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BILE ACID BINDING CAPACITY OF FISH PROTEIN HYDROLYSATES FROM DISCARD SPECIES IN THE WEST MEDITERRANEAN SEA

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ABSTRACT

Fish protein hydrolysates (FPH), produced from the six main discard species from the West Mediterranean Sea (sardine, horse mackerel, axillary seabream, bogue, small-spotted catshark and blue whiting) were tested for their bile acid-binding capacity. This capacity is directly linked to the ability to inhibit bile reabsorption in ileum and therefore to lower cholesterol levels in bloodstream. From each species, FPH were obtained by three different enzymatic treatments employing two serine endoproteases (subtilisin and trypsin) sequentially or in combination. The results show statistically significant differences among the fish species, attaining interesting average values of bile acid-binding capacity for blue whiting (27.32 % relative to cholestyramine on an equal protein basis) and horse mackerel (27.42% relative to cholestyramine on equal protein basis). The enzymatic treatment did not affect significantly the ability to bind bile acids of a given species. These results are similar to other protein sources such as soy protein or casein, of proven hypocholesterolemic effect. It can be concluded that fish protein hydrolysates from these discarded species are suitable as ingredients in the formulation of cholesterol-lowering supplements.

Keywords: Bile acid binding capacity, anticholesterolemic activity, discards, fish protein hydrolysates, enzymatic hydrolysis.

1. INTRODUCTION

Fish discards (portion of the catch which is returned to the sea) involve an underutilization of marine resources. Most of the fish dumped at sea is dead or dying, which provokes a negative impact on marine's food chains¹ and contributes to the spread of toxic compounds and parasites present in the fish tissues^{2,3}.

Fisheries in the West Mediterranean Sea present an average discard rate between 10 – 23%⁴, while fish captures in this area have been halved during the past decade. Such discards are potential raw materials for obtaining fish protein hydrolysates (FPH) exhibiting functional⁵ and bioactive properties such as antihypertensive, antioxidant, or antimicrobial, among others⁶.

Cholesterol is a lipid which plays a key role in the cell membrane formation, the synthesis of bile acids, vitamin D and steroid hormones. Despite its physiological function, abnormal cholesterol levels are strongly associated to the damage of coronary arteries, and therefore the development of various cardiovascular diseases. Total cholesterol levels in blood are the result of the dietary intake and intestinal reabsorption of bile acids. Under the form of bile acids, cholesterol is segregated to intestine at a rate of 800 – 1200 mg per day, where it joins cholesterol from dietary source (200 – 500 mg of cholesterol per day)⁷. Between 30 and 60% of total cholesterol reaching the intestine is absorbed entering blood stream. Dietary cholesterol is desesterified by cholesterol esterases, forming mixed micelles with plant sterols which can be absorbed through intestine wall. As for bile acids, 95% are reabsorbed in terminal ileum by active transport and recycled back to the liver. Therefore, the anticholesterolemic effect of a given compound can be displayed by inhibiting micellar solubilization of dietary cholesterol or by sequestering bile acids, which are excreted. Bile acid sequestration reduces the amount of bile acids recycled to liver, which therefore increases bile acid synthesis at the expense of removing cholesterol from bloodstream.

Most of the scientific works on bile acid sequestration agents are devoted to fiber components from vegetal sources, such as cereals^{8,9}, legumes¹⁰ or fruits¹¹. As for animal sources, Zhou et al.¹²

evaluated the binding capacity of chitosan from shrimp shell against cholic, deoxycholic and chenodeoxycholic acids. Some protein components have also shown hypocholesterolemic activity. For instance, Lanzini, et al.¹³ conducted in vivo studies on ileal resection patients demonstrating the bile binding capacity of caseins. Nagaoka et al.¹⁴ identified peptide fractions with hypocholesterolemic activity in casein and β -lactoglobulin hydrolysates. Among the few studies on proteins of marine origin, Wergedahl et al.¹⁵ tested the cholesterol-lowering effect of fish protein hydrolysates from Atlantic salmon (*Salmo salar*) on obese Zucker rats. Lin et al.¹⁶ evaluated the in vitro effect of freshwater clam (*Corvical sp.*) protein hydrolysate on both micellar solubilisation of cholesterol and bile acid-binding capacity. A recent study conducted by Hosomi¹⁷, demonstrated that protein hydrolysates from Alaska pollock exhibited higher bile acid-binding capacity and lower micellar solubility of cholesterol compared to casein hydrolysate.

Apart from the works mentioned above, studies concerning in vitro assays of fish protein hydrolysates for hypocholesterolemic activity are hardly found in the literature. Then, the aim of this work is to evaluate the *in vitro* bile acid binding capacity of protein hydrolysates from six fish discarded species, and thus, their potential use for lowering cholesterol. To this end, sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), axillary seabream (*Pagellus acarne*), bogue (*Boops boops*), small-spotted catshark (*Scyliorhinus canicula*) and blue whiting (*Micromesistius poutassou*) were chosen as raw material for the hydrolysates. The amount of discards from these species represents more than 90% of the yearly tonnage of finfish discarded in the West Mediterranean Sea¹⁸. Three protein hydrolysates were produced from each species, employing subtilisin and trypsin as enzymes. These proteases have been previously reported to yield protein hydrolysates exhibiting hypocholesterolemic activity when used independently. For instance, Nagaoka et al.¹⁴ obtained peptide fractions with trypsin from casein and β -lactoglobulin with hypocholesterolemic activity. In the case of subtilisin, Tiengo et al.¹⁹ used Alcalase 2.4L to obtain hydrolysates from plant protein (*Amaranthus cruentus*), which also exhibited bile acid binding capacity.

In this study, three reaction patterns were assayed in order to evaluate the effect of both enzymes when employed in consecutive or simultaneous mode. Such procedures using enzyme mixtures have been previously assayed to produce hydrolysates with ACE-inhibitory and antioxidant potencies. For example, Espejo-Carpio et al.²⁰ found that mixtures of trypsin and subtilisin enhanced the ACE-inhibitory activity goat milk protein hydrolysates, when compared to those produced using single enzymes. García-Moreno et al.²¹ also detected an increase in the antioxidant activity (measured by DPPH inhibition) of fish protein hydrolysates when subtilisin and trypsin were employed consecutively.

2. MATERIALS AND METHODS

2.1. Raw material

Raw sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), axillary seabream (*Pagellus acarne*), bogue (*Boops boops*), small-spotted catshark (*Scyliorhinus canicula*) and blue whiting (*Micromesistius poutassou*) were provided by the fishing harbour of Motril (Spain). All fish were kept in ice during the transportation and dewatered by means of a hydraulic press in the same day.

2.2. Proximate composition

Moisture and ash content were estimated gravimetrically by heating the samples until attaining constant weight at 103°C and 550°C, respectively²². Total nitrogen was determined by the Kjeldahl method and converted to crude protein by using a factor of 6.25²³. Lipid content was determined according to the Soxhlet semi-continuous extraction method.

2.2. Hydraulic pressing of the raw material

Raw material was partially dewatered by means of a hydraulic batch press (model ESP-K, Sanahuja, Spain). This operation reduces water content of the raw material and assures better preservation of the raw material against microbial spoilage. Prior to the pressing operation, the raw

material was preheated at 40°C during 30 minutes in a water bath. Three kg of pre-heated material were fed into a cylindrical press chamber, where it was submitted to three pressing cycles as described by García-Moreno et al.²⁴. Each cycle comprised a pressing steps at 120 bar intersected by relaxation periods where the pressure on the raw material decreased, favouring the release of press liquor from the press chamber. After completing the pressing procedure, a partially dewatered press cake was recovered, which was stored at -20°C prior to its use as substrate for protein hydrolysis.

2.3. Hydrolysis procedure

The press cakes were hydrolyzed employing two serine endoprotease enzymes, one of bacterial (subtilisin, EC 3.4.21.62) and other from animal origin (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 50°C, while enzyme-substrate ratio was set at 3% for both enzymes.

The press cake was grinded and then homogenised with demineralised water. Two hundreds mL of this suspension were fed into a jacketed reactor of volume capacity 250 mL. Three different enzymatic treatments were studied, coded as S+T, T+S and (S+T)₀ according to Table 1:

- S + T. Sequential treatment starting with hydrolysis with subtilisin for 2 hours. Then, trypsin was added to the reactor mixture and reaction continued for 2 extra hours.
- T + S. Sequential treatment starting with hydrolysis with trypsin for 2 hours. Then, subtilisin was added to the reactor mixture and reaction continued for 2 extra hours.
- (S+T)₀. Combined treatment employing a mixture 1:1 of both enzymes for 4 hours of reaction.

The degree of hydrolysis was monitored throughout the reaction by means of an automatic titrator (718 Stat Titrino, Metrohm, Switzerland), equipped of pH and temperature probes and employing NaOH 1 N as titrator agent. According to the pH-stat method, the degree of hydrolysis (DH) can be related with the amount of base consumed to keep pH constant throughout the reaction as follows:

$$DH = \frac{V_b \cdot N_b}{m_p} \cdot \frac{1}{\alpha} \cdot \frac{1}{h_{tot}} \quad (1)$$

where V_b (mL) is the amount of base consumed, N_b (eq/L) is the normality of the base, α is the average degree of dissociation of the α -NH₂ 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 milliequivalents of peptide bonds per gram of protein. Under the conditions of pH and temperature, α was 0.885, while h_{tot} was taken as 8.6 milliequivalents of peptide bonds per gram of protein, as listed by Adler-Nissen²³.

After completing 4 hours, reactions were stopped by heating the reaction mixture at 100 °C for 15 min. Then, hydrolysates were cooled down to room temperature and freeze-dried in a Labconco freeze drying system (Kansas City, MO, USA). The lyophilisate obtained was employed for the determination of the bile acid binding capacity.

2.4. Determination of the bile acid binding capacity

The in vitro bile acid binding capacity of the hydrolysates was determined in triplicate measurements according to the procedure described by Kahlon et al.⁸. Samples of 100 – 110 mg of hydrolysate powder were digested in 1 mL of 0.01 N HCl at 37 °C for 1 hour. This step simulates stomach digestion, which was stopped by readjusting pH at 6.3 with 0.1 N NaOH. At this stage, the predigested samples were subjected to intestinal digestion by adding 5 mL of porcine pancreatine (10 mg/mL) and 4 mL of 0.72 mM artificial bile preparation. The mixture was incubated under agitation at 1 h and 37 °C. Based on the composition of human bile, artificial bile acid mixture was formulated containing 75% of glycocholic bile acids and 25% of taurine bile acids. After intestinal digestion, the resulting mixture was centrifuged at 20,000 g for 30 min. Unbound bile acids in the supernatant were determined using a total bile acids determination kit (Diazyme, USA). This method is based in the first-order enzymatic oxidation of bile acids to 3-ketosteroids, which can be followed spectrophotometrically at 405 nm. The content of total bile acids was obtained using standard calibration curves prepared for pure cholic and chenodeoxycholic acids. The bile acid-

binding capacity was reported as the μmol of bile acids bound by gram of sample (i.e. retained in the sludge after centrifugation). A sample of 24 – 26 mg of cholesteramyne powder was employed as positive control, since it was able to sequestrate 93% of the total amount of bile acids contained in the artificial bile preparation.

2.5. Statistical analysis

A statistical program (Statgraphics 5.1.) was used for data processing and statistical analysis. Data were subjected to analysis of variance (ANOVA). The Tukey's multiple range test²⁵ was used to determine whether significant differences existed among enzymatic treatments and species. Differences among mean values were considered significant at a level of confidence of 95%.

3. RESULTS AND DISCUSSION

3.1. Proximate composition of the raw material

Table 1 shows the proximate composition of the six species employed for hydrolysis. The species present significant variability on the lipid content, which ranges from 0.8% (bogue) to 11.3% (sardine). Besides the average differences between lean and fat fish species, lipid content of a given fish species presents seasonal variations depending on the stage of maturity, as well as environmental and nutritional determinants of the animal^{18,26}. As for the protein content, no major differences were observed among the species, varying from 16% (sardine) to 20.3% (small-spotted catshark). These results are in accordance to previous works reported for species in the Mediterranean Sea²⁷⁻²⁹. It should be noted that the determination of crude protein content, based on the Kjeldahl method, may occur in overestimation due to quantification of nonprotein nitrogenous compounds (e.g. ammonia, urea). This error may be appreciable in elasmobranchs, such as small-spotted catshark, given its relatively high content of urea and trimethylamine oxide²⁹.

3.2. Hydrolysis curves and protein content of the hydrolysates

Fig. 1 illustrates an example of the hydrolysis reactions carried out in this work. It includes the three enzymatic treatments performed on the press cake of blue whiting. The time evolution of the degree of hydrolysis followed the general pattern described for enzymatic reactions^{23,30}. All the hydrolysis curves presented a high reaction rate after enzyme addition and then flattened progressively until reaching a stationary state where the degree of hydrolysis remained constant. This is attributable to the exhaustion of available peptide bonds, together with other phenomena which modulate the proteolytic activity such as thermal enzyme inactivation or product inhibition³⁰.

Table 2 lists the values of the final DH (i.e. after 4 hours of reaction), as well as the protein content of the corresponding freeze-dried hydrolysates. Every hydrolysate was given a sample code from 1 to 18. The degradability of proteins to the enzymes showed differences among the species studied. Indeed, the highest degrees of hydrolysis were attained by horse mackerel (samples 4-6), which presented a final DH between 18.2 and 21.0% depending on the enzymatic treatment. Contrarily, the lowest levels of final DH (below 15%) corresponded to sardine (samples 1-3).

With regard to the enzymatic treatments, the sequential treatment S+T led to the maximum DH for sardine, axillary seabream, bogue and small-spotted catshark. The rest of species presented the maximal extent of hydrolysis for the combined treatment (S+T)₀, where both enzymes were added simultaneously. In all the cases, the increase of DH observed after addition of trypsin was less marked than that obtained for subtilisin. This is explained by the narrow specificity of trypsin, which only cleaves peptide bonds containing the amino acids arginine and lysine. On the contrary, subtilisin is a serine endoprotease of wide spectrum, which cleaves preferably peptide bonds containing acid or neutral residues³¹.

The freeze-dried hydrolysates samples were analysed for their protein content, which is listed in Table 2. No correlation was observed between final DH and protein content ($r^2=0.0392$, $p > 0.05$). Indeed, no significant differences were observed between enzymatic treatments applied to the same species, except for sample 3 whose protein content was significantly higher than that of samples 1

and 2. According to the Tukey's multiple comparison test, protein content of non-fatty species (i.e. axillary seabream, bogue, blue whiting and small-spotted catshark) was similar (73.0 - 77.8%), except for small-spotted catshark hydrolysates (samples 13, 14 and 15) which presented higher levels of protein (87 - 89.5 %). As stated above, this value should be taken cautiously, given the significant levels of trimethylamine oxide in elasmobranch species. In contrast, the content of crude protein of fatty species (samples 1 to 6) presented significant differences with non-fatty species, ranging between 60.7 - 67.8%.

3.3. Bile acid binding capacity of the hydrolysates

This research employed the *in vitro* assay on six fish protein hydrolysates to test their abilities to sequester two individual bile acids, cholic and chenodeoxycholic acid, chosen because they were soluble in phosphate buffer in the range of temperatures assayed. *In vitro* assays have been widely chosen in the literature to evaluate bile acid-binding capacity of dietary fibers^{8,11,32} and, to a lesser extent, of polysaccharides¹² and protein fractions^{10,13,16}. Fig. 2 and 3 show the binding capacity of the 18 protein hydrolysates studied against cholic acid and chenodeoxycholic acid. Bile acid binding capacity was expressed in Fig. 2 as the amount of bile acids (μmol) retained by 100 milligrams of dry matter. Assuming that the major compounds responsible for the retention of bile acids were proteins, Fig. 3 lists the amount of bile acids bound by 100 milligrams of protein. Finally, the bile acid-binding capacity of the hydrolysates was related to cholestyramine in Table 3. This compound is assumed to bind 100% of bile acids, and it is commonly employed in literature as positive control^{10,11,16}. In our case, 100 mg of cholestyramine sample bound on average $10.44 \pm 0.02 \mu\text{mol}$ of the bile acids selected.

Overall, the bile acid binding capacity of the hydrolysates presented significant differences among the fish species studied, while the enzymatic treatment had low impact on this property, except for some particular cases. Blue whiting (samples 16 to 18) presented the strongest average binding capacity against cholic and chenodeoxycholic acid. According to the statistical analysis, enzymatic treatments had no influence on the average amounts of bile acids captured by blue whiting

hydrolysates, which were 2.16 ± 0.02 μmol of cholic acid and 2.00 ± 0.02 μmol of chenodeoxycholic acid per 100 mg of dry matter (Fig. 2). Considering the content of protein in the samples, these values rose to 2.85 ± 0.02 μmol of cholic acid and 2.64 ± 0.03 μmol of chenodeoxycholic acid per 100 mg of protein (Fig. 3). Related to cholestyramine, average binding capacities against cholic and chenodeoxycholic acids were 27.3% and 25.3% in protein basis, respectively (Table 3).

Horse mackerel hydrolysates obtained by the sequential treatment trypsin plus subtilisin (sample 5) presented the highest binding capacity against cholic acid in the experimental series (30.79% of bile acid-binding capacity related to cholestyramine), while the other treatments (samples 4 and 6), presented similar levels to those observed for blue whiting in Fig. 3. On the contrary, their ability to bind chenodeoxycholic acid was 30% - 50% lower. (Fig. 2 and 3).

On an equal protein basis, sardine hydrolysates bound an average value of 2.53 ± 0.03 μmol of bile acids per 100 mg of protein (Fig. 3). As observed for horse mackerel, sardine hydrolysates also exhibited less ability to sequester chenodeoxycholic acid (Fig. 2 and 3). The reverse trend was observed for bogue hydrolysates, where the sequential treatment trypsin plus subtilisin (sample 11) showed the maximal capacity to bind chenodeoxycholic acid (28.4 % related to cholestyramine in a protein basis) among the 18 samples analyzed (Table 3).

According to Fig. 3, axillary seabream (samples 7 to 9) and small spotted catshark (samples 13 to 15) hydrolysates presented the lowest values of bile acid-binding capacity. Axillary seabream exhibited higher average binding capacity for chenodeoxycholic acid (2.06 ± 0.02 μmol per 100 mg of protein) whereas small-spotted catshark did for cholic acid (1.84 ± 0.03 μmol per 100 mg of protein) (Fig. 2 and 3). Nevertheless, the amount of bile acids bound by small-spotted catshark hydrolysates were equivalent to those retained by sardine on equal dry basis (Fig. 2). The decrease in bile acid-binding capacity when reporting the results to protein basis was attributed to the high content of protein detected for small-spotted catshark hydrolysates (Table 2). Protein content in

these hydrolysates could have been overestimated given the presence of non-protein nitrogenous compounds in elasmobranchs²⁹.

The levels of bile acid-binding capacity obtained in this work are similar to those reported in literature. Kahlon et al.¹⁰ tested the in vitro binding capacity against cholic acid of some vegetable fractions, wheat gluten, soy protein, pinto and black beans, reporting values of 12, 17, 23 and 30%, respectively, when compared to cholestyramine on equal protein basis. The different affinity to bile acids was mainly attributed to the protein composition and structure, as well as the presence of other non-protein components such as dietary fibers. As for proteins of animal origin, Nagaoka et al.¹⁴ tested the in vitro taurocholate binding of casein and β -lactoglobulin hydrolysates, produced with trypsin at 0.4% by weight of protein for three hours. These hydrolysates were further purified by ultrafiltration and ion-exchange resins, reporting taurocholate binding capacities of 35% related to cholestyramine. Interestingly, this value is similar to that obtained for horse mackerel hydrolysates (31% to cholic acid related to cholestyramine), obtained by initial hydrolysis with trypsin under similar conditions (pH=8, 50°C).

Among the few studies on fish protein hydrolysates, Lin et al.¹⁶ extracted muscle protein from freshwater clams with hot water. The residual meat was then freeze-dried and diluted 10-fold with deionized water, obtaining a solution which was hydrolyzed with Protamex at 1% by weight of protein for 5 h. Both the hot water extract and the Protamex hydrolysate were tested for their in vitro bile-acid binding capacity against cholic acid. The authors reported mean values of 25% and 36% relative to cholestyramine on equal protein basis for the hot water extract and the hydrolysate, respectively. The higher affinity for bile acids shown by the protamex hydrolysate was explained by the release of hypocholesterolemic peptides in the course of the hydrolysis. Sugano et al.³³ suggested mean molecular weights between 1 and 10 kDa for hypocholesterolemic peptides, while other authors related bile acid sequestration to the presence of hydrophobic peptides³⁴. García-Moreno et al.²¹ determined the molecular weight distribution of sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark hydrolysates, concluding that the most abundant

species were small peptides below 1 kDa. Furthermore, it should be noted that subtilisin, employed as enzyme in this work, cleaves peptide bonds preferably containing hydrophobic residues.

4. CONCLUSIONS

FPH from six discarded species in the West Mediterranean Sea were tested for their *in vitro* bile acid-binding capacity. The enzymatic treatments in this work employed subtilisin and trypsin, sequentially or in combination. Both enzymes, employed individually, have been reported to produce hydrolysates with hypocholesterolemic potency, while their combined use has not been yet studied for this purpose. According to the multiple comparison method, the bile-acid binding capacity of the FPH studied presented significant differences ($p < 0.05$) among fish species, ranging from 17.21 to 30.79% relative to cholestyramine on equal protein basis. The results do not show a clear relationship between the enzymatic treatment and the bile acid-binding capacity of the hydrolysates. For instance, blue whiting hydrolysates (samples 16 to 18) presented important levels of bile acid-binding (27.3% on average relative to cholestyramine) regardless the enzymatic treatment or the bile acid standard employed, while horse mackerel hydrolysates (samples 4 to 6) exhibited a marked affinity for cholic acid, especially those produced by the sequential treatment T+S (30.79% to cholestyramine on equal protein basis). The levels of bile acid-binding detected in this work are comparable to other protein sources such as soy protein or casein, of proven hypocholesterolemic effect. These results pave the way for incorporation of FPH from these discarded species into the formulation of cholesterol-lowering supplements.

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Table 1. Proximate composition on a wet weight basis of the six fish species studied.

Species	Composition (wt%)			
	Moisture	Ash	Protein	Lipid
Sardine	64.6	3.0	16.0	11.3
Horse mackerel	73.5	3.6	16.9	5.0
Axillary seabream	73.1	5.5	17.6	3.7
Bogue	78.7	3.9	17.0	0.8
Small-spotted catshark	77.1	1.6	20.3	2.5
Blue whiting	77.6	2.9	19.0	2.5

Table 2. Final degree of hydrolysis and protein content on a dry weight basis of the hydrolysates studied. Protein content is expressed as mean and standard deviation of triplicate determination. Mean values with different superscript letters are significantly different ($P < 0.05$)

Species	Enzymatic treatment	Sample Code	DH (%)	Protein (wt%)
Sardine	S+T	1	14.9	61.5 ± 0.4 ^{ab}
	T+S	2	13.2	60.7 ± 0.4 ^a
	(S+T) ₀	3	13.7	66.4 ± 0.4 ^c
Horse mackerel	S+T	4	19.7	67.8 ± 0.2 ^c
	T+S	5	18.2	67.1 ± 0.2 ^c
	(S+T) ₀	6	21.0	62.5 ± 0.2 ^b
Axillary seabream	S+T	7	17.2	73.0 ± 0.3 ^d
	T+S	8	16.0	73.5 ± 0.3 ^{de}
	(S+T) ₀	9	16.3	74.9 ± 0.2 ^{ef}
Bogue	S+T	10	17.6	75.7 ± 0.7 ^{fg}
	T+S	11	17.0	76.8 ± 0.2 ^{gh}
	(S+T) ₀	12	15.3	76.4 ± 0.2 ^{gh}
Small-spotted catshark	S+T	13	19.2	87.0 ± 0.2 ⁱ
	T+S	14	18.3	89.5 ± 0.2 ^j
	(S+T) ₀	15	17.3	88.7 ± 0.5 ^j
Blue whiting	S+T	16	15.8	75.4 ± 0.2 ^{fg}
	T+S	17	13.2	77.8 ± 0.3 ^h
	(S+T) ₀	18	16.3	74.4 ± 0.5 ^{e-g}

Table 3. Bile acid-binding capacity of the hydrolysates relative to cholestyramine. Data represent mean and standard deviation of triplicate determination. Mean values with different superscript letters are significantly different ($P < 0.05$)

Sample	Binding relative to cholestyramine (%)			
	DM basis		Protein basis	
	Cholic acid	Chenodeoxycholic acid	Cholic acid	Chenodeoxycholic acid
1	15.4 ± 0.6 ^{c-e}	11.0 ± 1.0 ^{a-c}	25.0 ± 1.0 ^{g,h}	15.1 ± 1.3 ^{b,c}
2	13.9 ± 0.7 ^{a,b}	11.2 ± 0.3 ^{b,c}	22.9 ± 1.2 ^f	15.2 ± 0.5 ^c
3	16.4 ± 0.3 ^{e,f}	9.8 ± 0.3 ^a	24.7 ± 0.4 ^{g,h}	13.1 ± 1.1 ^a
4	16.4 ± 0.7 ^{e,f}	13.0 ± 0.1 ^{e,f}	24.1 ± 1.1 ^{f,g}	17.2 ± 0.1 ^d
5	20.7 ± 0.1 ^g	11.7 ± 0.1 ^{c,d}	30.8 ± 0.1 ^k	15.2 ± 0.2 ^c
6	17.1 ± 0.1 ^f	10.2 ± 0.7 ^{a,b}	27.3 ± 0.0 ^{i,j}	13.4 ± 0.9 ^{a,b}
7	14.2 ± 0.8 ^{a-c}	12.5 ± 0.7 ^{d,e}	19.5 ± 1.1 ^{c-e}	20.4 ± 1.2 ^e
8	14.4 ± 0.3 ^{a-d}	10.9 ± 0.8 ^{a-c}	19.6 ± 0.8 ^{d,e}	17.9 ± 1.2 ^d
9	13.3 ± 0.3 ^a	13.9 ± 0.6 ^{f,g}	17.7 ± 1.1 ^{a,b}	20.9 ± 0.8 ^e
10	15.8 ± 0.1 ^e	13.9 ± 0.7 ^{f,g}	20.9 ± 0.0 ^e	20.6 ± 1.0 ^e
11	14.6 ± 0.2 ^{b-d}	19.0 ± 0.1 ^h	19.0 ± 0.3 ^{b-d}	28.4 ± 0.2 ^h
12	13.5 ± 0.7 ^{a,b}	14.9 ± 0.1 ^g	17.7 ± 0.9 ^{a,b}	23.8 ± 0.2 ^f
13	15.5 ± 0.6 ^{d,e}	13.1 ± 0.8 ^{e,f}	17.8 ± 0.7 ^{a-c}	15.0 ± 0.9 ^{b,c}
14	16.1 ± 1.1 ^{e,f}	13.3 ± 1.3 ^{e,f}	17.9 ± 1.2 ^{a-c}	14.8 ± 1.5 ^{a-c}
15	15.3 ± 1.0 ^{c-e}	12.6 ± 0.7 ^{d,e}	17.2 ± 1.1 ^a	14.1 ± 0.8 ^{a-c}
16	20.7 ± 0.4 ^g	19.1 ± 0.5 ^h	27.5 ± 0.5 ^{i,j}	25.3 ± 0.6 ^{f,g}
17	20.5 ± 0.2 ^g	18.9 ± 0.2 ^h	26.3 ± 0.3 ^{h,i}	24.3 ± 0.2 ^f
18	20.9 ± 0.1 ^g	19.5 ± 0.2 ^h	28.2 ± 0.1 ^j	26.2 ± 0.2 ^g

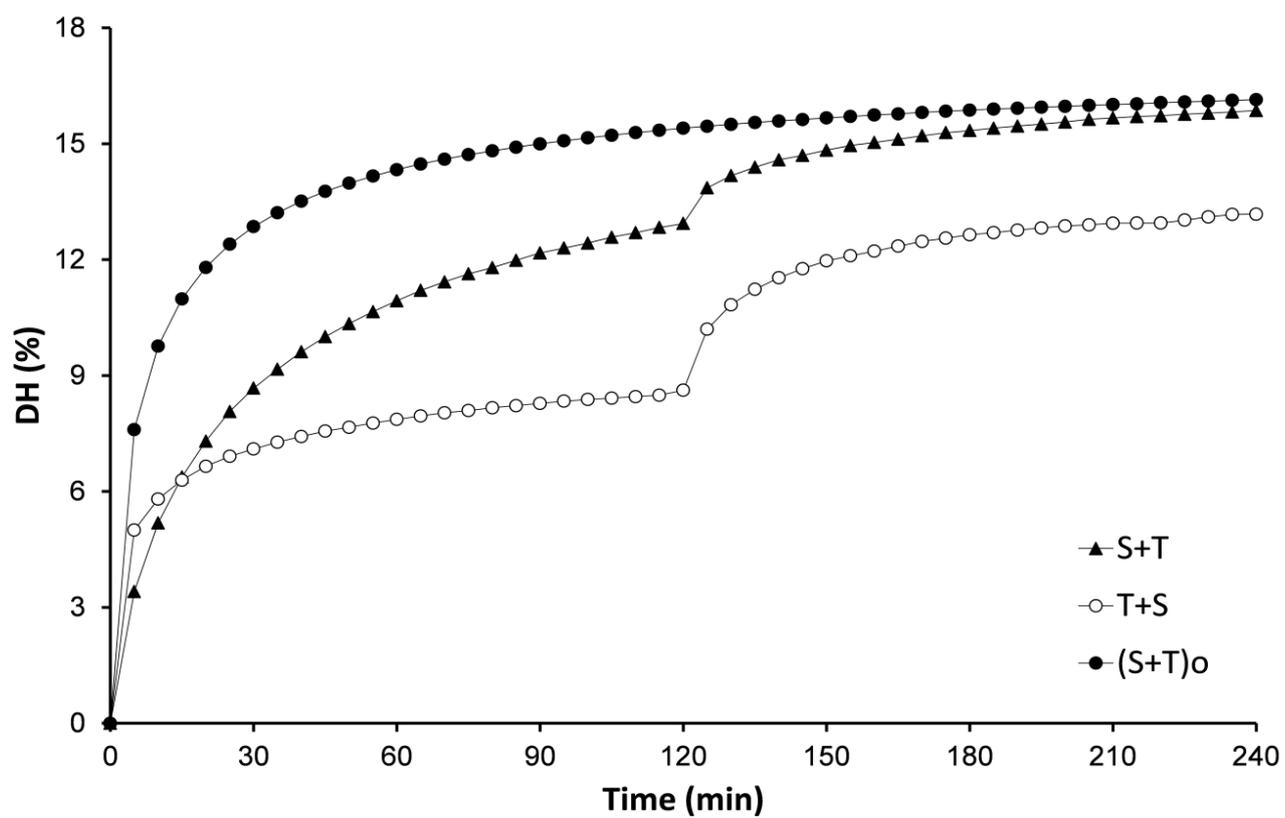
Figure 1. Hydrolysis curves of blue whiting for the three enzymatic treatments studied

Figure 2. Bile acid-binding capacity of the hydrolysate samples on an equal dry matter basis. Data represent mean and standard deviation of triplicate determination. Mean values with different superscript letters are significantly different ($P < 0.05$).

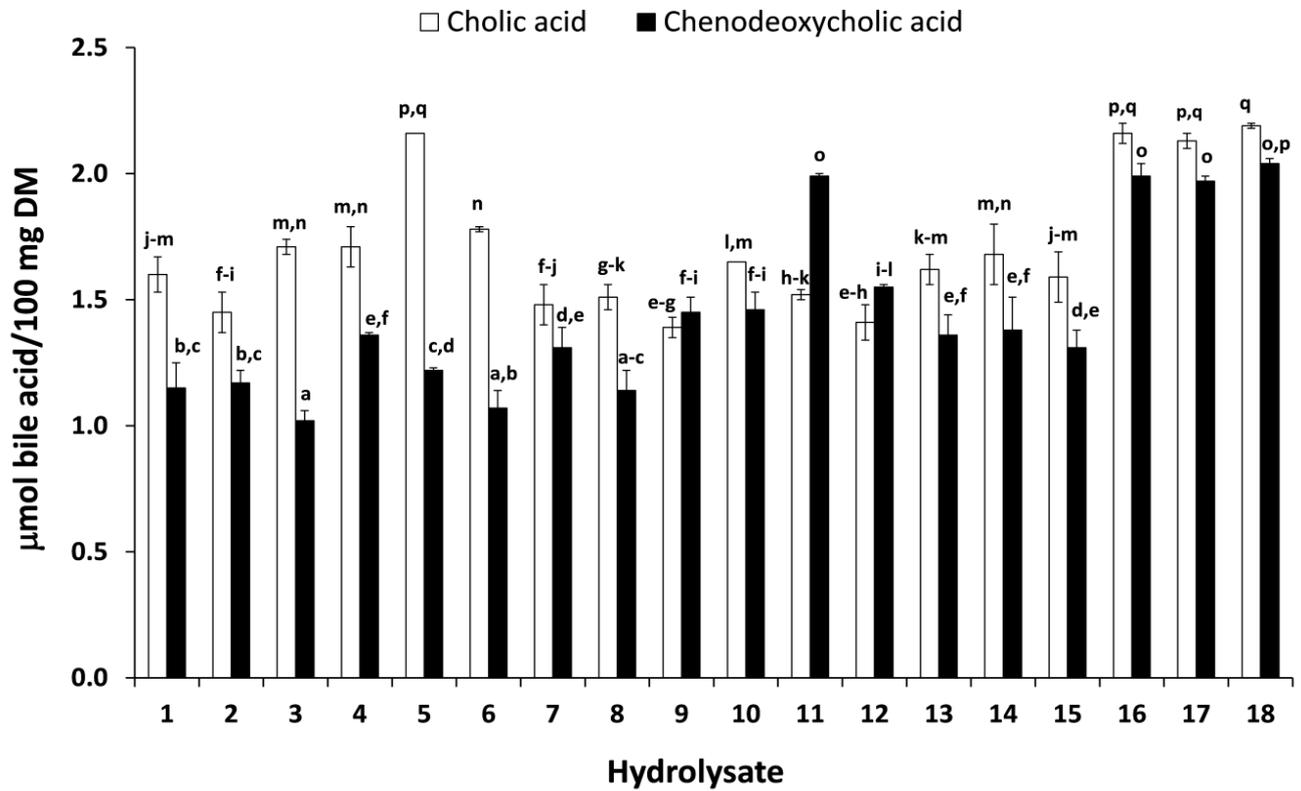


Figure 3. Bile acid-binding capacity of the hydrolysate samples on an equal protein basis. Data represent mean and standard deviation of triplicate determination. Mean values with different superscript letters are significantly different ($P < 0.05$).

