbance was read at 550 nm in a Beckman UV-VIS spectrophotometer (Beckman-Coulter, Brea, CA, USA).

3.2. Extraction of pepsin soluble collagen (PSC) from skin

Collagen from skin was extracted according to the methodology of Liu et al. [35] with minor modifications (figure 4). All procedures were performed at 4ºC. Skin pieces of blue shark and small-spotted-catshark were first treated with 0.1 N NaOH (1:15, w/v) and stirred for 24 h. Then, skins were washed with cold distilled water until neutral pH, and skin residues were extracted with 0.5 M acetic acid containing 0.1% (w/v) pepsin, at a sample solution ratio of 1:40 (w/v) for 24 h. Suspension was centrifuged at 6000 x g for 20 min, the residue discarded and the supernatant was salted-out by adding NaCl (final concentration of 2 M). The precipitate was dissolved in 0.5 M acetic acid and dialyzed against water using 12.000 Da cut-off membranes for 3 days. Aliquots were obtained and freeze-dried for analysis of Kjeldahl nitrogen, amino acid content, denaturation temperature and electrophoresis. The remaining liquid volume of dialyzed PSC was stored frozen at -20ºC until used for hydrolysis.

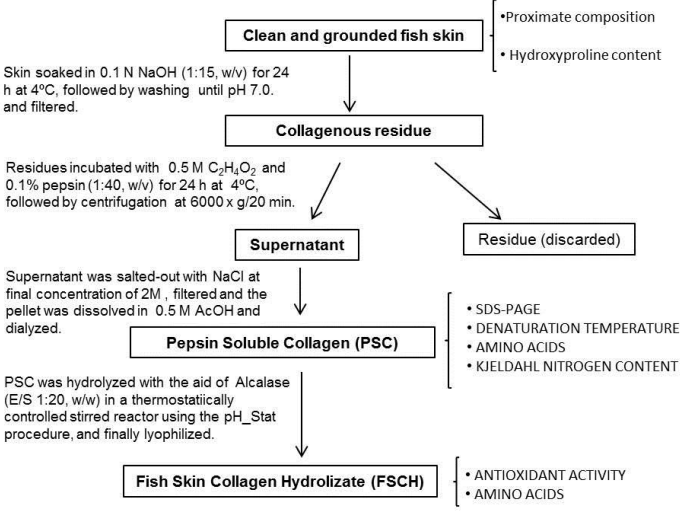


Figure 4. Scheme for the recovery of pepsin soluble collagen (PSC), preparation of the hydrolysate and analytical determinations.

The procedure used for swordfish and yellowfin tuna skin was slightly different than the one employed with sharks. Higher fat content in both swordfish and tuna skin required that after alkaline

treatment and before the acid pepsin extraction, samples were soaked in 10% butyl alcohol for 24 h to remove any remaining fat at a sample/ solid ratio of 1:10 (w/v), and then washed until neutral pH. Also de time for pepsin extraction of these skins was increased up to 3 days.

PSC yields were calculated using Kjeldahl nitrogen values (data not shown) in the collagen solution considering that collagen contains approximately 18.7% of nitrogen [53;54].

3.3. Characterization of Pepsin Soluble Collagen (PSC) from skin

3.3.1. Polyacrylamide Gel Electrophoresis

PSC samples for Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) were prepared according to methodology reported by Sotelo et al. [19]. Molecular weights of PSC subunits were estimated using high range molecular weight standards (BIO-RAD): Myosin (200 kDa); β-Galactosidase (116 kDa); phosphorylase B (97 kDa) and analyzing the gel with the software Quantity One (BIO-RAD).

3.3.2. Differential Scanning Calorimetry

Freeze-dried PSC samples were solubilized in 50 mM acetic acid (1 mg of freeze-dried sample/mL). Thermoestability of PSC solutions was measured in a DSC III microcalorimeter (Setaram, France) by differential scanning calorimetry (DSC). The samples were weighed accurately in a Mettler AE-240 balance, introduced into the calorimeter at 283.15° K and left for one hour to stabilize. Afterwards, temperature increase was set to 1° K/min up to 343° K. Denaturation temperature was calculated by difference with the apparent specific heat of ultrapure water.

3.3.3. Nitrogen content

PSC was analyzed in terms of nitrogen content by Kjeldahl method described in 3.2.1 considering a 5.4 factor to obtain the collagen content.

3.3.4. Amino acid composition

100 mg of lyophilized PSC samples were hydrolyzed using 6N hydrochloric acid under vacuum pressure at 110ºC for 24 hours. HPLC-fluorescence determination of amino acids, using AccQ-Tag Amino acid analysis column (Waters), was carried out after derivatization using the AccQ-Tag Chemistry kit (Waters- WAT052875).

3.4. Enzymatic hydrolysis of pepsin soluble collagen

Enzymatic hydrolysis was carried out according to the methodology of Liu et al. [35] with minor modifications. Prior to the hydrolysis process, the selected volume of each PSC collagen batch was thawed. Hydrolysates were prepared in a stirred and thermostated reactor connected to a pH electrode and a temperature probe, using the pH-Stat procedure, as described by Adler-Nissen [55]. Temperature and pH were recorded by a visual display at all time. Food-grade Alcalase provided by Novo Nordisk (Bagsvaerd, Denmark) was used for the hydrolysis. The 2 L of thawed PSC were introduced in the reactor and heated up to 55ºC (Alcalase optimum temperature), pH was adjusted to pH 8.0 with 1N NaOH and maintained constant during the hydrolysis reaction by automatically adding 1N NaOH. Hydrolysis started with the addition of enzyme (enzyme/protein ratio of 1:20 w/w). The hydrolysis reaction was allowed to continue for 3 h under constant stirring. At the end of hydrolysis, the enzyme was inactivated by heating at 90ºC for 5 min. The resulting hydrolysates were freeze-dried and kept frozen at -20ºC until characterization analysis.

3.4.1. Degree of hydrolysis

Degree of hydrolysis (DH) was obtained according to the following expression [55;56]where DH is the percent ratio between the total number of peptide bonds cleaved and the total number of peptide bonds in the initial protein.

$$DH(\%) = \frac{B \times N_b}{\alpha \times M_p \times h_{tot}} \quad (1)$$

where B is the volume (mL) of 1 M NaOH consumed during hydrolysis; N_b is the normality of NaOH; M_P is the mass (g) of initial protein (nitrogen x 5.4); ht_{ot} is the total number of peptide bonds available for proteolytic hydrolysis and α is the average degree of dissociation of the amino groups in the protein substrate and was calculated as follows:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \quad (2)$$

The pK value dependent on the temperature of hydrolysis was calculated according to the following expression, where T is the temperature (K):

$$pK = \left[7.8 + \frac{298-T}{298T}\right] \times 2400 \quad (3)$$

h_{tot} was calculated considering mean molecular weight of amino acids around 125 g/mol [57], and total content of amino acid in each PSC obtained from different species (PGLA: 78.4 g/100 g; SCAN: 96.02 g/100 g; TALB: 92.75 g/100 g; XGLA: 80.84 g/100 g). h_{tot} of PSC collagen were 6.8 meq/g protein, 8.3 meq/g protein, 8.06 meq/ g protein and 7.02 meq/ g protein for PGLA, SCAN, TALB and XGLA respectively.

3.5. Antioxidant capacity of pepsin soluble collagen hydrolysates

3.5.1. Ultrafiltration

To test the influence of molecular weight on antioxidant capacity, four grams of freeze-dried hydrolysates were dissolved in distilled water (1%) and ultrafiltrated in two steps using ultrafiltration centrifugal devices (Amicon Ultra-15 Unit, Merck Millipore) with molecular weight cut-off of 10 kDa and 3 kDa. After this process, fractions containing peptides with molecular weight between 10000-3000 Da (retentate fraction) and fractions containing peptides below 3000 Da (permeate fraction) were then freeze-dried and stored at -20ºC until subjected to antioxidant capacity analysis

3.5.2. Antioxidant activity determinations

β-carotene bleaching method

The β-carotene bleaching assay was performed according to Prieto et al. 2012 [58] with a microplate spectrophotometer. Reactions were performed combining in each well of a 96-well microplate, 25 μL of antioxidant (butyl hydroxytoluene (BHT) at 0–22.7 μM or hydrolysate samples) with 125 μL of β-carotene /linoleic emulsion. The microplate spectrophotometer (Multiskan Spectrum Microplate Spectrophotometer from Thermo Fisher Scientific) was programmed to record the absorbance at 470 nm and 45ºC every three minutes during a period of 200 min with agitation at 660 cycles/min (1 mm amplitude).

1,1-Diphenyl-2-picryhydrazyl (DPPH) radical-scavenging capacity

The antioxidant activity as radical-scavenging capacity was assessed with DPPH as a free radical, using an adaptation to microplate of the method described by Brand-Williams et al. [59] [60].

The decrease in the absorbance of hydrolysates and the BHT control (0-108 μM) was followed at 515 nm every 3 min during 200 min at 30ºC.

ABTS bleaching method

The ABTS (2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) radical scavenging activities were assessed according the protocol developed by Prieto et al. [60]. The absorbance at 414 nm and 30ºC (maintaining continuous agitation) of samples and BHT (0-9.1 μM) were measured each 3 min in the microplate reader.

In all methods the kinetics of reaction were performed in triplicate following the methodology of Amado et al. (2016) [61].

3.5.3. Amino acid composition

Hydrolysates were analyzed for amino acid content following the methodology described in 3.3.3 section.

3.5.4. Statistical analysis

Interspecific and intraspecific differences regarding antioxidant capacity between unfractionated hydrolysates (H) and 3 kDa MWCO ultrafiltrated fractions: permeates (P) and retentates (R) were tested by one-way analysis of variance (ANOVA). It was applied a Post hoc comparison test. Significance levels were set at $p \leq 0.05$. Statistical tests were performed with IBM SPSS 23 (IBM Corporation, Armonk, NY, USA).

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References

1. FAO. El estado mundial de la pesca y la acuicultura. Roma **2014**.
2. Wasswa, J.; Tang, J.; Gu, X. Utilization of fish processing by-products in the gelatin industry. *Food Rev Int* **2007a**, *23*, 159-174.
3. Gómez-Guillén, M.C.; Turnay, J.; Férnandez-Díaz, M.D.; Ulmo, N.; Lizarbe, M.A.; Montero, P. Structural and physical properties of gelatin extracted from different marine species: a comparative study. *Food Hydrocolloids* **2002**, *16*, 25-34.
4. Karayannakidis, P.D.; Chatziantoniou, S.E.; Zotos, A. Effects of selected process parameters on physical and sensorial properties of yellowfin tuna (*Thunnus albacares*) skin gelatin. *J Food Process Eng*, ***2014*** *37*(5), 461-473.
5. Wasswa, J.; Tang, J.; Gu, X.; Yuan, X.G. Influence of the extent of enzymatic hydrolysis on the functional properties of protein hydrolysate from grass carp (*Ctenopharyngodon idella*) skin. *Food Chem* **2007b**, *104*, 1698–1704.
6. Yongliang, Z.; Bafong, L.I; Xue, Z. The Scavenging of Free Radical and Oxygen Species Activities and Hydration Capacity of Collagen Hydrolysates from Walleye Pollock (*Theragra chalcogramma*) Skin. *J Ocean U China* **2009**, *8*(2), 171-176.
7. Nam, K.A;You, S.G.; Kim, S.M. Molecular and physical characteristics of squid (*Todarodes pacificus*) skin collagens and biological properties of their enzymatic hydrolysates. *J Food Sci* **2008**, *73*(4), 249-255.

8. Byun, H.G.; Kim, S.K. Purification and characterization of angiotensis I converting enzyme (ACE) inhibitory peptides from Alaska Pollack (*Theragra chalcogramma*) skin. *Process Biochem* **2001**, *36*, 1155-1162.
9. Chalamaiah, M.; Kumar, B.D.; Hemalatha, R.; Jyothirmayi, T. Fish protein hydrolysate: Proximate composition, amino acid composition, antioxidant activities and applications: A review. *Food Chem* **2012**, *135*, 3020-3038.
10. Jia, J.; Zhou, Y.; Lu, J.; Chen, A.; Li, Y.; Zheng, G. Enzymatic hydrolysis of Alaska Pollack (*Theragra chalcogramma*) skin and antioxidant activity of the resulting hydrolysate. *J Sci Food Agric*, **2010**, *90* (4), 635-640.
11. Vázquez, J.A.; Blanco, M.;Fraguas,J.;Pastrana,L.;Pérez-Martín,R.I. Optimisation of the extraction and purification of chondroitin sulphate from head by-products of *Prionace glauca* by environmental friendly process. *Food Chem*, **2016**, *198*, 28-35.
12. Autoridad Portuaria de Vigo. Memoria Anual 2015.
13. Blanco, M. Valorización de descartes y subproductos de pintarroja (*Scyliorhinus canicula*). Doctoral Thesis, Universidad de Vigo. December 2015.
14. Klompong, V.; Benjakul, S.; Kantachote, D.; Shahidi, F. Antioxidative activity and functional properties of protein hydrolyste of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chem* **2007**, *102*, 1317-1327.
15. Theodore, A.E.; Raghavan, S.; Kristinsson, H.G. Antioxidative activity of protein hydrolysates prepared from alkaline-aided channel catfish protein isolates. *J Agric Food Chem* **2008**, *56* (16), 7459-7466.
16. Kittiphattanabawon, P.; Benjakul, S.; Visessanguan, W.; Nagai, T.; Tanaka, M. Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Pricanthus tayenus*). *Food Chem* **2005**, *89*, 363-372.
17. Edwards, C.A.; O'Brien, W.D. JR. Modified assay for determination of hydroxyproline in a tissue hydrolyzate. *Clin Chim Acta* **1980**, *104*, 161-167.
18. Ahmad, M.; Benjakul, S.; Nalinanon S. Compositional and physicochemical characteristics of acid solubilized collagen extracted from the skin of unicorn leatherjacket (*Aluterus Monoceros*). *Food Hydrocolloids* **2010**, *24*, 588-594.
19. Sotelo, C.G.; Blanco, M.; Ramos-Ariza, P.; Pérez-Martín, R.I. Characterization of collagen from different discarded fish species of the West coast of the Iberian Peninsula. *J Aquat Food Prod T* **2015**, *25* (3), 388-399.
20. Benjakul, S.; Thiansilakul, Y.; Visessanguan, W.; Roytrakul, S.; Kishimura, H. ; Prodpran, T. Extraction and characterisation of pepsin-solubilised collagens from the skin of bigeye snapper (*Priacanthus tayenus and Prianthus macracanthus*). *J Sci Food Agric* **2010**, *90* (1), 132-138.
21. Zhang, M.; Liu, W.; Li, G. Isolation and characterisation of collagens from the skin of largefin longbarbel catfish (*Mystus macropterus*). *Food Chem* ***2009***, *115*, 826–831.
22. Lynn, A.K.; Yannas, I.V.; Bonfield, W. Antigenicity and immunogenicity of collagen. *J Biomed Mater Res B Appl Biomater* **2004**, *71*(B), (2), 343-354.
23. Morimoto, K.; Kunii, S.; Hamano,K.; Tonomura,B. Preparation and structural analysis of actinidain-processed atelocollagen of yellowfin tuna (*Thunnus albacares*). *Biosci Biotechnol Biochem* **2004**, *68*(4), 861-867.
24. Nagai, T.; Araki, Y.; Suzuki, N. Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chem* **2002**, *78*,173-177.
25. Jongjareonrak, A.; Benjakul,S.; Visessanguan, W.; Tanaka, M. Isolation and characterization of collagen from bigeye snapper (*Priacanthus macracanthus*) skin. *J Sci Food Agric* **2005a**, *85*,1203-1210.
26. Jongjareonrak, A.; Benjakul, S.;Visessanguan, W.; Tanaka,M. Isolation and characterization of acid and pepsin-solubilised collagens from the skin of browntripe red snapper (*Lutjanus vitta*). *Food Chem* **2005b**, *93*, 475-484.
27. Zelechowska, E.; Sadowska, M.; Turk, M. Isolation and some properties of collagen from the backbone of Baltic cod (*Gadus morhua*). *Food Hydrocolloids* **2010**, *24*, 325-329.

28. Borderías, A.J. ; Montero, P. Changes in fish muscle collagen during frozen storage. In *Storage lives of chilled and frozen fish and fish products* .International Institute of Refrigeration: Aberdeen, 1985;85-91.
29. Badij, F.; Howell, N. Elucidation of the effect of formaldehyde and lipids on frozen stored cod collagen by FT-Raman spectroscopy and differential scanning calorimetry. *J Agric Food Chem* **2003**, *26*, 51(5), 1440-1446.
30. Foegeding, E.A.; Lanier, T.C.; Hultin, H.O. Collagen. Characteristics of edible muscle tissues. In *Food Chemistry*, O. R. Fennema ,Eds.; New York: Marcel Dekker, Inc.,1996, pp. 902–906.
31. Kittiphattanabawon, P.; Benjakul, S.; Visessanguan, W.; Kishimura, H.; Shahidi, F. Isolation and characteridsation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). *Food Chem* **2010**, *119(4),* 1519-1526.
32. Woo, J.; Yu, S.; Cho, S.; Lee, Y.; Kim, S. Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin. *Food Hydrocolloids* **2008**, *22 (5)*, 879-887.
33. Love, R.M.; Yamaguchi, K.; Créach, Y.; Lavéty, J. The connective tissues and collagens of cod during starvation. *Comp Biochem Physiol B Biochem mol* **1976**, *55* (4), 487-492.
34. Sikorski, Z.E.; Kolakowska, A.; Pan, B.S. The nutritive composition of the major groups of marine food organisms. In: *Seafood: Resources, nutritional composition and preservation*, Sikorski, Z.E. (Ed.). CRC Press, Inc., Boca Raton, Florida,1990, pp: 29-54.
35. Liu, D.; Liang, L.; Regenstein, J.M.; Zhow, P. Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). *Food Chem* **2012**,*133*, 1441-1448.
36. Nishimoto, M.; Sakamoto, R.; Mizuta, S.; Yoshinaka, R. Identification and characterization of molecular species of collagen in ordinary muscle and skin of the Japanese flounder *Paralichthys olivaceus*. *Food Chem* **2005**, *90 (1-2)*, 151-156.
32.
37. Nagai, T.; Suzuki, N. Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo*, Linnaeus) uter skin. *Food Chem* **2002**, *76*, 149-153.
38. Singh, P.; Benjakul, S.; Maqsood, S.; Kishimura, H. Islation and characterization of collagen extracted from the skin of the striped catfish (*Pangasianodon hypophtalmus*). *Food Chem* **2011**, *124 (1),* 97-105.
39. Nomura, Y.; Toki, S.; Ishii, Y.; Shirai, K. The physicochemical property of shark type I collagen gel and membrane. *J Agric Food Chem* **2000**, *48,* 2028-2032.
40. Wong, D.S. Mechanism and theory in food chemistry. Van Nostrand Reinhold, New York, 1989.
41. Bougatef, A.; Nedjar-Arroume, N.; Manni, L.; Ravallec, R.; Barkia, A.; Guillochon, D.; Nasri, M. Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. *Food Chem* **2010**, *118*, 559–565.
42. Kristinsson, H.G. ; Rasco, B.A. Fish protein hydrolysates: Production, biochemical and functional properties. *Crit Rev Food Sci Nutr* **2000**, *40*, 43-81.
43. Quian, Z-J.; Jung, W.K.; Kim, S.K. Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana Shaw*. *Bioresour Technol* **2008**, *99*, 1690-1698.
44. Harman, L.S.; Mottley, C.; Mason, R. Free radical metabolites of L-Cysteine oxidation. *The journal of biological chemistry* **1984**, 259 (9), 5606-5611.
45. Sarmadi, B.H.; Ismail, A. Antioxidative peptides from food proteins: A review. *Peptides* **2010**, *31*, 1949-1956.
46. Chi, C-F.; Wang, B.; Deng, Y.Y.; Wang, Y.M.; Deng, S.G. ; Ma, J.Y. Isolation and characterization of three antioxidant pentapeptides from protein hydrolsates of monkfish (*Lophius litulon*) muscle. *Food Res Int* **2014**, *55*, 222-228.
47. Vélez-Alavez, M. Evaluación de los indicadores de estrés oxidativo asociados a las características de nado en elasmobranquios y teleósteos. Doctoral Thesis, Centro de investigaciones Biológicas del Noroeste, S.C, 2015

48. Lundblad, R. Techniques in protein modification. Boca Raton, FL, CRC Press. 1994.
49. Barth, D.; Kyrieleis, O.; Frank, S.; Renner, C.; Moroder, L. The role of cystine knots in collagen folding and stability. Part II. Conformational properties of (Pro-Hyp-Gly) model trimers with N-and C-terminal collagen type III cystine knots. *Chemistry* **2003**, *9*, 3703-3714.
50. Blanco, M.; Perez-Martin, R.I.; Sotelo, C.G. Identification of Shark Species in Seafood Products by Forensically Informative Nucleotide Sequencing (FINS). *J Agric Food Chem* **2008**, *56*, 9868-9874.
51. A.O.A.C. Methods of analysis (15th ed.), Association of Official Analytical Chemistry. Washington, D.C., 1997.
52. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can J Biochem Phsiol* **1959**, *37*, 8, 911-917.
53. Muyonga, J.H.; Cole, C.G.B;Duodu,K.G. Characterisation of acid soluble collagen from skins of Young and adult Nile perch (*Lates niloticus*). *Food Chem* **2004**, *85*,81-89.
54. Eastoe, J; Eastoe,B. A method for the determination of total nitrogen in proteins. *British Gelatine and Glue Research Association research reports* **1952**, series B, 5, 1-17.
55. Adler-Nissen J. Control of the proteolytic reaction and of the level of bitterness in protein hydrolysis processes. *J Chem Technol Biotechnol* **1984**, *34(B)*, 215-222.
56. Camacho, F.; González-Tello,P.; Páez-Dueñas, M.P.; Guadix, E.M.; Guadix,A. Correlation of base consumption with the degree of hydrolysis in enzymic protein hydrolysis. *J Dairy Res* **2001**, *68*, 251-265.
57. Nielsen, P.M.; Petersen, D.; Dambmann, C. Imporoved method for determining food protein degree of hydrolysis. *J Food Sci* **2001**, *66* (5).
58. Prieto, M.A; Rodríguez-Amado,I.; Vázquez, J.A.; Murado,M.A. β-Carotene assay revisited. Application to characterize and quantify antioxidant and prooxidant activities in a microplate. *J Agric Food Chem* **2012**, *60*, 8983−8993.
59. Brand-Williams, W.; Cuvelier M.E.; Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci Technol* **1995**, *28*,25–30.
60. Prieto, M.A.; Curran, T.P.; Gowen, A.; Vázquez, J.A. An efficient methodology for quantification of synergy and antagonismin single electron transfer antioxidant assays. *Food Res Int* **2015**, *67*,284–298.
61. Amado, I.R; González, M.P.; Murado, M.A.; Vázquez, J.A. Shrimp wastewater as a source of astaxanthin and bioactive peptides. *J Chem Technol Biotechnol* **2016**, *91*,793-805.