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GLOSSARY OF ACRONYMS

Acronym	Definition
AO	Antioxidant
Ash	Ashes
AU	AnsonUnit
ACE	Angiotensin I-converting enzyme
BW	Blue Whiting
DGLA	Dihomo-linolenic acid
DHA	Docosahexaenoic acid
Dig	Digestibility
DPA	Docosapentaenoic acid
DPPH	Diphenyl-2-picryhydrazyl
EPA	Eicosapentaenoic acid
FPHs	Fish Protein Hydrolysates
GC	Gas-Chromatography
GPC	Gel Permeation Chromatography
H	Heads
H_m	Maximum Hydrolysis
kDa	Kilodalton
Lip	Lipids
LO	Landing Obligation
Mo	Moisture
MRCS	Minimum Reference Conservation Size
OM	Organic Matter
PD	Polydispersity
PDI	Polydispersity Index
pH_{opt}	Optimal pH
Prs	Soluble Protein
Pr-tN	Proteins as Total Nitrogen
RSM	Response Surface Methodology
RT	Rainbow Trout
RT_H	Heads of Rainbow Trout
RT_TF	Trimmings and Frames of Rainbow Trout
SB	Seabream
SB-B	Seabream by-products
SB-H	Seabream Heads
SB-S	Seabream Skins and Bones
S_H	Heads of Salmon
S:L	Solid:Liquid
S_TF	Trimmings and Frames of Salmon
T	Temperature
TF	Trimmings+Frames

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Acronym	Definition
T_{opt}	Optimal Temperature
TS	Total sugars
V_{dig}	Yield of Digestion
Y	Dependent variable

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Executive Summary

In the present deliverable, different aquaculture by-products were evaluated as substrates for the production of fish protein hydrolysates (FPHs), potentially adequate as protein ingredients of aquaculture feeds. The most relevant results were:

a) Rainbow trout. The optimal conditions for the production of hydrolysates from by-products (heads, H, and trimmings+frames, TF) using Alcalase as biocatalyst, were: 0.1% (v/w) of enzyme concentration, pH=8.27, T=56.2ºC, ratio (Solid:Liquid=1:1), 3 h of hydrolysis and agitation of 200 rpm. These data obtained at 100 mL-reactor scale were then validated at 5L-reactor scale. The hydrolytic capacity of Alcalase and the protein quality of FPHs were excellent in terms of complete digestion of wastes, high degrees of hydrolysis, high concentration of soluble protein, good balance of amino acids and almost full *in vitro* digestibility. Additionally, fish oils were also recovered from wastes jointly with FPHs. The production of dried hydrolysates from trout by-products is already ended.

b) Atlantic salmon. The optimal values of Alcalase to digest salmon wastes (H and TF) were 0.2% (v/w) of enzyme, pH8.98, T64.2ºC, 200 rpm, 3 h of hydrolysis and S:L=1:1. Yields of FPHs production were very high and high quality hydrolysates were generated. The salmon FPHs from TF showed the higher protein content in comparison to the rest of FPHs from salmonids. Average molecular weights from salmonid-FPHs ranged from 1.4 to 2.0 kDa. The production of dried hydrolysates from salmon by-products is also finished.

c) Seabream and seabass. Preliminary experiments of hydrolysis were performed, in order to select the most adequate commercial endo and exoproteases. Alcalase showed a better performance working with skin and bones substrates but working with heads the combination of Alcalase and Flavorpro and the Protamex enzyme showed better yields. The protein recovery yields were, however, quite low in all cases: therefore, the enzymatic hydrolysis must be optimized previous to pilot plant productions, to increase protein yields, using for instance higher enzyme percentage or extending the time of the hydrolysis.

d) As regards blue whiting (BW) discards, the optimization of Alcalase hydrolysis led to the following best conditions of operation: enzyme concentration of 1% (v/w), pH8.6, T60ºC, S:L=1:2, 4 h of hydrolysis and agitation of 200 rpm. As in the previous salmonids cases, the production of FPHs at 5L-reactor confirmed the results obtained at lab scale and the valuable chemical properties described for salmon and trout. The production of dried hydrolysates at pilot plant scale (300L-reactor) is now in progress.

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1.Introduction

Fish meal may be obtained using a thermal process of fish and aquaculture discards and by-products, but the environmental impacts (air pollution, odours, high water consumptions, etc.) of plants using such process is huge. This process involves coagulation of the protein and its separation from the oil. Fish meal production jointly with fish oil recovery is the most common process for the use of fish and aquaculture by-products and discards but the biomass undergoes a low valorisation level (generally obtaining low quality products). Thus, alternatives for a better use of these biomasses, aimed at maximising the production of compounds of high commercial interest for diverse sectors of application, must be investigated.

Valorisation processes focused to the enzymatic hydrolysis to produce fish protein hydrolysates (FPHs), including the recovery of essential nutrients and bioactive compounds (Blanco et al., 2015, Halim et al., 2016), could be an excellent and feasible practice to efficiently upgrade these substrates. The preparation and characterization of FPHs using different fish species, enzymes, or hydrolysis conditions have been extensively studied (Chalamaiah et al., 2012; Vázquez et al., 2017a). In these studies, FPHs have demonstrated excellent functional properties as antioxidants against free radicals (Batista et al., 2010), antihypertensive pharmacological agents, specifically, as inhibitors of the angiotensin-I converting enzyme (Nasri et al., 2013) and antimicrobial properties (Wang et al., 2018).

On the other hand, since FPHs are rich in soluble proteins and with high digestibility, they can be also employed as ingredient of aquaculture feeds and pet-food (Ospina-Salazar, 2016; Swanepoel and Goosen, 2018) with very promising results. Finally, it must be mentioned that FPHs could be also used as substrate to obtain peptones (mixture of polypeptides and free amino acids) useful as ingredient of culture media for microbial growth (Pleissner and Venus, 2016). A great percentage of microbial bioproduction costs are due to the price of peptones (Shi et al., 2018), so the search of new protein fractions from food wastes is an essential research issue (Pleissner and Venus, 2016).

One GAIN objective is to demonstrate that aquafeed can be obtained from sustainable sources, implementing the principles of circular economy. Using aquaculture by-products for valorising protein-rich fractions resulting from processing farmed fish for the production of aquafeeds contribute to improve circularity and reduce waste.

Besides, since 2013 the European Common Fisheries Policy has established the Landing Obligation (LO) in the Regulation EU No. 1380/2013 of the European Parliament and the Council, 11th of December 2013. The full implementation of this regulation demands the managing of biomasses previously discarded by the fishing fleet at landing ports. The LO established that "*all the species which are subject to catch limits shall be brought and retained on board the fishing vessels, recorded, landed and counted against the quotas*". In the case of individuals with size below the minimum reference conservation size (MRCS) the

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use of these biomasses is restricted by the LO and cannot be used for direct human consumption; therefore, alternative uses such as fish meal, pet food or FPHs production, among others, should be employed. In the case of blue whithing *(Micromesistius poutassou)*, in some fisheries the total capture of some fishing trips cannot be fully absorbed by the demand for human consumption due to amount and/or quality and so it can be used for the production of fish protein hydrolysate as ingredient of aquafeed.

Therefore, two kinds of substrates have been selected to generate FPHs as feed ingredients: 1) by-products generated by aquaculture fish processing, and 2) whole fish individuals discarded by fishing activity. For the first case, heads, trimmings and frames produced in the preparation of fillets from farmed salmonids (rainbow trout and salmon) and seabream/seabass have been explored. For the second substrate (fish discard), individuals of blue whiting (BW) were chosen because it is the species most discarded by the fishing fleets that work in the Northeast Atlantic (Egerton et al., 2018; Uhlmann et al., 2019).

The objective of this report is to present the results of the optimization of the steps and conditions to produce protein-rich ingredients to be incorporated in aquaculture feeds as ingredient for replacing fish meals in aquafeeds. The production of these ingredients is achieved by the enzymatic hydrolysis of fish substrates using optima experimental conditions and resulting in FPHs which will be incorporated in aquafeeds. These will be evaluated in the fish trials to be performed in GAIN project WP1.

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2.Materials and Methods

Fish material processing

Heads, trimmings together with frames of salmonids, rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmon salar*) (Figure 1), were kindly supplied by a Galician Company (Isidro 1952, S.L., Cambre, A Coruña) that processes gutted Norwegian salmon and grows trout in its farms. These by-products (45-50 kg of each by-product and origin) were frozen and kept at -18ºC until processing. Initially, the 4 types of substrates: heads of rainbow trout (RT_H), trimmings and frames of rainbow trout (RT_TF), heads of salmon (S_H) and trimmings and frames of salmon (S_TF) were ground in a meat mincer (Figure 1E-F are examples of by-product minces before hydrolysis).

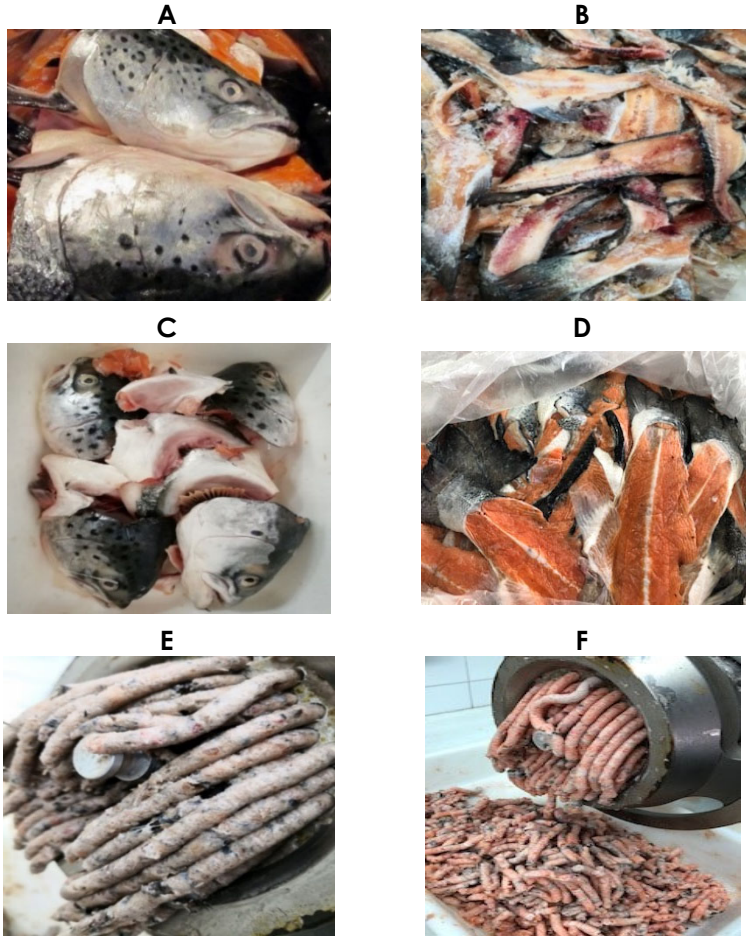

Figure 1. Pictures of the salmonids by-products processed in the IIM-CSIC: A) heads of rainbow trout, B) trimmings and frames of rainbow trout, C) heads of salmon and D) trimmings and frames of salmon. E) minced of rainbow trout by-products and F) minced of salmon by-products.

Discarded individuals of blue whiting (BW, *Micromesistius poutassou*) were captured in the Northeast Atlantic (Galician coast, Area IXA), separated from other commercial species on board, and kept in ice until landing and arriving to our lab in IIM-CSIC (less than 48 h). Once there, discards were immediately homogenised by grinding and stored at -18ºC until use.

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The main by-products from Culmárex (Aguilas, Murcia, Spain) were sent to ANFACO to be used in the project. They mainly work with seabass (*Dicentrarchus labrax*) and seabream (SB, *Sparus aurata*) for filleting, and the by-products of their company consist on heads, skins and bones and viscera. They sent to ANFACO approximately 400 kg of these different by-products in 24 boxes (Figure 2). All the substrates could be included in the survey; however, the viscera probably will have more lipids than proteins and due to this they can cause possible problems of separation that should be avoided.

Figure 2. By-products of seabream generated in Culmárex after filleting and sent to ANFACO. A) skins and bones B) viscera and C) heads.

2.1 Enzymes used for the production of the hydrolysates

The commercial proteases selected for this study were: Alcalase 2.4L (2,4 AnsonUnit/g, AU/g enzyme) and Protamex (1,5 AU/g enzyme) purchased to Novozymes (Nordisk, Bagsvaerd, Denmark) as well as Flavorpro™ 795MDP (Biocatalysts Limited, Cardiff, Wales, UK). The most relevant variables that must be taken into account and optimized in order to maximize the proteolytic capacity of these proteases are: pH, temperature, time of hydrolysis and amount of enzyme per initial substrate weight. Table 1 shows the most suitable working ranges of pH and temperature for these enzymes indicated by the commercial producers. This information is merely indicative since it was established for the hydrolysis of pure proteins such as casein and albumin but not for substrates as complex as the ones to be investigated in GAIN. This is the main reason why the optimal condition for the hydrolysis of each species and substratum must be determined by means of a well-designed set of experiments. Table 1 also shows the costs of these enzymes per kg, which is important for further scaling-up.

Table 1. Ranges of best conditions to and industrial cost of the enzymes used.

	Protamex®	**Alcalase 2.4L, FG**	**Flavorpro™ 795MDP**
Optimum range of T (ºC)	35-60	30-65	45-55
Optimum range of pH	5-7	7-9	5.0-7.0
Cost as €/kg (Less than 500 kg)	47	33	-

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2.2 Experimental design for the optimization of enzyme hydrolysis of salmonid by-products and BW discards

The combined effect of *pH* and temperature (*T*) on the Alcalase digestion of RT_H, RT_S and BW were evaluated by means of rotatable second order designs (with 5 replicas in the center of the experimental domain) (Box et al., 2005). Alcalase 2.4L was chosen due to its excellent capacity of proteolysis when it was applied to several marine substrates as squid pens, fish cartilages, crustacean shells and other fish tissues and by-products (Vázquez et al., 2016a, 2017a, 2017b, 2018, 2019). The rest of the experimental conditions remained constant: agitation, (S:L) ratio and enzyme concentration. Codified and natural values for all experimental conditions tested in the factorial designs together with constant independent variables are summarized in Table 2. The responses (dependent variables, *Y*) evaluated were the concentration of soluble protein (Prs), the maximum hydrolysis (H_m) and the yield of digestion (V_{dig}). Orthogonal least-squares calculation on factorial design data were used to obtain empirical equations describing the different response assessed (*Y*) in function of the independent variables:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \qquad [1]$$

where: *Y* is the dependent variable evaluated, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of combined effect, b_{ii} is the coefficient of quadratic effect, *n* is the number of variables and X_i and X_j are the independent variables studied in each case. Student's t-test (α=0.05) was employed to determine statistical significance of coefficients. Coefficient of determination (R^2) and adjusted coefficients of determination (R^2_{adj}) were used to establish goodness-of-fit and the following mean squares ratios from Fisher F test (α=0.05) were calculated to define model consistency: *F1* = Model/Total error, being the model acceptable when $F1 \geq F_{den}^{num}$; and *F2* = (Model + Lack of fitting)/Model, being the model acceptable when $F2 \leq F_{den}^{num}$. F_{den}^{num} are the theoretical values to α=0.05 with corresponding degrees of freedom for numerator (num) and denominator (den).

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Table 2. Experimental domain and coding of the independent variables in the factorial design executed to study the joint effect of pH and temperature on the Alcalase hydrolysis of salmonid by-heads and BW discards.

	Natural values	
Coded values	**pH**	**T (ºC)**
-1.41	6.0	30.0
-1	6.6	37.3
0	8.0	55.0
+1	9.4	72.7
+1.41	10.0	80.0

Codification: $Vc = (Vn-V_0)/\Delta Vn$ — Decodification: $Vn = V_0 + (\Delta Vn \times Vc)$
Vn = natural value of the variable to codify — ΔVn = increment of Vn for unit of Vc
V_0 = natural value in the centre of the domain — Vc = codified value of the variable

Constant conditions
For RT_H and S_H: Agitation= 200 rpm; r (S:L)= 1:1; [Alcalase]= 0.5% (v/w) or 12 AU/kg of heads.
For BW discards: Agitation= 200 rpm; r (S:L)= 1:2; [Alcalase]= 1% (v/w) or 24 AU/kg of BW.

These experiments were carried out in a pH-Stat system equipped with a 100 mL enzyme reactor with temperature and agitation control present in the IIM-CSIC. After hydrolysis, FPHs were quickly heated (90ºC/15 min) for enzyme inactivation.

Secondly, the individual effect of enzyme concentration was studied using the same experimental equipment and maintaining constant (in the optimal values obtained in the previous factorial plans), the rest of experimental conditions. In the same way, the individual effect of (S:L) ratio on salmonid heads hydrolysis was also finally tested. In all optimisation experiments, after hydrolysis (3 h) the content of mini reactors were centrifuged (15000 x g/20 min) and the sediments (mainly bones) and supernatants quantified.

2.3 Production of salmonids and BW hydrolysates at lab scale

Lab-scale hydrolysis were carried out in a controlled pH-Stat system with a 5 L glass-reactor (suspending 1 or 2 kg of milled discards in 2 L of distilled water, (S:L) ratio of 1:1 and 1:2 w/v, respectively) using 5M NaOH as alkaline reagent for pH-control (equipment presents in the IIM-CSIC). Optimal conditions obtained in previous section for each studied substrate was used in this step. At the end of the hydrolysis (3 or 4 h), the content of the reactors was filtered (100 □m) to remove bones, the liquid hydrolysates were centrifuged (15000 x g/20 min) to recover oils (adding a step of decantation for 15 min) and final FPHs were quickly heated (90ºC/15 min) for enzyme deactivation. A schematic flowchart of FPHs processing from by-products and fish discards is shown in Figure 3.

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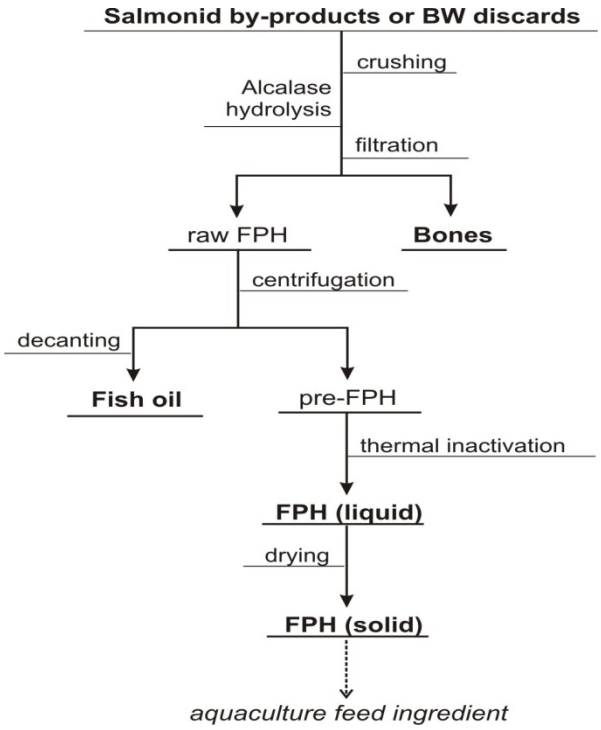

Figure 3. Schematic flowchart of by-products and discards processed through enzymatic hydrolysis.

2.3 Preliminary studies of enzyme hydrolysis of seabream by-products

The hydrolysis of triturated samples of skins and bones (SB-S) and heads (SB-H) from seabream were investigated. The lab-assays were carried out in flasks of 1L of volume in an INNOVA 40 (New Brunswick Scientific, Edison, New Jersey, USA orbital shaker, Figure 4) located in ANFACO using the experimental conditions showed in Table 3. Moreover, a blank assay was performed to quantify the protein extraction by seabream endogenous enzymes. The aim of these assays was to compare the yield obtained with the different enzymes, in similar operation conditions of pH and temperature with a (S:L) ratio of 1:2 recommended by the supplier. Besides, reaction time (1 h) and enzyme concentration used were selected in order to minimize the hydrolysis costs. After the hydrolysis, enzymes were inactivated by temperature maintaining the flasks at 90ºC during 10 min. Finally, samples were centrifuged and filtrated to remove the solid phase and to study the protein content of liquid one.

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Table 3. Preliminary enzymatic hydrolysis of seabream by-products performed in ANFACO.

Assay	Enzyme concentration (%, v/w)			T (ºC)	pH
	Protamex	Flavorpro	Alcalase		
SB-S1	1	-	-	55	7.0
SB-S2	-	1	-	55	7.0
SB-S3	-	-	1	55	9.0
SB-S Blank	-	-	-	55	7.0
SB-H1	1	-	-	55	7.0
SB-H2	-	1	-	55	7.0
SB-H3	-	-	1	70	9.0
SB-H4	-	0.5	0.5	55	8.0
SB-H Blank	-	-	-	70	9.0

Figure 4. Orbital shaker Innova 40 presents in ANFACO showing seabream hydrolysates.

2.4 Chemical and biological analysis

The proximal composition of by-products and discards was determined by means of: 1) water, ash and organic matter content (AOAC, 1997), 2) total nitrogen (AOAC, 1997) and total protein as total nitrogen x 6.25 and 3) total lipids (Bligh and Dyer, 1959). The profile of fatty acids from fish oil was analysed by GC-chromatography after chemical methylation (Lepage and Roy, 1986). The basic analyses of FPH were: 1) total soluble protein (Lowry et al., 1951); 2) total sugars (Dubois et al., 1956); 3) total protein as total nitrogen x 6.25 (AOAC, 1997); 4) proximal composition (as previously cited), 5) amino acids content (quantified by ninhydrin reaction, using an amino acid analyser (Biochrom 30 series, Biochrom Ltd., Cambridge, UK), according to the method of Moore et al. (1958); and 6) *in vitro* digestibility

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(pepsin method: AOAC Official Method 971.09 following the modifications reported by Miller et al., (2002). Molecular weights of FPH were determined by Gel Permeation Chromatography (GPC). The system used was an Agilent 1260 HPLC consisting of quaternary pump (G1311B), injector (G1329B), column oven (G1316A), refractive index (G1362A), diode array (G1315C) and dual-angle static light scattering (G7800A) detectors. Standard and samples were eluted with a 0.15M ammonium acetate/0.2M acetic acid buffer at pH 4.5 pumped at 1 mL/min through four columns (PSS, Germany): Proteema precolumn (5 µm, 8 x 50 mm), Proteema 30Å (5 µm, 8 x 300 mm), Proteema 100Å (5 µm, 8 x 300 mm) and Proteema 1000Å (5 µm, 8 x 300 mm) after a 100 µL injection. Column oven and light scattering detector were kept at 30ºC and refractive index detector was maintained at 40ºC. Detectors were calibrated with a polyethylene oxide standard (PSS, Germany) of 106 kDa (Mw) and polydispersity index 1.05. Absolute molecular weights were estimated with refractive index increments (dn/dc) of 0.185.

Antihypertensive and antioxidant (AO) activities were also determined in final FPH samples obtained at the end of hydrolysis. Briefly, *in vitro* Angiotensin I-converting enzyme (ACE) inhibitory activity (I_{ACE}) was based on the protocol defined by Estévez et al. (2012) and IC_{50} values (protein-hydrolysate concentration that generates a 50% of I_{ACE}) were calculated according to dose-response modelling as previously reported (Amado et al., 2013). The antioxidant capacity of FPH were analysed by three methods: a) 1,1-Diphenyl-2-picryhydrazyl (DPPH) radical-scavenging ability following the microplate protocol developed by Prieto et al. (2015a); b) ABTS (2,2´-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) bleaching method according the microplate protocol recently published (Prieto et al., 2015a); c) Crocin bleaching assay using an optimised microplate report (Prieto et al., 2015b). All antihypertensive and AO determinations were done in triplicate employing FPH samples at concentration of 1 g/L of soluble protein.

2.5 Numerical and statistical analyses

Data fitting procedures and parametric estimations were carried out by minimisation of the sum of quadratic differences between observed and model predicted values, using the non-linear least-squares (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel spreadsheet. Confidence intervals from the parametric estimates (Student's t test) and consistence of mathematical models (Fisher's F test) were evaluated by "SolverAid" macro.

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3. Results and Discussion

3.1 FPHs from salmonid by-products

The proximate composition of salmonids raw materials is summarized in Table 4. The moisture of these samples ranged between 66% and 70% and the organic matter was higher in salmon than rainbow trout and also superior in trimmings+frames compared to heads by-products. TF showed greater amount of proteins and lower level of total lipids. S_H was the fattest by-product (Table 4). Optimization of salmonid heads hydrolysis was studied following the factorial designs defined in Table 1 using the mentioned pH-stat system (100 mL reactor).

Table 4. Proximate composition of salmonids by-products in terms of moisture (Mo), organic matter (OM) and ashes (Ash). Total lipids (Lip), proteins (Pr-tN, as total nitrogen x 6.25) and proteins after degreasing samples (Pr-tN*) were determined using dried substrates. Error bars are the confidence intervals for n=3-4 (samples from different batch) and α=0.05.

	Mo (%)	OM (%)	Ash (%)	Lip (%)	Pr-tN (%)	Pr-tN* (%)
RT_H	69.6±0.2	27.7±0.2	2.7±0.1	47.7±0.4	35.2±1.0	66.1±3.4
RT_TF	66.5±0.9	30.6±0.4	3.0±0.5	44.0±0.9	45.1±2.3	84.5±2.5
S_H	62.6±1.0	34.7±1.2	2.7±0.6	54.3±0.3	30.1±1.0	78.3±4.0
S_TF	63.1±0.5	34.0±1.0	2.9±0.3	46.2±0.9	44.8±2.0	82.9±3.0

Figures 5 and 6 (A, B and C plots) show the experimental data and the corresponding theoretical surfaces for the three responses of RT_H and S_H hydrolysis. Empirical equations were calculated from factorial data and optima values determined for each case studied (Table 5). From a statistical point of view, the degrees of correlation between the experimental data and predicted by the equations (degree of explicability of the equations) were ranging from 76% to 91% for the three responses and the two substrates evaluated. Both equations were also statistically robust since Fisher tests (F1 and F2) were satisfied (data not shown). The average data of pH_{opt} and T_{opt} were 8.27 and 56.2ºC for RT_H and 8.98 and 64.2ºC for S_H.

Based on these conditions, the concentration of protease and the S:L ratio that maximizes the production of FPHs was subsequently evaluated in one-factor-at-a-time method (Figure 5D-I). For RT_H, the values of H_m increased at higher (S:L) but not significant difference were found among ratios for the responses V_{dig} and Prs ($p>0.05$). The effect of Alcalase concentration followed a similar trend: higher H_m value at larger enzyme added but similar response for V_{dig} and Prs results. Thus, (1:1) ratio and 0.1% (v/w) of Alcalase were selected as the best conditions to digest the trout by-products therefore reducing the costs of the hydrolyzation.

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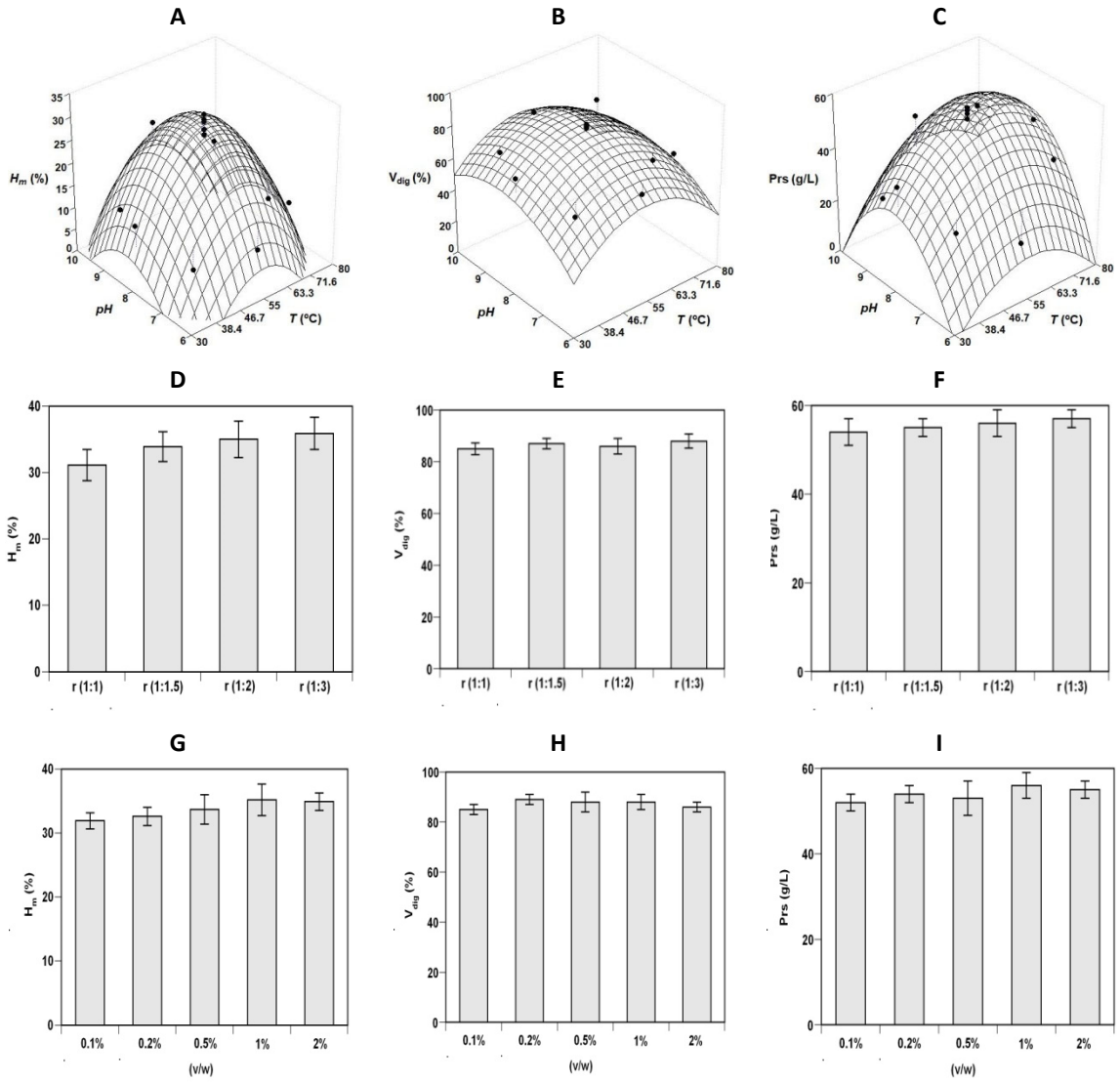

Figure 5. Optimization studies of RT_H hydrolysis by Alcalase. Experimental data and predicted response surfaces describing the joint effect of pH and *T* on H_m response (A), V_{dig} response (B) and Prs response (C) as defined in Table 6. D: Individual effect of Alcalase concentration over H_m. E: Individual effect of Alcalase concentration over V_{dig}. F: Individual effect of Alcalase concentration over Prs. G: Individual effect of S:L ratio over H_m. H: Individual effect of S:L ratio over V_{dig}. I: Individual effect of S:L ratio over Prs. Error bars are the confidence intervals for n=2 (replicates of different hydrolysates) and α=0.05.

In a similar way, the single effect of solid:liquid ratio and Alcalase concentration on S_H hydrolysis were tested maintaining constant the average values of pH_{opt} and T_{opt} previously defined. The three responses are displayed in Figure 6 (D-I plots) indicating the lack of significant differences between the ratios studied. All responses from hydrolysis (H_m, V_{dig} and Prs) rised with the increase in the protease used up to an Alcalase concentration of 0.2%

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v/w. Thus, ratio of (1:1) and 0.2% of enzyme were the conditions chosen for carrying out the hydrolysis of salmon wastes to produce aquaculture feed ingredients.

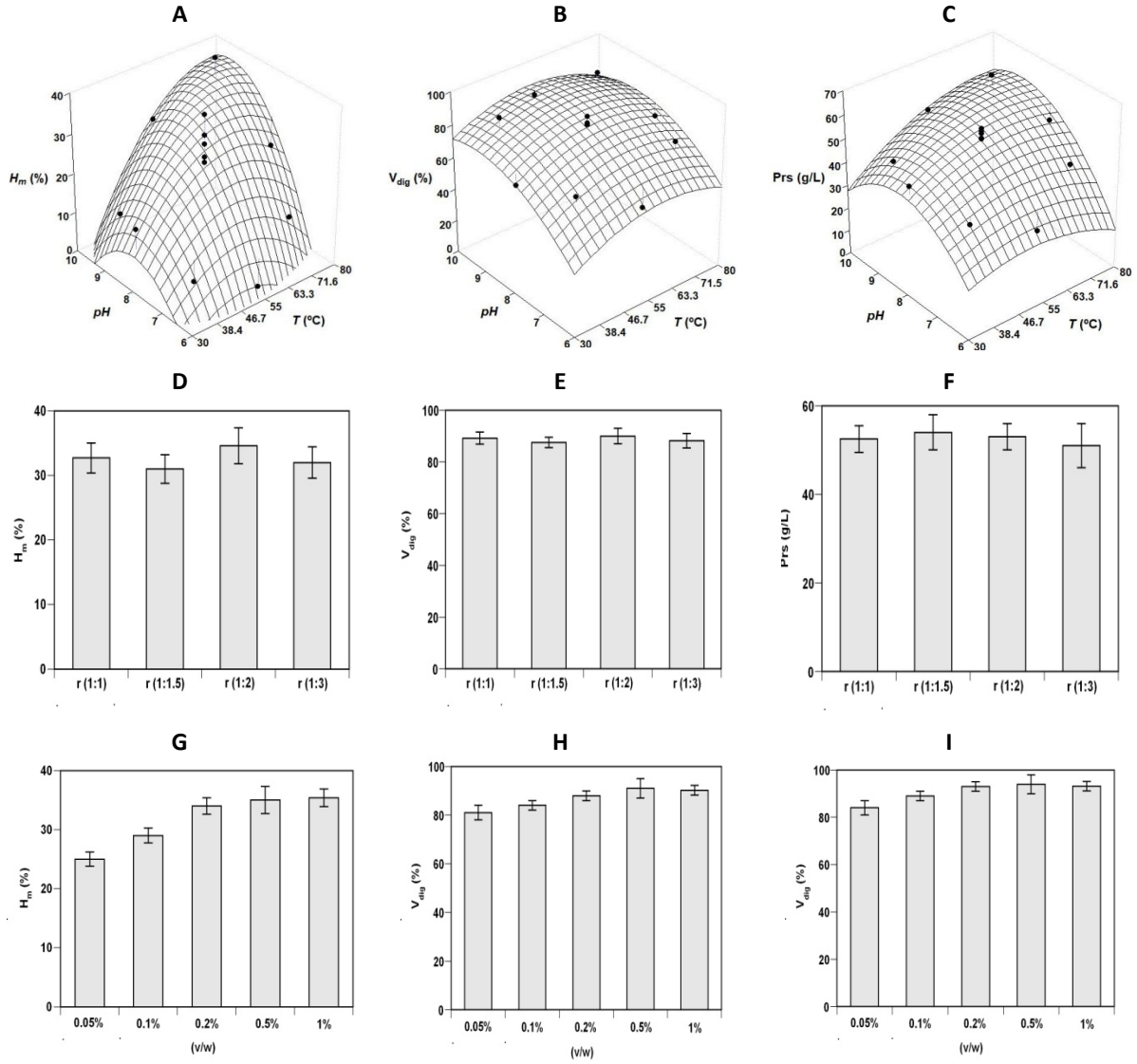

Figure 6. Optimization studies of S_H hydrolysis by Alcalase. Experimental data and predicted response surfaces describing the joint effect of pH and *T* on H_m response (A), V_{dig} response (B) and Prs response (C). D: Individual effect of Alcalase concentration over H_m. E: Individual effect of Alcalase concentration over V_{dig}. F: Individual effect of Alcalase concentration over Prs. G: Individual effect of S:L ratio over H_m. H: Individual effect of S:L ratio over V_{dig}. I: Individual effect of S:L ratio over Prs. Error bars are the confidence intervals for n=2 (replicates of different hydrolysates) and α=0.05.

In summary, the productions of FPHs from salmonids were then performed in optimal and with the following experimental conditions: 1) Alcalase 0.1%, pH8.27, T56.2ºC, 200 rpm, 3 h of hydrolysis and S:L=1:1 for RT_H and RT_TF; 2) Alcalase 0.2%, pH8.98, T64.2ºC, 200 rpm, 3 h of hydrolysis and S:L=1:1 for S_H and S_TF. All productions were done, among 18-20

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batches, in a 5L-pH-stat reactor with 2 kg of ground initial raw material, as it has been shown before, in this case the time-course hydrolysis was accurately described by Weibull equation (Table 6, Figure 7).

Table 5. Polynomial equations describing the joint effect of temperature (*T*) and *pH* on Alcalase hydrolysis of RT_H and S_H. Optima values of the two variables (T_{opt}, pH_{opt}) to achieve the theoretical maximum responses (Y_{max}) from the empirical equations was also calculated.

	Second order models	R^2_{adj}	T_{opt} (ºC)	pH_{opt}	Y_{max}
RT_H	H_m (%) = 27.10 + 2.72 *pH* – 9.16 T^2 – 6.39 pH^2	0.909	55.0	8.30	30.1%
	V_{dig} (%) = 83.34 – 2.35 *T* + 3.21 *pH* – 12.17 T^2 – 6.86 pH^2	0.759	53.3	8.33	83.8%
	Prs (g/L) = 55.41 + 4.42 *T* + 3.60 *pH* + 2.25 *T pH* – 8.19 T^2 – 13.1 pH^2	0.869	60.2	8.17	56.3 g/L
S_H	H_m (%) = 29.44 + 5.30 *T* + 8.54 *pH* – 5.36 T^2 – 7.30 pH^2	0.803	63.8	8.83	33.3%
	V_{dig} (%) = 85.72 + 2.11 T + 8.76 *pH* – 5.62 T^2 – 5.62 pH^2	0.779	58.4	9.10	89.3%
	Prs (g/L) = 54.71 + 3.49 T + 6.77 *pH* + 3.25 *T pH* – 3.30 T^2 – 6.82 pH^2	0.902	70.5	9.00	58.6 g/L

Table 6. Weibull equation applied to describe FPHs kinetics and corresponding parameters definition.

$H = H_m\left\{1-\exp\left[-\ln 2\left(\frac{t}{\tau}\right)^{\beta}\right]\right\}$ $v_m = \frac{\beta H_m \ln 2}{2\tau}$	*H* is the degree of hydrolysis (%); *t* the time of hydrolysis (min); H_m the maximum degree of hydrolysis (%); β a parameter related with the maximum slope of muscle hydrolysis (dimensionless); v_m the maximum rate of hydrolysis (% min^{-1}) and τ the time required to achieve the semi-maximum degree of hydrolysis (min).

The agreement between experimental and simulated data was total (determination coefficients higher than 0.999) and the statistical feasibility and robustness of equation was also confirmed by F-Fisher test (p<0.005) (Table 7). The maximum degrees of hydrolysis were slightly greater in salmon by-products whereas the maximum rates of hydrolysis were slower on trout wastes. In addition, these numerical values of parameters were similar to those obtained in 100 mL-reactor and reported in Table 6 and Figures 5 and 6.

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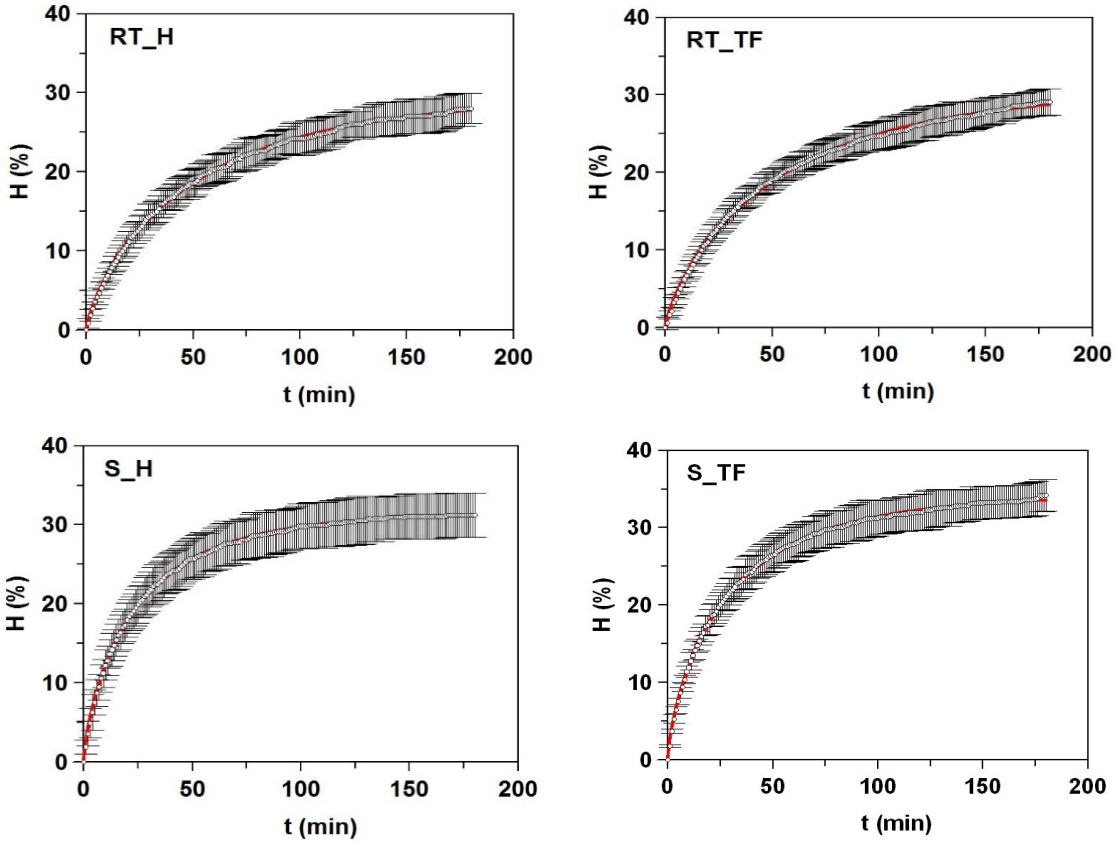

Figure 7. Alcalase hydrolysis of aquaculture by-products: RT_H: heads of rainbow trout, RT_TF: trimmings and frames of rainbow trout, S_H: heads of salmon and S_TF: trimmings and frames of salmon. Experimental data of kinetics (symbols) were fitted to the Weibull equation (continuous line). Error bars are the confidence intervals for n=18-20 (replicates of different hydrolysates) and α=0.05.

Table 7. Kinetic parameters and confidence intervals obtained from Weibull equation modeling the time course of the hydrolysis degree (*H*) of salmonid by-products catalysed by Alcalase. Determinaton coefficients (R^2) of fittings and p-values are also shown.

	H_m (%)	β (dimensionless)	τ (min)	v_m (% min^{-1})	R^2	p-values
RT_H	29.66±0.17	0.801±0.008	32.07±0.37	0.257±0.004	0.999	<0.005
RT_TF	30.94±0.25	0.807±0.011	34.19±0.56	0.253±0.005	0.999	<0.005
S_H	31.55±0.06	0.770±0.006	15.85±0.11	0.531±0.004	1.000	<0.005
S_TF	34.27±0.10	0.756±0.007	18.41±0.14	0.488±0.005	0.999	<0.005

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The processing of salmonid hydrolysates was performed using the protocol showed in Figure 3. Figure 8 presents different photographs related with the production of FPH and other resulting products.

Figure 8. Different sequences of enzymatic hydrolysis of salmonid wastes in a 5L-pH-stat reactor (A) with the differential recovery of clean bones (B) and fish oils (F) together with the production of liquid (G) and dried FPHs (I) by means of a freeze-drying equipment (H). The rest of the images show the cooling of hydrolysates (C) prior centrifugation (D) and decantation of FPHs to separate fish oils (E). Processes performed in the IIM-CSIC.

For example, one by-product of the FPH production are bones, the percentage recovered in filters after hydrolysis was around 9-12% (w/w of initial substrate) and the yield is higher in

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salmon than in trout (Table 8). Besides, the recovered bones did not present a significant amount of residual muscle or organic material. Oil was also separated from FPH, in this case the yield was around 9-11% (v/w of initial substrate) and the highest volume of fish oil was found in S_H. The digestion of these solid by-products by Alcalase was lower than those reported for BW, but in all cases higher than 84%. The composition in fatty acids of the oils was summarized in Table 9. Oleic acid (more than 50%) and linoleic acid (more than 12%) were the main fatty acids present in oils and the amount of DHA and EPA, the most relevant from their biological properties, did not exceed 3%. Omega-3/omega-6 ratios for salmonid oils were lower or equal than 0.5 revealing their low potential as ingredients for nutraceutical applications (Simonopoulos and DiNicolantonio, 2017).

Table 8. Mass balances and proximal analysis of the products obtained from Alcalase hydrolysates of salmonid by-products. Showed errors are the confidence intervals for n=18-20 (replicates of different hydrolysates) and α=0.05. m_b: percentage of the bones recovered; V_{oil}: percentage of oil recovered; V_{dig}: percentage of digestion/liquefaction of the solid by-products to the liquid phase; Prs: Total soluble protein determined by Lowry; TS: Total sugars; Dig: Digestibility; Pr-tN: Total protein determined as total nitrogen x 6.25.

FPH	m_b (%)	V_{oil} (%)	V_{dig} (%)	Prs (g/L)	Pr-tN (g/L)	TS (g/L)	Dig (%)
RT_H	9.98±1.31	9.36±0.75	88.4±1.2	47.8±4.8	53.1±1.9	1.40±0.10	92.5±3.2
RT_TF	9.43±0.52	10.63±0.42	84.4±1.1	53.9±5.1	58.4±2.7	1.22±0.10	93.2±2.5
S_H	11.13±1.36	11.37±0.60	89.8±0.7	61.0±1.3	64.2±3.1	1.29±0.10	93.0±2.2
S_TF	11.59±0.44	9.30±0.12	86.3±1.1	69.7±2.1	71.1±2.6	1.50±0.10	94.1±2.8

Depending on the method used for the quantification of proteins, the levels of protein material present in aquaculture FPHs ranged from 48-69 g/L for Prs, 53-71 g/L for Pr-tN and 52-73 g/L for Pr (Σaa). TF substrates led to a larger concentration of protein in comparison to heads, regarding species, salmon FPHs showed higher protein concentration than those obtained with trout. The *In vitro* digestibility (Dig) of FPHs was excellent, in all cases, it has been found values higher than 92% without significant differences between FPHs ($p>0.05$).

Regarding amino acids content in FPHs, the main ones are Glu, Asp and Gly but all the essential amino acids (Ile, Leu, Val, Lys, Met, Phe, Thr, His and Arg) are included in the salmonid hydrolysates produced here (Table 10). These two hydrolysates protein and amino acid contents and digestibilities shown in Tables 6 and 10 were in concordance with the chemical, functional and nutritional properties necessary for their utilization in animal feed and microbial culture media (Martínez-Álvarez et al., 2015; Vázquez et al., 2016b).

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Table 9. Fatty acids content (as % of total fatty acids) in the fish oils recovered from RT_H, RT_TF, S_H and S_TF, complementary to the production of FPHs. Errors are the confidence intervals for n=6 (samples from different hydrolysates) and α=0.05.

Formula	Fatty acids	RT_H	RT_TF	S_H	S_TF
C8:0	Caprylic acid	0.03±0.03	-	-	-
C10:0	Capric acid	0.07±0.01	-	-	-
C12:0	Lauric acid	0.12±0.01	-	-	-
C13:0	Tridecanoic acid	0.06±0.01	-	-	-
C14:0	Myristic acid	0.74±0.01	0.69±0.01	2.23±0.25	2.29±0.16
C14:1	Myristoleic acid	0.10±0.03	0.01±0.00	-	-
C15:0	Pentadecanoic acid	0.13±0.01	0.05±0.01	-	-
C15:1	Pentadecenoic acid	4.10±0.11	4.42±0.05	-	-
C16:0	Palmitic acid	6.13±0.09	6.58±0.03	7.49±0.82	6.65±0.73
C16:1n7c	Palmitoleic acid	2.77±0.18	3.49±0.17	2.09±0.24	2.06±0.67
C17:0	Heptadecanoic acid	0.17±0.09	0.06±0.00	-	-
C17:1	Heptadecanoleic acid	0.69±0.51	0.07±0.01	-	-
C18:0	Stearic acid	1.80±0.05	1.87±0.02	2.13±0.11	2.07±0.17
C18:1n9c,t	Oleic acid	52.85±0.46	57.48±0.16	44.56±1.31	40.90±2.20
C18:2n6c,t	Linoleic acid	12.06±0.27	13.19±0.14	22.66±2.20	22.31±1.42
C20:0	Arachidic acid	0.42±0.22	0.09±0.02	-	-
C18:3n6	□-Linolenic acid	0.31±0.04	0.24±0.00	-	-
C18:3n3	Linolenic acid	2.04±0.06	2.13±0.03	5.83±0.85	6.79±1.68
C20:1n9	Eicosenoic acid	1.88±0.68	2.23±0.27	6.41±2.21	10.52±3.06
C21:0	Henicosanoic acid	0.50±0.01	0.50±0.03	-	-
C20:2n6	Eicosadienoic acid	0.51±0.07	0.95±0.15	1.01±0.20	1.16±0.08
C22:0	Docosanoic acid	0.16±0.02	0.06±0.01	-	-
C20:3n6	Dihomo-linolenic acid (DGLA)	0.32±0.04	0.30±0.03	-	-
C20:4n6	Arachidonic acid	0.17±0.08	0.22±0.01	-	-
C23:0	Tricosanoic acid	0.11±0.01	0.03±0.01	-	-
C21:4n3	Heneicosatetraenoic acid	1.86±0.14	0.59±0.09	1.88±0.36	2.09±0.18
C22:2n6	Docosadienoic acid	0.18±0.13	0.01±0.00	-	-
C20:5n3	Eicosapentaenoic acid (EPA)	0.63±0.04	0.55±0.02	0.34±0.08	0.38±0.05
C24:0	Lignoceric acid	0.26±0.02	-	-	-
C24:1n9	Nervonic acid	6.72±0.74	1.95±0.80	-	-
C22:6n3	Docosahexaenoic acid (DHA)	2.14±0.09	2.27±0.07	3.36±0.23	2.78±0.45
	DHA+EPA (%)	2.77±0.13	2.82±0.08	3.70±0.27	3.16±0.45
	r: ω-3 / ω-6	0.49±0.01	0.37±0.01	0.49±0.06	0.51±0.06

On the other hand, the average molecular weight (Mw) of peptides in salmonid FPHs were (Table 10): 1944±264 Da (index of polydispersity, PD: 2.11) for RT_H, 1682±65 Da (PD: 1.58) for RT_TF, 1945±136 Da (PD: 1.57) for S_H and 1442±51 Da (PD: 1.53) for S_TF. In the case of the number average molecular weight (Mn) of peptides present in the hydrolysates, the

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results obtained from gel permeation chromatography (GPC) were: 920±110 Da for RT_H, 1067±152 Da for RT_TF, 1235±91 Da (PD: 1.57) for S_H and 944±40 Da (PD: 1.53) for S_TF. The percentage of protein material included into different ranges of sizes is also indicated in Table 11. A representation of GPC-profiles of such peptides distribution from FPHs is displayed in Figure 9.

Table 10. Amino acids content of FPH (% or g/100 g total amino acids) from salmonid by-products. OHPro: hydroxyproline. Pr: protein concentration calculated. in g/L. as the total sum of amino acids present in FPH. Errors are the confidence intervals for n=16-20 (replicates of different hydrolysates) and α=0.05.

Amino acids	RT_H	RT_TF	S_H	S_TF
Asp	9.78±0.19	10.32 ±0.20	9.61±0.30	10.33±0.06
Thr	4.38±0.22	4.44±0.15	3.83±0.39	2.95±0.04
Ser	5.00±0.20	4.83±0.06	4.98±0.05	4.98±0.11
Glu	13.89±0.14	14.98±0.34	13.42±0.45	13.23±0.08
Gly	9.93±1.10	8.94±2.94	12.49±1.11	11.08±0.27
Ala	7.19±0.31	6.98±0.22	7.92±0.45	8.45±0.03
Cys	0.76±0.09	0.74±0.05	0.75±0.11	0.83±0.03
Val	4.35±0.33	4.24±0.22	3.39±0.16	3.44±0.30
Met	3.16±0.10	3.33±0.15	3.13±0.28	3.82±0.33
Ile	3.22±0.29	3.21±0.23	2.28±0.22	2.02±0.16
Leu	7.09±0.33	7.19±0.07	6.17±0.33	6.36±0.22
Tyr	3.36±0.18	3.39±0.22	3.37±0.45	4.40±0.17
Phe	4.38±0.25	4.09±0.15	4.93±0.89	7.15±1.13
His	2.20±0.49	2.18±0.02	2.00±0.13	2.11±0.17
Lys	7.78±0.42	8.60±0.13	7.04±0.46	7.96±0.37
Arg	5.97±0.10	5.96±0.14	5.69±0.40	4.44±0.03
OHPro	2.25±0.37	1.86±0.38	2.85±0.62	2.00±0.14
Pro	5.30±0.34	4.72±0.16	6.15±0.83	4.45±0.28
Pr (Σaa) (g/L)	51.96±2.83	66.08±1.72	66.08±3.72	72.71±2.13

The production of FPHs from salmonids is completed, we have still to analyse the antioxidant and antihypertensive properties of the hydrolysates and to finish the drying of some batches of liquid S_TF hydrolysates. Additionally, the production of FPHs from turbot (*Scophthalmus maximus*) by-products to provide as ingredient for GAIN project WP1 was not initially included in the GAIN proposal since at that time turbot was rather sold unprocessed both in the Spanish and Portuguese markets. However, the most important turbot producer company in Europe (Stolt Sea Farm, A Coruña, Spain) has recently started filleting turbot for other European markets and, therefore, it is now generating significant amounts of by-products such as heads, trimmings, frames and viscera. We have just received turbot by-products and will begin in the next few days with factorial designs to optimize the best conditions for the production of enzymatic hydrolysates. We hope to finish all aquaculture FPHs in July 2019 to supply SPAROS with these feed ingredients. In the annexe, an example of the technical data sheet for FPHs is shown. This template will be filled for each FPH, with

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all the analytic results incorporated, when final FPHs is supplied.

Table 11. Average molecular weights (as Mn and Mw) and associated confidence intervals for n=5 (samples from different hydrolysates) and α=0.05. Percentage of peptides distribution between molecular weight ranges was also determined. PDI: polydispersity index.

FPH	Mn (Da)	Mw (Da)	PDI	<1800 Da (%)	<3000 Da (%)	1000-3000 Da (%)
RT_H	920±110	1944±264	2.113	73.0±4.5	86.0±2.9	38.0±3.9
RT_TF	1067±152	1682±65	1.576	74.4±1.1	89.6±1.5	47.6±8.1
S_H	1235±91	1945±136	1.574	64.6±3.6	85.0±2.0	52.0±3.9
S_TF	944±40	1442±51	1.528	77.8±1.4	91.7±1.0	43.3±2.3

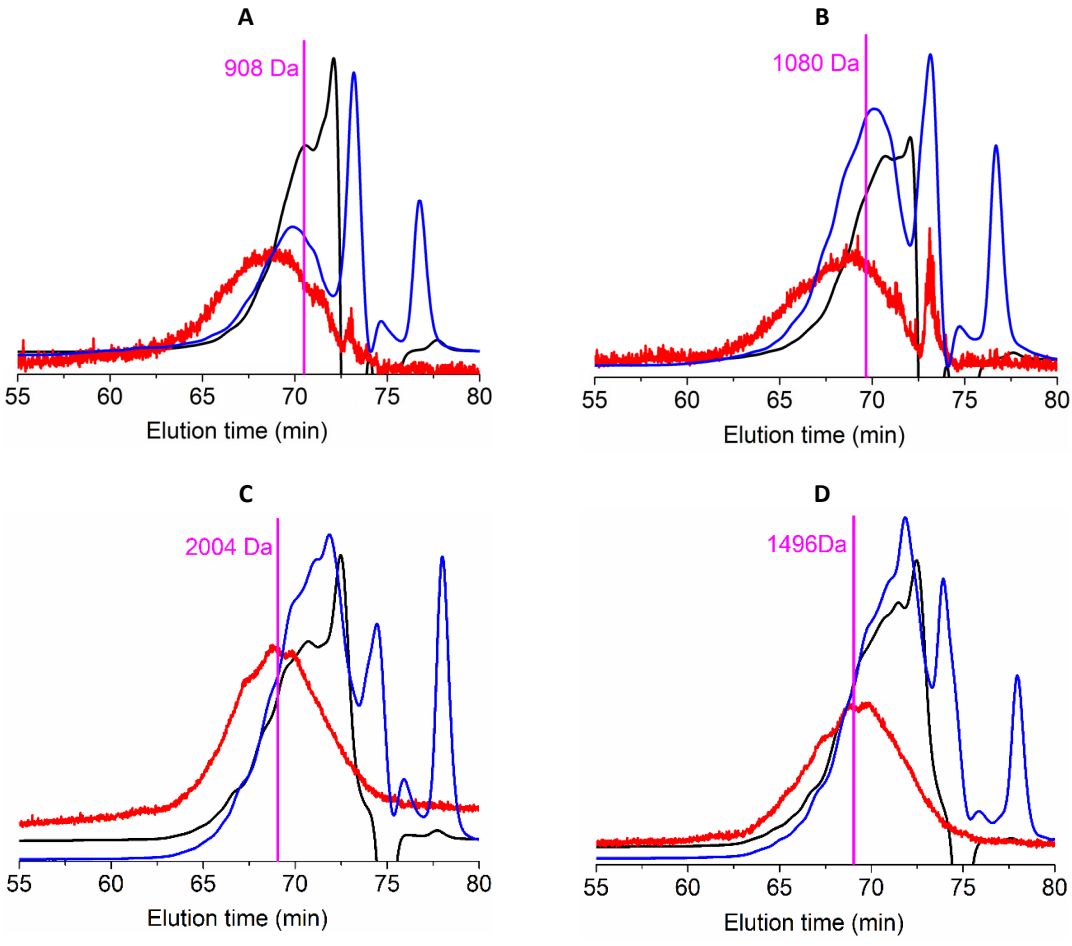

Figure 9. GPC eluograms of FPHs (A: RT_H, B: RT_TF, C: S_H and D: S_TF). Black line: refractive index; blue line: UV (232 nm); red line: right angle light scattering; vertical lines: number average molecular weight (Mn).

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3.2 FPHs from seabass and seabream by-products

Skins and bones (SB-S) and heads (SB-H) from seabream were chemically characterized (Table 12), further lipid characterization was performed and in Table 13 the complete fatty acids composition is shown.

Even in by-products such as skins and viscera, the lipid content is almost equal to that of proteins: therefore, it seems difficult to obtain fat-free hydrolysates from seabream. The other by-products will be characterized and used once the lab-scale experiments with Skin and bones will be finished.

Table 12. Overview of chemical characterization of the seabream skins and bones (SB) and heads (SH) by-product. Errors are standard deviation.

	SB-S	**SB-H**
Humidity (%)	61 ± 4	58 ± 4
Proteins (%, wet basis)	16 ± 1.2	14.8 ± 1.1
Lipids (%, wet basis)	14.4 ± 0.9	18.3 ± 1.1
Ashes (%, wet basis)	8.1 ± 0.7	8.6 ± 0.8
Fatty acids: saturated (%, wet basis)	3.0 ± 0.9	4.0 ±1.4
Fatty acids: monounsaturated (%, wet basis)	6	8
Fatty acids: polyunsaturated (%, wet basis)	4.1	5.1
EPA+DHA (%, wet basis)	1.1	1.36
Omega 3 (%, wet basis)	1.87	2.29
Omega 6 (%, wet basis)	2.10	2.67
Trans (%, wet basis)	0.12	0.15

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Table 13. Fatty acids composition of seabream by-products (SB-B and SB-S).

Fatty acid	Name	Concentration	
		SB-S (%)	SH-H (%)
C14:0	Myristic acid	2.37	2.46
C14:1	Myristoleic acid	0.11	0.14
C15:0	Pentadecanoic acid	0.28	0.30
C16:0	Palmitic acid	13.93	15.22
C16:1T	Trans-palmitoleic acid	0.23	0.24
C16:1 (n-7+n-9)	Palmitoleic acid	5.52	6.04
C17:0	Heptadecanoic acid	0.29	0.27
C17:1	Heptadecanoleic acid	0.58	0.59
C18:0	Stearic acid	3.04	3.16
C18:1T	Trans-Oleic acid	0.22	0.24
C18:1n-9	Oleic acid	33.13	33.60
C18:1n-7	cis-Vaccenic acid	2.79	2.82
C18:2T	Trans- Linoleic acid	0.40	0.40
C18:2n-6	Linoleic acid	14.07	14.07
C18:3n-6	□-Linolenic acid	0.00	0.16
C20:0	Arachidic acid	0.47	0.28
C18:3n-3	Linolenic acid	2.49	2.41
C20:1	Eicosenoic acid	1.85	1.72
C18:4n-3	Octadecatetraenoic acid	0.18	0.15
C21:0	Henicosanoic acid	0.68	0.51
C20:2	Eicosadienoic acid	0.90	0.59
C20:3n-6	Dihomo-linolenic acid (DGLA)	0.47	0.36
C22:0	Docosanoic acid	0.00	0.00
C20:3n-3	Eicosatrienoic acid	1.26	1.16
C22:1	Erucic acid	0.00	0.00
C20:4n-6	Arachidonic acid	0.42	0.40
C23:0	Tricosanoic acid	0.52	0.51
C22:2	Docosadienoic acid	0.00	0.00
C20:5n-3	Eicosapentaenoic acid (EPA)	2.32	2.23
C24:0	Lignoceric acid	0.00	0.00
C24:1	Nervonic acid	0.50	0.44
C22:4n-6	Adrenic acid	0.29	0.28
C22:5n-3	Docosapentaenoic acid (DPA)	1.60	1.63
C22:6n-3	Docosahexaenoic acid (DHA)	5.72	5.51

The objective of this lab-scale study is to find the most promising enzyme or combination of enzymes with protease activity to be used in enzymatic hydrolysis process to reach maximum protein content in final dried products. The interest for animal-protein hydrolysates to be used in fish diets seems to be related with the increase of the nonspecific immunity of fishes. Moreover, these hydrolysates will be used as a good source of amino acids and the peptides can present antimicrobial, antioxidant or other bioactive properties, which can be interesting from fish farms. Therefore, they seem to be a promising source of bioactive peptides of considerable interest for animal care, always considering existing legislation (Khan et al., 2011; Santos et al., 2013; Zhang et al., 2013).

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A bibliographic review was done in order to select the appropriate enzymes for enzymatic hydrolysis of seabream by-products in order to obtain final protein hydrolysates with good effects in aquaculture feeding. Errore. L'origine riferimento non è stata trovata. 14 shows some interesting results.

Table 14. Bibliographic review of enzymes used and positive effect in aquaculture feeding.

Source	***Enzyme***	***Positive effect***	***Reference***
Tuna viscera	Alcalase	Growth	Chotikachinda et al. (2013)
Pollock	Protamex	Growth	Refstie et al. (2004)
Pollock	Alcalase+ Flavourzyme	Growth, feed efficiency	Zheng et al. (2012)
Pacific hake	Alcalase	Growth	Ho et al. (2014)

The lab-assays were carried out in flasks of 1L of volume and a blank assay was performed to investigate the protein extraction by seabream endogenous enzymes. The aim of these assays was to compare the yield obtained with the different enzymes, in similar operation conditions of pH and temperature, in the S:L ratio of 1:2 recommended by the supplier. Besides, reaction time (1 h) and enzyme concentration used were selected in order to minimize the hydrolysis costs. As mentioned, after the hydrolysis enzymes were inactivated by temperature maintaining the flasks at 90ºC during 10 min. Finally, samples were centrifuged and filtrated (Figure 10) to remove the solid phase and to study the protein content of liquid one (using the method of Lowry).

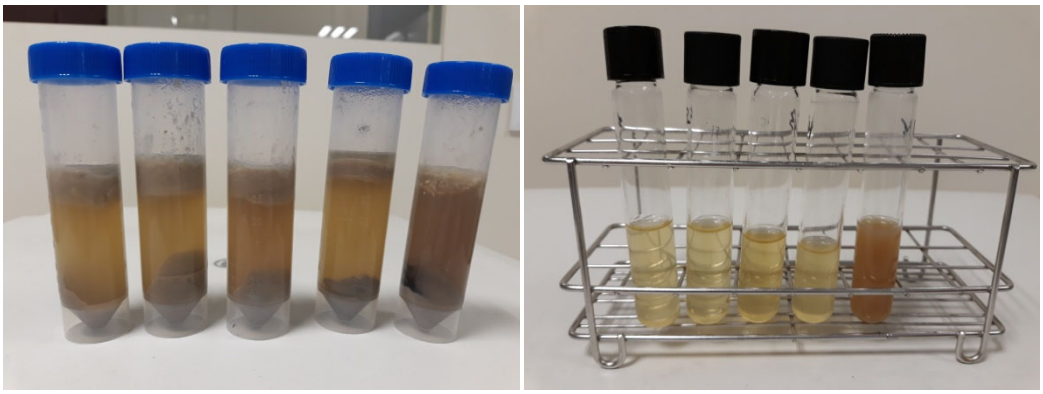

Figure 10. Aspect of hydrolyzed samples (from left to right, SB-H1. SA-H2, SB-H3, SB-H4 and SB-H Blank) after centrifugation (left) and filtration (right).

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Table 15 shows the protein yields obtained. They were calculated as the ratio of solubilized protein and the initial protein of the substrates considering the preliminary characterization and the quantity include in the experimental assays.

Table 15. Lab-scale assays with SB_S and SB_H by-products.

	Prs (g/L)	***Yield (%)***
SB-S1	25	31
SB-S2	27	34
SB-S3	29	36
SB-S Blank	13	16
SB-H1	28	38
SB-H2	23	31
SB-H3	26	36
SB-H4	28	38
SB-H Blank	20	20

Alcalase showed a better performance working with skin and bones substrates (
11) reaching almost 30%, but working with heads the combination of Alcalase and Flavorpro and the Protamex enzyme show better yields. In the assay with heads and Alcalase (SB-H3) the use of 70ºC (its theoretical temperature to reach optimal activity) did not show an important increase respect other enzymes. Moreover, the combination of Alcalase and Flavorpro slightly increase the yield over the individual use of Alcalase or Flavorpro, specially respect to the last one, where the yield increases over 20%.

In the assays carried out with heads, SB-H, blank assay, i.e., without enzyme, the protein recovery achieved was slightly higher than working with skins and bones, SB-S. This would be due to the blank samples in the heads showed high turbidity (that could affect to the Lowry analysis) but also due to the endogenous enzymes in heads or high temperature (70ºC respects to 55ºC) could increase the protein content in this sample (SB-H Blank).

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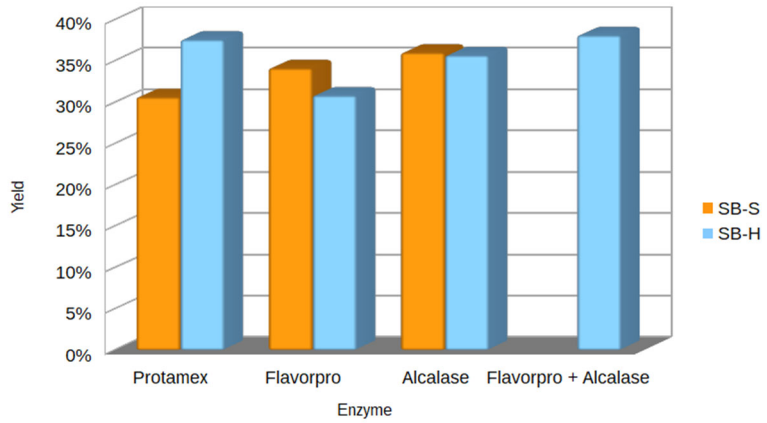

Figure 11. Yield values for protein extraction depending on the enzyme used for SB-S and SB-H by-products.

However, considering these data of FPH for by-products used, the protein recovery yields with all selected enzymes at these specific experimental conditions were quite low, since the thermal treatment or the endogenous enzymes acting in blank assays were enough to liberate almost half of the total protein content obtained. Therefore, the enzymatic hydrolysis must be optimized previous to pilot plant assays, to increase protein yields, using for instance higher enzyme percentage or extending the time of the assays.

Once the lab-scale assays are finished, the best conditions will be selected to be reproduced at pilot-scale in order to reach the amount of protein hydrolysate, to be processed in Task 1.2.

Valorisation of fishery discards as aquafeed ingredients

As in the case of salmonids, the optimization of the enzyme hydrolysis kinetics was also carried out by means of factorial design experiments (Table 1) and statistically analyzed by response surface methodology (RSM). The results of the optimization plan are presented in Figure 12. Experimental data and predicted surfaces for both responses were shown in Figure 12A and B.

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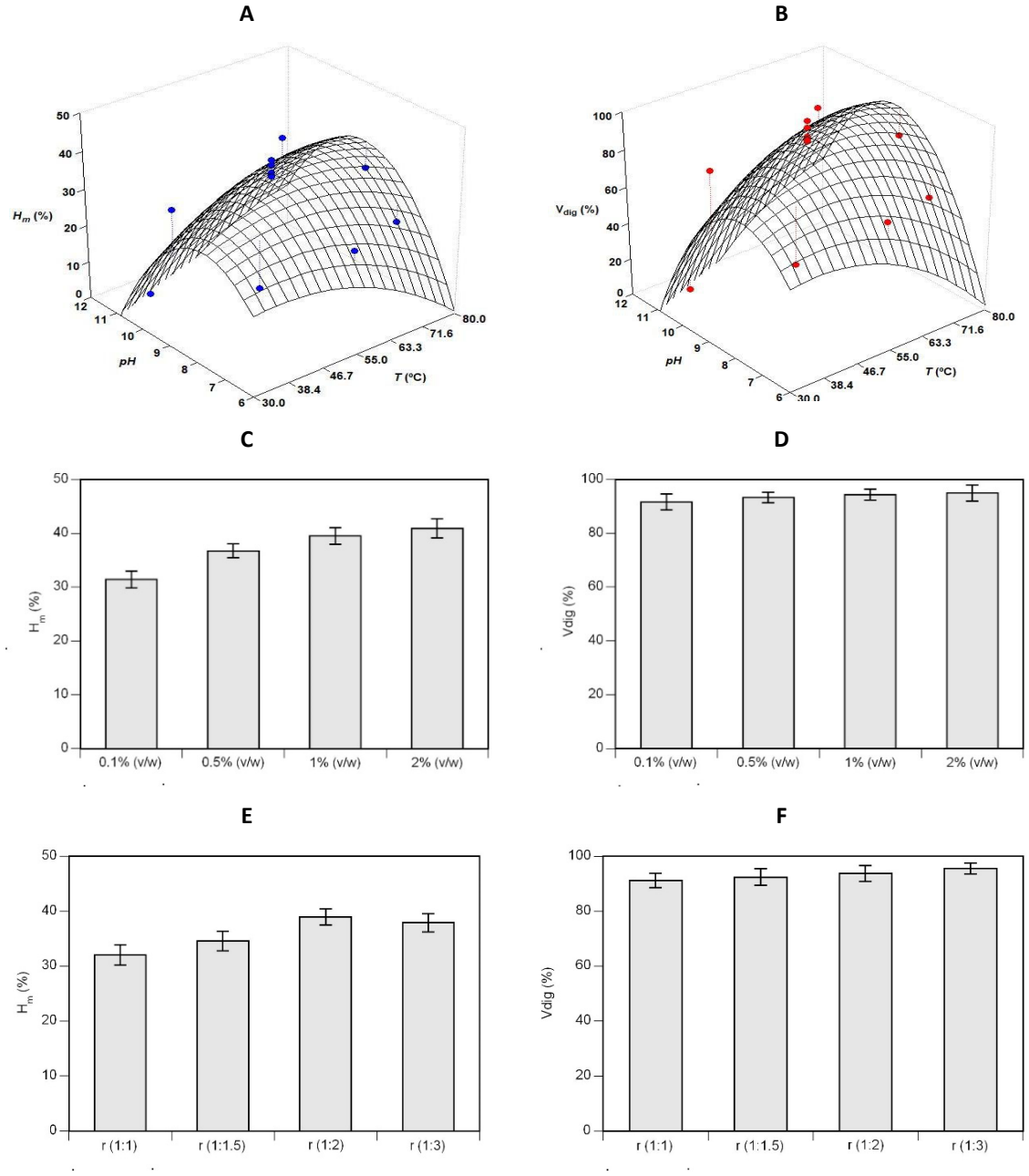

Figure 12. Optimization studies of Alcalase hydrolysis of BW discards. A: Experimental data and predicted response surfaces describing the simultaneous effect of pH and T on H_m response. B: Experimental data and predicted response surfaces describing the simultaneous effect of pH and T on V_{dig} response. C: Individual effect of Alcalase concentration over H_m. D: Individual effect of Alcalase concentration over V_{dig}. E: Individual effect of S:L ratio over H_m. F: Individual effect of S:L ratio over V_{dig}. Error bars are the confidence intervals for n=2 (replicates of different hydrolysates) and □=0.05.

Polynomial equations calculated from RSM analysis and the corresponding optima values of pH and T (pH_{opt} and T_{opt}) for both responses (H_m and V_{dig}), obtaining by numerical derivation, are summarized in Table 16. The goodness of fitting of the experimental data to the

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equation (as R^2_{adj}) and the predicted data of H_m and V_{dig} (maximum responses, Y_{max}) at the values of pH_{opt} and T_{opt} were also shown Table 16. Then, and using the average values (60ºC, pH 8.65), the individual effects of Alcalase concentration and solid:liquid ratio (S:L ratio) on the hydrolysis process were evaluated (Figure 12C-F).

Table 16. Polynomial equations describing the joint effect of temperature (T) and pH on Alcalase hydrolysis of BW. Optima values of the two variables (T_{opt}, pH_{opt}) to achieve the theoretical maximum responses (Y_{max}) from the empirical equations was also calculated.

Second order models	R^2_{adj}	T_{opt} (ºC)	pH_{opt}	Y_{max} (%)
H_m (%) = 37.04 + 2.88 T – 6.35 pH + 6.64 T pH – 3.22 T^2 – 12.56 pH^2	0.822	59.5	8.61	38.0%
V_{dig} (%) = 92.6 + 7.44 T – 13.8 pH + 16.7 T pH – 8.06 T^2 – 29.3 pH^2	0.814	60.5	8.69	94.7%

The difference between the concentrations of Alcalase 1% and 2% (39.5±1.6% and 40.9±1.8% for H_m and 94.3±2.0% and 95.0±3.0% for V_{dig}, respectively) were not statistically significant (p>0.05) but they were higher for H_m response and equal for V_{dig} response than employing 0.1% and 0.5% of Alcalase. Taking into account V_{dig} as dependent variable, the effect of increasing (S:L) ratios was not significant (p>0.05). For H_m, 1:2 and 1:3 ratios led to higher degrees of hydrolysis than 1:1 and 1:1.5 ratios.

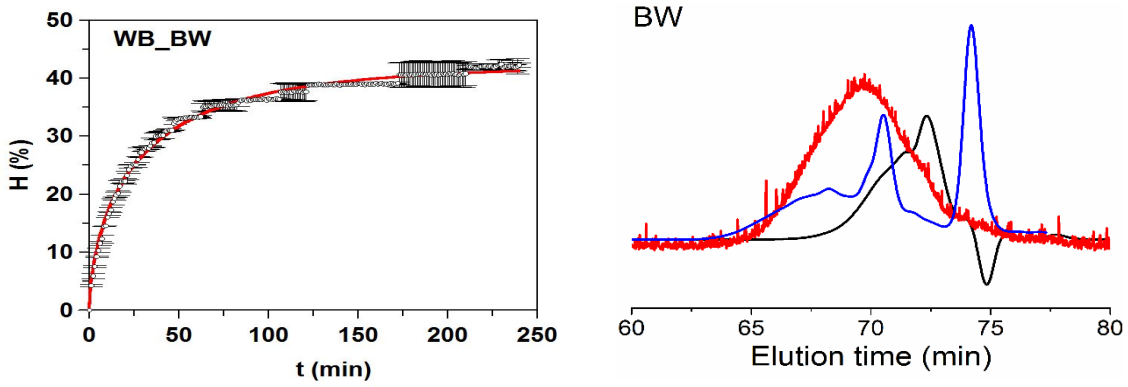

Figure 13. Left: Alcalase hydrolysis of whole bodies (WB) from BW discards. Experimental data of kinetics (symbols) were fitted to the Weibull equation (continuous line). Error bars are the confidence intervals for n=3 (replicates of different hydrolysates) and α=0.05. Right: Distribution of molecular weights of FPH analyzed by gel permeation chromatography (GPC), right angle light scattering detector (red); refractive index detector (black); UV detector (280 nm) (blue).

To check the hydrolysis scaling, the production of FPHs from BW were assayed in a 5L-pH-stat reactor using the prior defined conditions (Alcalase 1%, pH8.6, T60ºC, S:L=1:2, agitation of 200 rpm). The ability of Weibull equation for describing the kinetic profiles (Figure 13) was almost perfect with R^2= 0.992 and the consistency of the equation was statistically

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demonstrated (p<0.005). The numerical values of parameters were: H_m= 42.13±0.33%, β = 0.639±0.017, τ = 16.65±0.44 min and v_m= 0.56±0.02% min^{-1}. These results agreed with those found in optimization studies at lower scale (100 mL-reactor). Comparing with salmonid results, H_m values were significantly higher in FPHs of BW discards (p<0.05).

Table 17. Mass balance after hydrolysis and chemical characteristics of fish protein hydrolysates (FPHs). m_b: percentage of recovered bones; V_{oil}: percentage of the oil recovered; V_{dig}: percentage of the digestion/liquefaction of the solid by-products to the liquid phase; Prs: Total soluble protein determined by Lowry method; TS: Total sugars; Dig: Digestibility; Pr-tN: Total protein determined as total nitrogen x 6.25. Pr-Σaa: protein concentration calculated as the total sum of amino acids present in FPHs. Antioxidant activities determined by DPPH, ABTS and Crocin methods. I_{ACE}: *in vitro* Angiotensin I-converting enzyme (ACE) inhibitory activity. Error bars are the confidence intervals for n=3 (replicates of different hydrolysates) and α=0.05.

Matter balance and chemical composition of FPHs					
m_b (%)	6.7±0.3	**V_{oil} (%)**	0.95±0.07	**V_{dig} (%)**	93.4±0.8
Prs (g/L)	47.8±4.8	**Pr-tN (g/L)**	49.9±1.7	**TS (g/L)**	1.2±0.1
Dig (%)	97.2±0.4	**Pr-Σaa (g/L)**	49.9±2.7	-	-
Amino acids composition of FPHs					
Asp (%)	10.39±0.10	**Cys (%)**	0.54±0.02	**Phe (%)**	4.79±0.19
Thr (%)	4.40±0.16	**Val (%)**	4.52±0.02	**His (%)**	2.02±0.07
Ser (%)	5.13±0.13	**Met (%)**	3.63±0.15	**Lys (%)**	8.52±0.11
Glu (%)	14.90±0.27	**Ile (%)**	3.72±0.07	**Arg (%)**	6.00±0.08
Gly (%)	5.96±0.18	**Leu (%)**	8.36±0.07	**OHPro (%)**	3.04±0.52
Ala (%)	7.26±0.14	**Tyr (%)**	3.57±0.16	**Pro (%)**	3.72±0.28
Bioactivities of FPHs					
DPPH (%)	19.81±0.52	**ABTS (μg/mL)**	8.29±0.85	**Crocin (μg/mL)**	3.89±0.45
I_{ACE} (%)	39.55±1.85	-	-	-	-

The mass balances of recovered products after BW hydrolysis and the chemical characteristics of FPHs are defined in Table 17. The inorganic parts, basically bones almost completely free of organic matter, were separated by filtration from FPHs and were approximately 7% of the initial weight of the raw material. The yield of digestion (value of V_{dig}) of initial fish discards by the Alcalase was of 93.4% and the recovered oil, after centrifugation and decantation of FPHs, achieved a value of 0.95% (v/w of substrate). The main fatty acids present in this fish oil were (Table 18), in the following order, oleic acid (24.6±0.9%), docosahexaenoic acid (DHA) (20.0±0.8%), palmitic acid (13.7±0.5%), pentadecenoic acid (9.2±0.7%) and eicosapentaenoic acid (EPA) (7.6±0.3%). The ratio omega-3/omega-6 for BW oil was 5.92±0.09%.

The protein concentration in BW hydrolysates were 48 g/L and 50 g/L, quantified as soluble proteins by the Lowry method and by total nitrogen x 6.25 or total sum of amino acids,

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respectively. On the other hand, the *in vitro* digestibility (Dig) of hydrolysates was almost complete (97%).

The most abundant amino acids are Glu and Asp followed by Leu and Lys. Essential amino acids (Ile, Leu, Val, Lys, Met, Phe, Thr, His and Arg) are also significantly present in BW-FPHs. These levels of amino acids together with the high digestibility of BW hydrolysates reveal its extraordinary nutritional value as potential ingredient for: 1) healthy food supplements (Nikoo et al., 2016), 2) aquaculture feed and pet food diets (Swanepoel and Goosen, 2018) and 3) microbial culture media (Vázquez et al., 2016b). In addition, antioxidant and antihypertensive properties of BW hydrolysates were also determined. In general, the obtained activities were not especially remarkable in comparison with other FPHs reported in literature (Aissaoui et al., 2015; Teixeira et al., 2016). Regarding the molecular weight, the average size of peptides present in the hydrolysates is around Mw= 900 Da (Figure 13, right). All these results are part of an article submitted for publication in *Journal of Cleaner Production* (Vázquez et al., accepted in press).

Table 18. Fatty acids content (as % of total fatty acids) in the fish oils recovered from whole body of blue whiting (WB_BW) complementary to the production of FPHs. Errors are the confidence intervals for n=3 (samples from different hydrolysates) and α=0.05.

Formula	Fatty acids	WB_BW	Formula	Fatty acids	WB_BW
C8:0	Caprylic acid	0.01±0.01	**C18:3n6**	-Linolenic acid	1.35±0.09
C10:0	Capric acid	0.04±0.03	**C18:3n3**	Linolenic acid	1.02±0.10
C12:0	Lauric acid	0.10±0.02	**C20:1n9**	Eicosenoic acid	3.13±0.20
C13:0	Tridecanoic acid	0.06±0.01	**C21:0**	Henicosanoic acid	0.07±0.01
C14:0	Myristic acid	2.69±0.47	**C20:2n6**	Eicosadienoic acid	0.24±0.02
C14:1	Myristoleic acid	0.06±0.05	**C22:0**	Docosanoic acid	0.13±0.03
C15:0	Pentadecanoic acid	0.53±0.12	**C20:3n6**	Dihomo-linolenic acid (DGLA)	0.34±0.07
C15:1	Pentadecenoic acid	9.15±0.67	**C20:4n6**	Arachidonic acid	0.73±0.05
C16:0	Palmitic acid	13.71±0.51	**C23:0**	Tricosanoic acid	0.09±0.01
C16:1n7c	Palmitoleic acid	5.55±0.19	**C21:4n3**	Heneicosatetraenoic acid	1.21±0.08
C17:0	Heptadecanoic acid	0.41±0.08	**C22:2n6**	Docosadienoic acid	0.32±0.06
C17:1	Heptadecanoleic acid	0.44±0.03	**C20:5n3**	Eicosapentaenoic acid (EPA)	7.58±0.31
C18:0	Stearic acid	3.01±0.25	**C24:0**	Lignoceric acid	0.53±0.05
C18:1n9c,t	Oleic acid	24.57±0.92	**C24:1n9**	Nervonic acid	0.68±0.08
C18:2n6c,t	Linoleic acid	2.06±0.12	**C22:6n3**	Docosahexaenoic acid (DHA)	19.99±0.77
C20:0	Arachidic acid	0.22±0.02			
	DHA+EPA (%)	27.57±0.82			
	r: ω-3 / ω-6	5.92±0.09			

Nevertheless, in order to reduce the potential economic cost of FPHs from BW a new set of experiments were conducted using lower concentration of Alcalase (0.2%), lower S:L ratio (1:1) and shorter time of hydrolysis (3 h). Under these conditions, the value of H_m was lower

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than the previously found (32.1±1.3%) but V_{dig} was however similar to those ones (92.6±1.1%). A summarized representation of the step performed for BW hydrolysate production is shown in Figure 14.

The production of 30 kg of dried BW-FPH, according to the requirements for the preparation of aquafeed for fish trials to be performed in WP1, is being carried out at the IIM pilot plant. This production involves a previous step of concentration of the FPH liquid (by thin film evaporator) and a final drying in a spray-dryer equipment. Finally, as a summary of the yields of bioproductions from hydrolysis of salmon and rainbow trout by-products and blue whiting are presented in Figures 15-19.

Figure 14. Different sequences of enzymatic hydrolysis of BW discards (A) in a 5L and 300 L-pH-stat reactors (D and F, respectively). B and C: crushing of BW discards; E: clean bones and BW hydrolysate; G: Alcalase hydrolysis in 300 L reactor; H: spray-dryer: I: dried hydrolysate. Processes performed in the IIM-CSIC.

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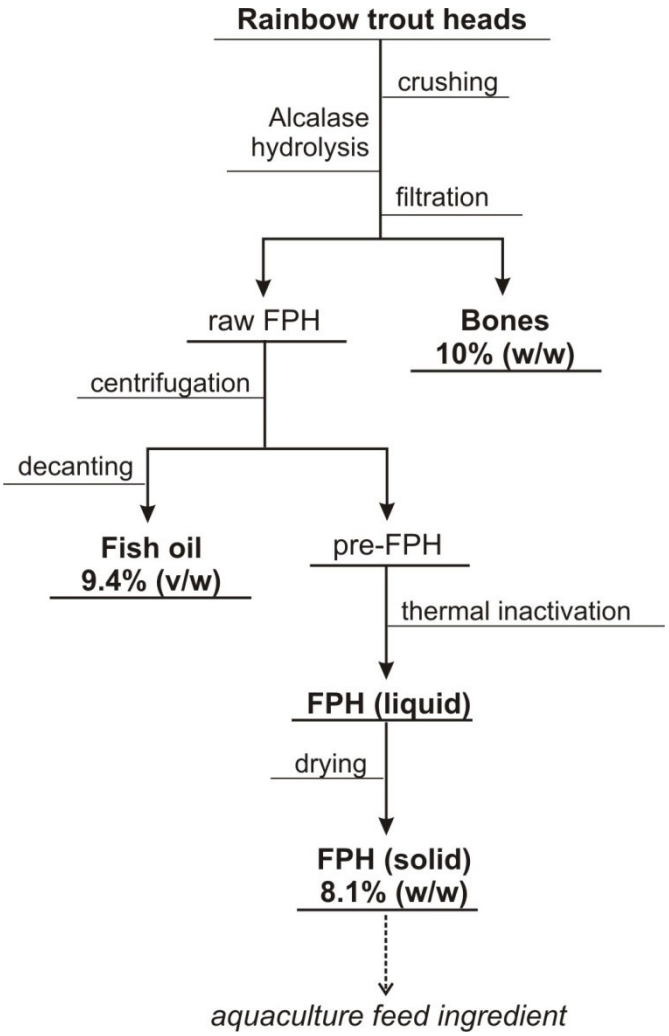

Figure 15. Flowchart of rainbow trout heads hydrolysis including yield of bioproductions (bones, oil and solid FPH).

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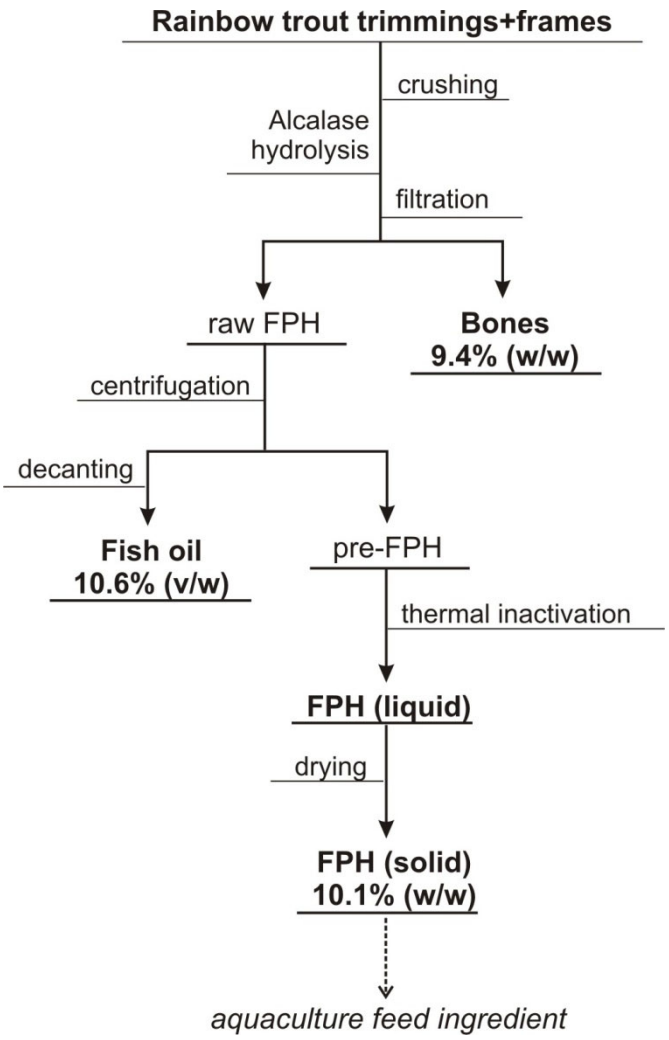

Figure 16. Flowchart of rainbow trout trimmings+frames hydrolysis including yield of bioproductions (bones, oil and solid FPH).

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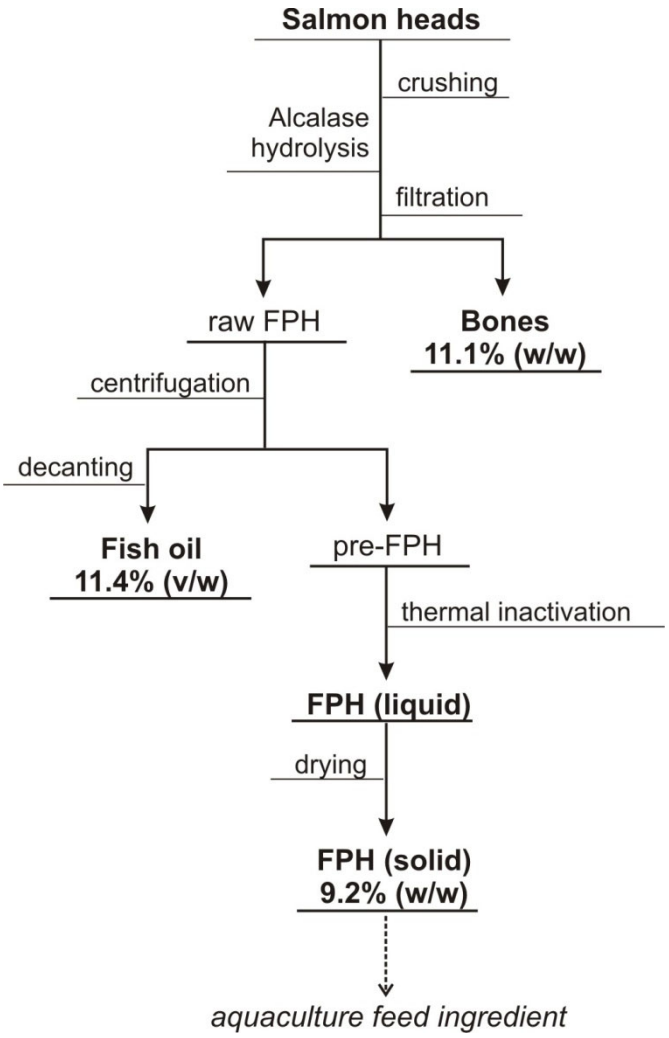

Figure 17. Flowchart of salmon heads hydrolysis including yield of bioproductions (bones, oil and solid FPH).

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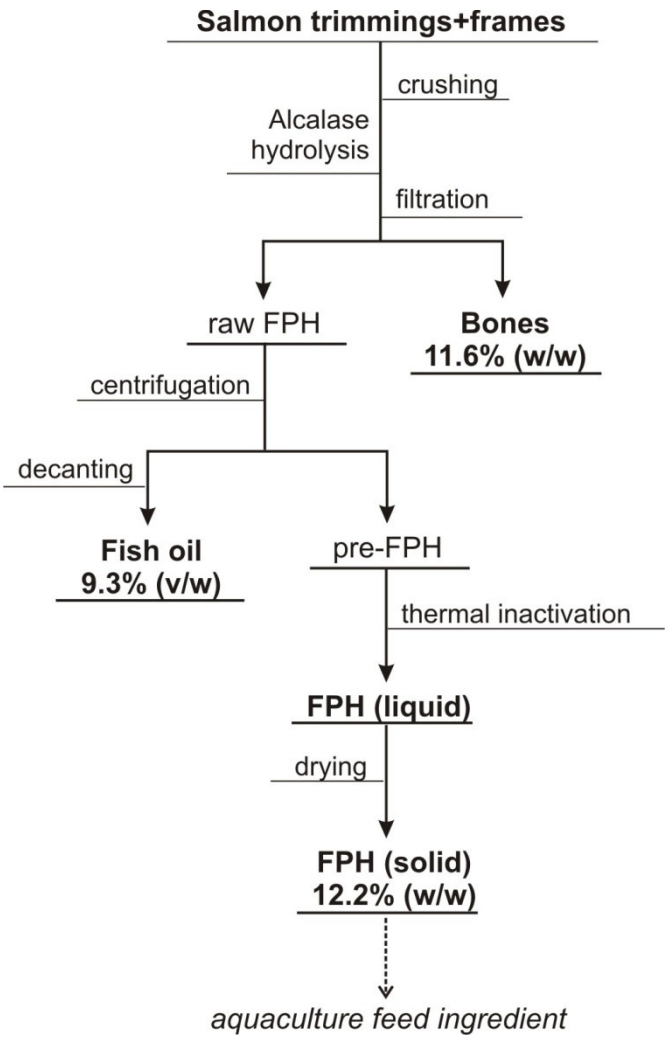

Figure 18. Flowchart of salmon trimmings+frames hydrolysis including yield of bioproductions (bones, oil and solid FPH).

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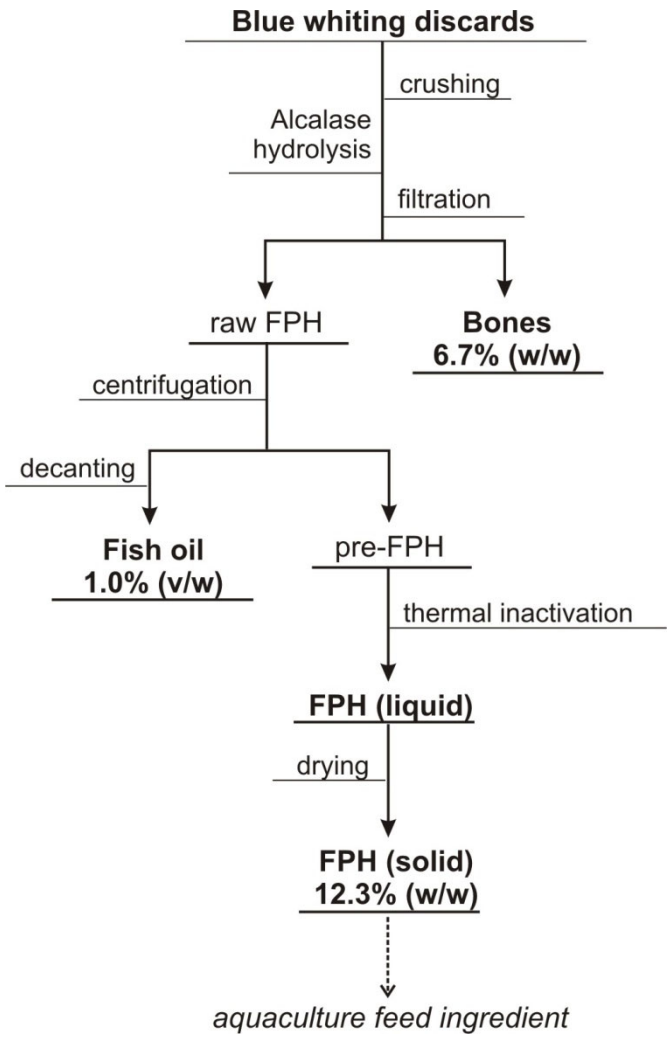

Figure 19. Flowchart of blue whiting discards hydrolysis including yield of bioproductions (bones, oil and solid FPH).

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4. Conclusions

The optimal conditions of Alcalase 2.4L hydrolysis on by-products of salmonids aquaculture (heads, trimmings and frames) and blue whiting discards (the most discarded species by Galician and Portugal fishing fleets) were stablished: a) [Alcalase]= 0.1% (v/w), pH8.27, T56.2ºC, S:L=1:1, t_h= 3 h and 200 rpm for trout, b) [Alcalase]= 0.2% (v/w), pH8.98, T64.2ºC, S:L=1:1, t_h= 3 h and 200 rpm for salmon and c) [Alcalase]= 1% (v/w), pH8.6, T60ºC, S:L=1:2, t_h= 4 h and 200 rpm for BW. The selection of this commercial endoprotease was made based on our expertise and knowledge in the application of several endo- and exoproteases (see literature cited) on the digestion of multiple marine wastes.

The suitability of this choice was experimentally confirmed here in terms of its high proteolytic capacity and the profiles of peptide sizes present in the resulting FPHs (more than 30% below 1 kDa and 86% below 3 kDa). The recovery of fish oils in the same process of FPHs production was also addressed mainly for the case of the salmonid by-products. In addition, preliminary experiments of hydrolysis for seabream/seabass by-products were also conducted at lab-scale evaluating the hydrolytic response of three proteases (Alcalase 2.4L, Protamex and Flavorpro). Initial yields of soluble protein released were low and the improvement and optimization of production conditions are currently being developed.

The production of dried FPHs from salmonids is almost finished but the production of hydrolysates from BW discards at pilot plant scale is still in progress. Moreover, we are now hydrolysing by-products from turbot recently provided by Stolt Sea Farm. The final supply of hydrolysates from all substrates to the formulation of feeds in WP1 will be thus carried out in the expected time.

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ANNEX

FISH PROTEIN HYDROLYSATE of BLUE WHITING (FPH-BLUE WHITING)

Description:	Brownish fine powder
Species:	Blue whiting (*Micromesistius poutassou*)
Source:	Whole body
Grade:	Food/Animal feed
Solubility:	Water (>80%)
Country of origin:	Galicia (Spain)

Description	**Results**
Color	Brownish
Odor	Fish smell
Texture	Fine powder
Taste	Fishy flavor

Analytical	**Results**
Moisture (%)	Will be determined (Wbd)
Organic matter (%)	Wbd
Ash (%)	Wbd
Protein by total nitrogen (%/db)	Wbd
Protein by sum of amino acids (%/db)	Wbd
Total Lipids (%/db)	Wbd
In vitro Digestibility (%)	Wbd
Hydrolysis degree (%)	Wbd

Heavy metals	**Results**
Arsenic	Will be determined (Wbd)
Cadmium	Wbd
Mercury	Wbd
Lead	Wbd

Microbiology	**Results**
Total plate count	Wbd
Yeast and molds	Wbd
Enterobacterial count	Wbd
Salmonella	Wbd
Total Coliforms	Wbd

Total amino acids composition of FPH (% or g/100 g)					
Asp	Wbd	Cys	Wbd	Phe	Wbd
Thr	Wbd	Val	Wbd	His	Wbd
Ser	Wbd	Met	Wbd	Lys	Wbd
Glu	Wbd	Ile	Wbd	Arg	Wbd
Gly	Wbd	Leu	Wbd	OHPro	Wbd
Ala	Wbd	Tyr	Wbd	Pro	Wbd

Bioactivities of FPH					
DPPH (%)	Wbd	ABTS (µg/mL)	Wbd	Crocin (µg/mL)	Wbd
IACE (%)	Wbd	-	-	-	-

Molecular weight distribution (% of peptides) and average molecular weights (Da)					
<0.5 kDa	Wbd	<0.5-1 kDa	Wbd	1-3 kDa	Wbd
<1.8 kDa	Wbd	<3 kDa	Wbd		Wbd
Mw	Wbd	Mn	Wbd		Wbd

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