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1   **Optimised protein recovery from mackerel whole fish by using sequential acid/alkaline**

2   **isoelectric solubilization precipitation (ISP) extraction assisted by ultrasound**

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9   **Abstract**

10   The growing fishery industry needs to find new green-processes in order to provide a solution to the

11   huge amount of wastes and by-products that such industrial activity produces. Currently, around a

12   40% of the total weight of the mackerel is considered a by-product, because just the fillets are used in

13   the food market. ISP method has been revealed as a useful tool for protein recovering, however the

14   yield of this process is traditionally lower than enzymatic methods. In present work, the use of

15   sequential acid/alkaline extraction and alkaline extraction assisted by ultrasound, have been

16   implemented in order to increase the yield of the process. It has been demonstrated that (i) sequential

17   extraction is able to recover practically 100% of total protein, and (ii) applying ultrasound to alkaline

18   extraction is possible to recover more than 95% of total protein from mackerel by-products. Extracted

19   proteins were characterized according to their size, and the amino acid profile of final product was

20   determined.

21   **Highlights:**

22   - Isoelectric extraction method has been modified for fish protein processing

23   - Modified extraction method achieves higher yields than those of traditional method

24   - Ultrasound increased the amount of protein recovered using acid or alkali extraction

25   - Amino acid profile of extracted proteins was not modified by extraction methods

26   **Keywords:**

27   Fish by-products, isoelectric solubilization-precipitation, protein recovery, ultrasound

28   **1   Introduction**

29   According to the Food and Agriculture Organization of the United Nations (FAO) statistics

30   (http://www.fao.org/fishery/statistics/global-capture-production/en), in 2015 almost 200 million tons

31   of fish were produced between captures (93.7 million tonnes) and aquaculture (106 million tonnes).

32   One of the most challenging and important issues to handle is the supply of high quality proteins to all

33   the growing population. United Nations and FAO claim that more than 10% of population lives in

34   constant malnutrition. It is therefore necessary to unlock the potential of our marine bio resources as a

35   source of protein. About 25% of the total production is considered inedible (i.e.by-products).

36   However, the nutritional value of the by-products is similar to that of the edible parts. If not used, this

37   biomass would be discarded either as waste or as low value by-products, which would generate

38   additional waste disposal and environmental problems. The recovery of proteins from fishery by-

39   products would therefore, be of great importance since it would not only alleviate the serious concerns

40   related to the management of visceral waste but would also help produce novel low-cost proteins for

41   industrial application (Simpson, Nayeri, Yaylayan, & Ashie, 1998).

42   The by-product of mackerel fish processing which represents around a 40% of fish total weight and

43   has a protein content comparable to the fillet 14-16% (Ramakrishnan, Ghaly, Brooks, & Budge,

44   2013). Mackerel is one of the most discarded fish species, ranging from 16 to 37% of total mackerel

45   catches between 2003 and 2012. In 2015, it was reported that more than ten thousand tonnes of

46   mackerel were discarded in Spain and Germany (ICES, 2016). Since, 2015 onwards a landing

47   obligation for European Union fisheries was introduced for small pelagic fish including mackerel (EC

48   No 1393/2014). Therefore, it is expected and increase in the volumes of landed whole mackerel, not

49   suitable for direct commercialisation, but which has the potential to be employed as source of

50   proteins. Technological interventions can assist in recovery of protein from unmarketable whole

51   fishes. Solid fish wastes have been utilized as a source of proteins and essential amino acids of high

52   nutritional value during the last decade (Benhabiles, Abdi, Drouiche, Lounici, Pauss, Goosen, et al.,

53   2012; Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; Ferraro, Carvalho, Piccirillo,

54   Santos, Castro, & Pintado, 2013). Enzymes can be applied to recover proteins from mackerel wastes

55 yielding a good result, almost a 80% of total protein can be recovered in form of peptides and free

56 amino acids (Ramakrishnan, Ghaly, Brooks, & Budge, 2013), but time employed to reach this

57 recovery yield (4 hours) is quite high when compared with ISP technique that only needs 10 minutes

58 to be completed. Additionally, due to final molecular weight of the peptides gelling and textural

59 properties are practically lost.

60 Isoelectric solubilisation-precipitation (ISP) enables protein recovery from a variety of sources. Such

61 processing allows selective, pH-induced water solubility of muscle proteins with concurrent

62 separation of lipids and removal of materials not intended for human consumption such as bones,

63 scales, skin, etc.(Tahergorabi, Beamer, Matak, & Jaczynski, 2012). This method is based on the fact

64 that solubility of muscle proteins is reduced at the isoelectric point, usually around at pH 5.5, while

65 becoming gradually more soluble as the pH is more acidic or basic than this value (Tahergorabi,

66 Beamer, Matak, & Jaczynski, 2011; Taskaya, Chen, Beamer, Tou, & Jaczynski, 2009). Using low

67 temperatures along the process (2 – 4 °C) avoids the protein degradation and enables recovery of

68 proteins with good textural properties (Tahergorabi, Beamer, Matak, & Jaczynski, 2012), gelation

69 ability (Liang & Hultin, 2005; Undeland, Kelleher, & Hultin, 2002), furthermore   nutritional quality

70 is maintained (Gehring, Gigliotti, Moritz, Tou, & Jaczynski, 2011). Comparisons between acidic and

71 alkaline pH, regarding protein yield, have been made employing different raw materials. Kristinsson,

72 Theodore, Demir, and Ingadottir (2005), compared the yields achieved from acid and alkaline

73 solubilisation from a variety of fish species. Using acid solubilisation obtained protein yields of

74 71.5% from catfish, 73.6% from Spanish mackerel, 81.2% from croaker and 78.7% from mullet, when

75 alkaline precipitation was used the recovered protein was 70.3, 69.3, 58.9 and 65.0% respectively.

76 Yields reported in the literature vary in the range of 42% and 90% and generally alkaline

77 solubilisation usually results in higher protein recoveries than acidic treatments (Gehring, Gigliotti,

78 Moritz, Tou, & Jaczynski, 2011). Besides, it has been studied how ISP technique can be applied as

79 mild non-thermal pasteurization (Lansdowne, Beamer, Jaczynski, & Matak, 2009).

80 ISP can be assisted by ultrasound to increase the yield of protein recovery, as it was reported for other

81 valuable compounds (Rastogi, 2011). Ultrasound waves are mechanical waves which can be

82 propagated by rarefactions and compression through solid, gas and liquid media. Such expansion

83 cause negative pressures in liquids, leading to the formation of vapour bubbles when the pressure

84 exceeds the tensile strength of the liquid. Vapour bubbles undergo implosive collapse which is known

85 as bubble cavitation (Luque-García & Luque de Castro, 2003). Cavitation generates macro-

86 turbulence, high-velocity inter-particle collisions and micro-porous which improve the permeability of

87 the matrix concluding in a best permeation of the solvent into cell the matrix, allowing target

88 compounds to interact with solvent and making easier the extraction. (Both, Chemat, & Strube, 2014;

89 Li, Pordesimo, & Weiss, 2004). This effect increases the efficiency of extraction by increasing mass

90 transfer and internal diffusion mechanisms (Vilkhu, Manasseh, Mawson, & Ashokkumar, 2011).

91 This work demonstrates the efficiency of ultrasound treatment in increasing the extraction yield of

92 protein and application of sequential ISP extraction to increase the protein from discarded whole

93 mackerel compared to traditional ISP methods.

94 **2   Material and methods**

95 **2.1   Isoelectric solubilization precipitation**

96 Fish samples were supplied by the Cashelmara Company. The whole fresh fish were blended using a

97 blender *(Robot Coupe R4 1500)*, vacuum packed and stored in -20^{0}C for further use. Twenty grams of

98 homogenized fish were mixed with different acid (HCl) and alkali (NaOH) solutions at several

99 concentrations (0.1M, 0.2M, 0.3M and 0.4M) at sample/solvent 1:10 ratio. Mixture was homogenized

100 for 30 seconds using a laboratory homogenizer (T25 digital ULTRA-TURRAX®).

101 Homogenized samples were immediately placed in the stirrer allocated in a cold room

102 at 4^{0}C. After ten minutes of extraction the samples were then centrifuged using a laboratory

103 batch centrifuge (Sigma 6K10 and Sigma 2-16 PK) at 9000g for 20 minutes. Proteins in supernatant

104 were then precipitated by shifting the pH value to 5.5 by adding HCl or NaOH at 1M or 0.1M for fine

105 adjustment. Precipitates obtained were then weighed and the protein content was determined using

106 the Dumas method (LECO FP628, 3000 Lakeview Avenue, St. Joseph, MI 49085).

2.2 Ultrasound assisted extraction

Ultrasound studies were carried out using 20 g of minced whole mackerel placed in jacketed beaker coupled to a temperature controlled water bath with 200 mL of solvent (HCl 0.1 M or NaOH 0.1 M). Temperature was maintained at 4^0C. Extraction process was carried out with a 750 W ultrasound processor (VC 750, Sonics and Materials, Inc., Newton, USA) operating at a frequency of 20 kHz. In a previous work (Kadam, Tiwari, Smyth, & O'Donnell, 2015) the ultrasonic intensity was calculated: 20% of amplitude (22.8 µm or 7.00 W/cm^2) and 60% of amplitude (68.4µm or 35.61 W/cm^2). The extraction was conducted for 10 min, using a cycle of pulses of 5 seconds on and 5 seconds off, i.e., total time of ultrasound was 5 minutes. In the case of ultrasonic bath the device employed (Branson B3510) has a constant frequency of 40 kHz and the extraction was conducted for 60 minutes.

After extraction, the samples were centrifuged using same parameters as above. Proteins present in the supernatant, were precipitated by adjusting the pH of the solution to a value of 5.5 by adding either NaOH or HCl 2M solution and stored until analysis: and pellet was used for a second step of extraction using a different solution; i.e. if first step was done using an acid solution the second step was done using alkaline solution and vice versa. A scheme of the flow process is shown in Figure 1.

2.3 Determination of protein recovery

Protein content in pellet and supernatant was determined by Leco FP628 (LECO Corp., MI, USA) protein analyzer based on the Dumas method (Simone et al., 1997) which determines the nitrogen in a variety of materials. Sample extract of around 0.200 g was exactly weighed into tin foil cup. The sample with tin foil cup was kept in auto-sampler at least duplicate measurements were taken for each sample.

2.4 Molecular weight distribution of recovered protein

SEC chromatographic analyses were carried out to determine the molecular size of the hydrolysates. Phosphate buffer (pH 7.5, 0.1 M) was used as mobile phase with a flow of 0.85 mL/min in a Waters HPLC (2795 Separation Module) coupled to two serial-connected columns: Zorbax GF-250 (4.5 µm

132 particle size, 150 Å pore size) and Zorbax GF-450 (6 µm particle size and 300 Å pore size). Injection

133 volume was of 20 µL. The result was monitored at 254 nm in a Photodiode Array Detector (Waters

134 2996) and the area of each peak was evaluated using the Empower Pro 2 software (Waters

135 Corporation). A calibration curve was made using albumin (66kDa), carbonic anhydrase (29 kDa),

136 cytochrome C (12.4 kDa), aprotinin (6.5 kDa), angiotensin II acetate, (Asp-Arg-Val-Tyr-Ile-His-Pro-

137 Phe; 1046 Da) and leucine enkephalin, (Tyr-Gly-Gly-Phe-Leu; 555 Da).

138 **2.5   Amino acid profile**

139 Proteins were hydrolyzed in 6M HCl at 110°C for 23 hours and the resulting hydrolysates analyzed on

140 the amino acid as per free amino acids method (Hill, 1965). Samples were deproteinised by mixing

141 equal volumes of 24% (w/v) tri-chloroacetic acid (TCA) and sample, these were allowed to stand for

142 10 minutes before centrifuging at 14400 x g (Microcentaur, MSE, UK) for 10 minutes. Supernatants

143 were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2 to give approximately 250 nM of

144 each amino acid residue. Samples were then diluted 1 in 2 with the internal standard, norleucine, to

145 give a final concentration of 125 nm/mL. Amino acids were quantified using a Jeol JLC-500/V amino

146 acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na+ high performance cation

147 exchange column.

148 **2.6   Statistical analysis**

149 All experiments were carried out by duplicate and analyses were carried out on all samples. In order

150 to see difference between the groups an ANOVA with multiple comparison of Games-Howell (no

151 parametric test) was performed using SPSS version 17.0. Values were considered significant at

152 $p<0.05$

153 **3 Results and discussion**

154 **3.1 Protein recovery using ISP at different acid and alkali concentration**

155 In order to determine which concentration of acid or alkali yields the best protein recovery,

156 concentrations from 0.1M up to 0.4 M of HCl and NaOH were tested. The ratio employed of

157 samples/solution was of 1:10, it has been demonstrated that best yields are achieved when this high

158 solution to sample ratio since the ionic strength of the medium is lower(Torres, Chen, Rodrigo-

159 Garcia, Jaczynski, & Shahidi, 2007). Although at the same alkali or acid concentration the solution

160 has the same ionic strength, the higher the ratio of solution to sample the diluted the salts from the

161 sample and therefore the ionic strength is lower. The results obtained are shown in Table 1. Protein

162 yields obtained in this study were significantly higher with alkaline compared to acidic condition

163 extraction.

164 Protein extraction was remarkably better when alkaline solution was employed compared to

165 acid solubilisation; NaOH solution concentration of 0.1 to 0.3 M showed differences ($p<0.05$)

166 between them, however the highest percentage of recovered protein (74%) was obtained using 0.4 M

167 NaOH for extraction. Yields reported in the literature vary in the range of 42% and 90% and generally

168 alkaline solubilisation usually results in higher protein recoveries than acidic treatments (Gehring,

169 Gigliotti, Moritz, Tou, & Jaczynski, 2011). When HCl is employed as extraction solution, the highest

170 yield was found at lowest acid concentrations (49.5%); on the other hand the lowest yield (19.3%)

171 was founded when the highest acid (0.4 M) concentration was employed. This effect can be due to the

172 different behaviour of Na^+ and Cl^- ions. As a result of increased ionic strength (IS) the myofibrillar

173   proteins precipitate at a lower pH; and therefore, pH must reflect the changing pI in order to achieve

174   maximum protein precipitation (i.e., recovery efficiency) during continuous ISP processing. Increased

175   IS causes a shift in the pI to a lower pH because the Cl^- binds positively charged amino acids (AA) to

176   a greater extent than the Na^+ that binds negatively charged AA (Ockerman, 1996).The lower recovery

177   yields with acidic solution are likely due to the pH of the isoelectric point being lowered and the pH

178   of 5.5 used for precipitation of solubilized proteins no longer being the pH of minimum solubility of

179   the soluble proteins . Because of this factor a negative effect can be seen when concentration of

180   chloride ions is increased.

181   **3.2   ISP assisted by ultrasonics**

182   After assessing the influence of acid and alkaline on the extraction yield of mackerel proteins,

183   ultrasound was employed to assist the extraction in order to achieve a highest recovery yield. A

184   control experiment was carried employing distilled water as solution and testing different ultrasonic

185   treatments: extraction by stirring, ultrasound bath, ultrasound probe at 20% of amplitude and

186   ultrasound probe at 60% of amplitude. In all cases the pH was 6.0 with no significant changes in pH

187   were detected after extraction process. Extraction times, centrifugation process and sample/solution

188   ratio employed were the same as previous section. The results obtained are showed in Table 2.

189   At this pH, only 5.9% of total protein can be recovered as soluble protein; it has been reported

190   that at this pH sarcoplasmic proteins are completely insoluble, meanwhile myofibrillar proteins are

191   slightly soluble (Torres, Chen, Rodrigo-Garcia, Jaczynski, & Shahidi, 2007). However, a significant

192   increase ($p > 0.05$) in protein recovery was achieved following extraction with ultrasound. This fact

193   confirms that, ultrasound leads to a better interaction between extraction solutions and proteins. As

194   the ultrasonic intensity is increased the protein recovered is higher (13.6% at 60% of amplitude). In

195   such case the amount of energy applied to the samples is higher and the cavitation processes leads to a

196   higher level of matrix degradation (Ito, Tatsumi, Wakamatsu, Nishimura, & Hattori, 2003), allowing

197   the extracting solution to be in contact with proteins thereby increasing the yield of the process(Kim,

198   Kim, Kim, Park, & Lee, 2012).

3.3   ISP-ultrasound assisted extraction at acidic and alkaline conditions

When the first experiments using ISP assisted by ultrasound were carried out, a notable amount of pellet remained after the extraction, additionally a different protein profile was observed in the size exclusion chromatograms; which meant that different proteins where extracted, thus probably proteins extracted at alkaline pH were not extracted at acidic pH, and vice versa. In order to increase the overall process, yield it was proposed to use the remaining pellet for a second extraction process; using the remaining insoluble material (still rich in protein) for a second extraction process employing acid or basic conditions depending on which one was employed in the first step. This sequential extraction was carried out following the tradition ISP methodology assisted by external stirring, or assisted by ultrasonic (probe and water bath). Results of soluble protein obtained after each step are showed in Table 3.

It has been demonstrated that ISP sequential extraction (acid followed by alkaline) was able to recovery the 98.6% of the proteins; to the best of our knowledge this is the first time that this method is reported achieving practically the total protein recovery by means of ISP methodology. When alkaline extraction is followed by acid extraction, the yield is notably lower (83%). These results support the evidence that certain proteins which remain insoluble at acidic pH can be effectively solubilized under alkaline conditions, and vice versa.

When the extraction process is carried out in conjunction with ultrasound the yield is remarkably increased in the first extraction step. The amount of protein recovered under acidic conditions ranged from 60.3% to 74.6% when 20% and 60% of amplitudes was respectively used. When alkaline solution was employed the percentage of protein recovered ranged from 87.6% (20% of amplitude) to 94.7% (60% of amplitude). Depending on the solution employed ultrasound bath has different performance compared to the other methods; a positive effect in acid extraction when compared to traditional and ultrasound assisted extraction at 20%; and negative effect in alkaline extraction when compared to ultrasound at 20% and 60% ($p < 0.05$).

224   As it was mentioned, sequential extraction (acid-alkaline) was able to recover almost the 100%

225   protein contented in the fish sample, so it is not necessary to use ultrasound assisted extraction to

226   improve yield. On the other hand, when alkaline extraction is followed by acid extraction the total

227   yield achieved was 83.3%, extraction assisted by ultrasound increased the yield up to 92.5% and the

228   97.3% of the proteins when 20% and 60% of amplitude was employed. It is a significant improvement

229   ($p < 0.05$) when compared to traditional ISP. It implies that ultrasound is a green technology able to

230   increase the recovery yield in ISP process, open new possibilities for protein recovery from fisheries

231   by-products or developing new fish-based products. The possibility that solvent/sample ratio can be

232   reduced, keeping the same high yield, is now open; that technology can lead to a more sustainable fish

233   protein industry.

234   The fact that more proteins can be extracted in the second step of the process could be as a

235   consequence of a higher degree of matrix degradation, due to a longer exposure to ultrasound. It was

236   reported that ultrasound can lead to some degree of protein hydrolysis at mild pH (6.5) (Ito, Tatsumi,

237   Wakamatsu, Nishimura, & Hattori, 2003; Kim, Kim, Kim, Park, & Lee, 2012), such hydrolysis could

238   be more intense at extreme pH, as used in present work, this may make protein extraction easier as

239   the structural proteins of the muscle (titin, nebulin, and collagen) are broken down.

240   **3.4   Molecular weight of recovered proteins**

241   Size exclusion chromatography was employed to evaluate variation of the protein profile after

242   different extraction processes. Chromatograms are showed in Figure 2 and the weight distribution of

243   the extracted proteins is shown in Figure 3.

244   Fish myosin, the major myofibrillar protein, is a 520 kDa hexamer consisting of two 220 kDa

245   polypeptides referred to as myosin heavy chains (MHC). Each MHC is non-covalently attached to two

246   18–25 kDa light chains (LC)(Kristinsson & Hultin, 2003). It has been recently reported that titin (the

247   largest protein known to date), nebulins (in the range of 600-900 kDa) and collagen β, α1 and α2 (250

248   and 120 kDa) can be extracted using ISP, however these protein remains practically unaltered and

249   they can be detected using SDS-PAGE electrophoresis. However, when the   same samples are

250 extracted using alkaline pH assisted by ultrasound, the bands corresponding to these very high

251 molecular weight proteins were less evident (Tian, Wang, Zhu, Zeng, & Xin, 2014) due to a process

252 of degradation.

253   When acidic or alkaline solutions are employed for extraction, high molecular weight proteins

254 can be observed, those proteins corresponds to titin, nebulin and heavy chain of myosin, based on the

255 peak corresponding to protein >250kDa acid extraction is more effective in solubilizing larger

256 molecular weight proteins. However, the main peak observed is that corresponding to 35-41 kDa, that

257 is composed by actin (41 kDa), and tropomyosin α and β (37 and 33 kDa). This peak shows that   the

258 40% and 29% of total proteins extracted in acid or alkaline conditions respectively are in this range

259 Furthermore , a small peak (13 and 14% of total area) which corresponds to collagen chains (120

260 kDa) can be detected. Such profile obtained is in agreement with results published by Tian et als,

261 2014. Even some traces of short peptides, in the range of 1-5 kDa could be detected, being higher

262 (around 10% of total protein extracted) when ISP is conducted at alkaline pH. An unidentified peak

263 in the size of 13 kDa, accounting for 16.7-20% of total extracted protein, is present in with alkaline

264 extraction; however such peak was not detected after acidic extraction, with either traditional ISP or

265 with ultrasound assisted process. This peak is an indication that the alkaline extraction process is more

266 effective; probably an alkaline soluble protein is being extracted.

267   When protein size was analysed after ultrasonic assisted extraction, it was found that the area of

268 the peaks corresponding to larger proteins (100 to more than 500 kDa) was slightly lower; meanwhile

269 the area corresponding to those proteins within the range of 10 and 40 kDa was higher. This fact,

270 together the increase in yield, suggests that the largest proteins are still extracted at the same time that

271 some hydrolysis process is taking place. It has been reported how titin and collagen can be degraded

272 after being exposed to ultrasonic treatments (Kim, Kim, Kim, Park, & Lee, 2012), and when these

273 structural proteins are solubilized, the extraction of more actin and myosin can be achieved; which

274 explains how ultrasound enhances the yield in protein recovery

275   The molecular weight of the proteins recovered in the second step of sequential acid/alkaline

276   or alkaline/acid extraction was also determined. Those peaks corresponding to myosin, titin, nebulin

277   and collagen are absent; indicating that these proteins were completely recovered in the first

278   extraction step of the process, regardless the pH employed. However, small amounts of actin,

279   collagen and peptides can be still recovered.

280   **3.5   Amino acidic profile of recovered protein**

281   High quality protein (i.e., complete protein) is determined based on the presence of all the nine

282   essential amino acids (EAA) in adequate quantities to support human or animal health. To assess

283   protein quality, it is important to determine the amount of EAA in proteins recovered with ISP, to this

284   end the amino acid profile of recovered proteins was characterized. Regardless of the method

285   employed for protein extraction, amino acid profile was constant and no significant changes were

286   detected. In the case of sequential extraction both pellets were mixed before obtaining the profile.

287   Table 4 shows the relative amount of each amino acid compared to total amount, results have been

288   compared with US National Nutrient Database for Standard Reference (USDA, 2017) and other

289   profiles reported in literature(Leu, Jhaveri, Karakoltsidis, & Constantinides, 1981). Main difference

290   seen in the present work is a low recovery of tyrosine; its regular value is within the range of 3.2-

291   3.5%, while in the present work values of 0.9% were detected. On the contrary, histidine content was

292   found in levels twice as high as those reported in the compared references. It is remarkable how

293   histidine yields the 42% of free amino acid in final product.

294   Data suggest that ISP-US assisted and sequential ISP extraction are feasible, fast and simple

295   method to recover high quality protein that can be applied for multiple purposes. The quality of

296   proteins recovered by ISP, although lower than that of egg protein, is comparable to or higher than

297   that of soy protein and milk protein. The nutritional quality of proteins recovered from whole krill

298   using ISP has been determined (Chen, Tou, & Jaczynski, 2007).

299

300 **4   Conclusions**

301   A new sequential extraction process, based on ISP technique has been developed; applying this

302 new method it was possible to increase the recovery yield from 49% (using HCl 0.1M) or 64% (using

303 NaOH 0.1M) to 100% (using 0.1M HCl/NaOH sequential extraction). Besides, the influence of

304 ultrasound on the process yield was determined, it was found that 60% of amplitude for ten minutes in

305 0.1M NaOH solution was able to recover the 94% of total protein in a single extraction step. It was

306 shown that lower amplitudes (20%) or ultrasonic bath increases the yield of the extraction when

307 compared to traditional ISP. Alkaline extraction was shown   to solubilize a broader range of proteins

308 than acid extraction, including a non-identified 13 kDa protein which can explain the differences in

309 yield between acid and alkaline extractions. From a nutritional point of view, the proteins recovered

310 by the methods explored in this work, are suitable for food purposes due to its high content en

311 essential and non-essential amino acids.

312   That implies that ISP-US assisted extraction is a promising tool in order to develop greener

313 extraction process, since the amount of water and reagents can be decreased; however further research

314 is needed in order to optimize the process.

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413

Figure captions:

415 Figure 1: Flow chart of sequential extraction assisted by ultrasound.

416 Figure 2: SE-HPLC chromatograms of extracted proteins: (a) HCl 0.1M (stirring); (b) NaOH 0.1 M

417 (stirring); (c) HCl 0.1 M (ultrasound assisted, 20% amplitude); (d) NaOH 0,1 M (ultrasound assisted,

418 20% amplitude); (e) second supernatant after sequential acid-alkali extractions (ultrasound assisted,

419   20% of amplitude) and (f) second supernatant after alkali-acid (ultrasound assisted, 20% of

420   amplitude).

421   Figure 3: Molecular weight profile of proteins extracted by means of different extraction processes.

422

Table 1: Percentage of recovered protein after precipitate the supernatant, and not recovered protein (pellet) after ISP extraction using different concentrations of NaOH or HCl. Different superscripts (a to f) denote significant differences (p<0.05).

	% of initial protein		Mass balance (%)
	Not recovered protein	Recovered protein	
HCl 0.1	44.84±0.39[a]	49.48±0.84[a]	94.32
HCl 0.2	43.99±0.38[a]	48.08±0.04[a]	92.07
HCl 0.3	57.11±0.67[b]	42.20±0.17[b]	99.31
HCl 0.4	77.63±0.51[c]	19.30±0.26[c]	96.93
NaOH 0.1	33.61±5.85[d]	64.05±0.09[d]	97.66
NaOH 0.2	33.8±0.15[d]	65.45±0.06[e]	99.25
NaOH 0.3	33.08±3.87[d]	63.06±0.08[d]	96.14
NaOH 0.4	23.2±0.16[e]	74.25±0.16[f]	97.45

Table 2: protein solubilized at pH 6 using different ultrasonic conditions: 20% and 60% of amplitude. Different small superscripts (a to c) denote significant differences ($p < 0.05$).

	% of initial protein		
	Not recovered protein	**Recovered protein**	**Mass balance**
Control no US	86.83 ± 4.54^{a}	5.93 ± 0.58^{a}	92.76
Control US 20% of amplitude	75.56 ± 0.58^{a}	9.37 ± 1.48^{b}	84.93
Control US 60% of amplitude	70.55 ± 3.51^{b}	13.64 ± 0.59^{c}	84.20
Control US Bath	86.58 ± 1.63^{a}	11.47 ± 1.00^{c}	98..05

Table 3: Percentage of protein recovered using sequential extraction process. Different small superscripts (a to d) denote significant differences in first extraction, second extraction and total extracted protein, either for acid-alkaline or alkaline-acid process ($p < 0.05$).

	Acid-Alkaline extraction			Alkaline-Acid extraction		
	1st extraction HCl 0.1 M	2nd extraction NaOH 0.1 M	Total extraction	1st extraction NaOH 0.1 M	2nd extraction HCl 0.1 M	Total extraction
Traditional ISP	49.48±0.84[a]	49.23±1.51[a]	98.6%[a]	64.05±0.09d	19.27±1.19b	83.3%c
20% of amplitude 10 min	60.31±0.66[b]	35.27±8.18[a]	95.5%[a]	87.59±3.3e	4.86±0.80d	92.5%b
60% of amplitude 10 min	74.66±5.25[c]	19.00±3.49[b]	93.6%[b]	94.71±0.82f	2.62±2.30d	97.3 %a
US Bath 1h	69.34±2.82[d]	30.21±0.82[c]	98.5%[a]	78.95±0.88[c]	n.d.	n.d.

n.d.: not determined.

Table 4: Amino acid profile, referred as percentage of the total amino acids; and percentage of free amino acid composition, found in combined protein extracted after sequential alkaline-acid extraction.
a: US National Nutrient Database for Standard Reference.

EAA	Free	Total	NNDSR[a]	Leu et al., 1981	NEAA	Free	Total	NNDSR[a]	Leu et al., 1981
Asp	1.50	9.28	10.72	11.06	Ile	1.75	5.05	4.83	5.15
Tyr	2.37	0.89	3.53	3.28	Leu	2.76	9.39	8.51	8.81
Ser	1.93	3.47	4.27	4.22	Met	0.50	3.42	3.10	2.53
Glu	9.71	15.02	15.63	14.81	Lys	7.35	9.42	9.61	7.40
Gly	5.93	4.33	5.03	5.62	Thre	1.88	4.21	4.59	5.34
Ala	10.84	6.12	6.34	7.22	Phe	2.78	4.40	4.09	3.94
Cys	2.91	0.43	1.36	-	Val	4.69	5.78	5.40	7.31
Arg	2.60	6.24	6.26	7.12	His	42.18	6.23	3.08	4.22
					Pro	-	3.01	3.70	1.41

Figure 1

Figure 1

Figure 2

Figure 3

Moleular weight distribution of extracted proteins

Legend:
- HCl 0.1 M, stirring
- NaOH 0.1 M stirring
- HCl 0.1 M 7.00 W/cm2
- NaOH 0.1 M 7.00 W/cm2
- acid-alkali 7.00 W/cm2
- alkali-acid 7.00 W/cm2

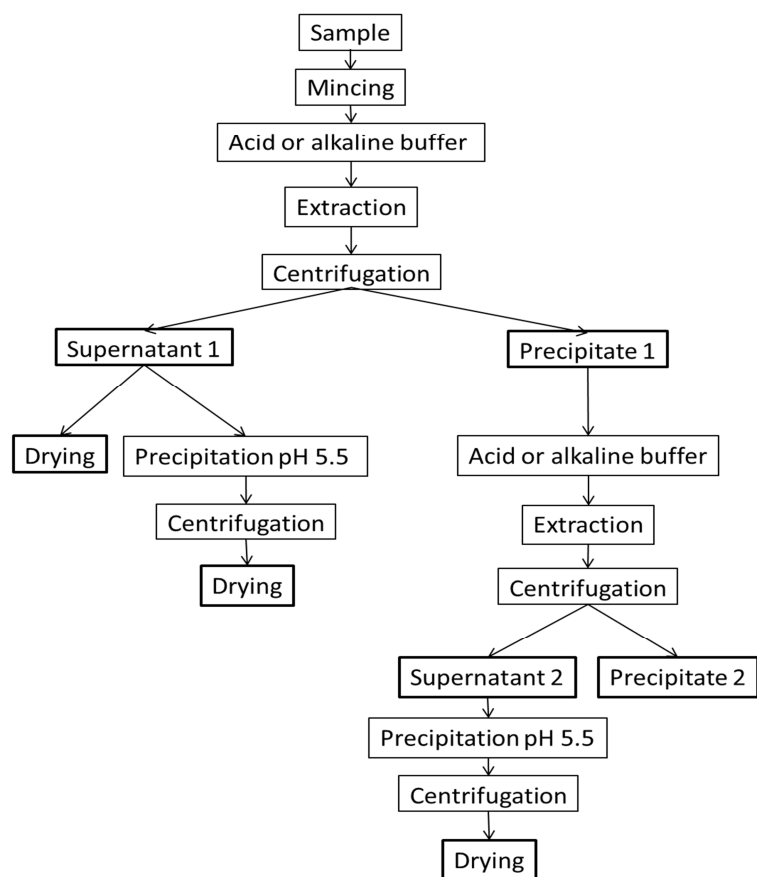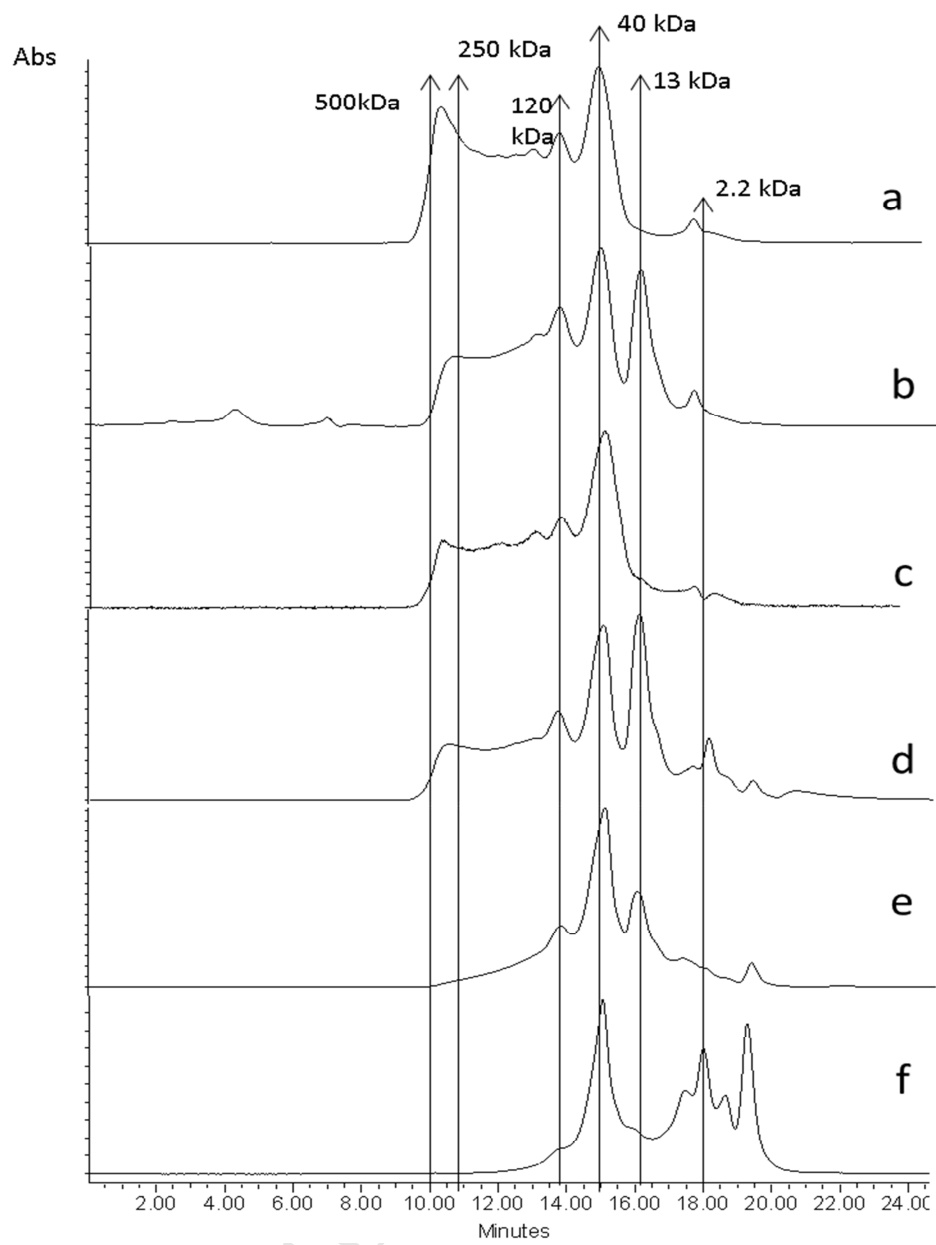Y-axis: % of total protein (0 to 50)

X-axis categories: >250, 250 to 120, 120, 40, 13, <2.2 (kDa)

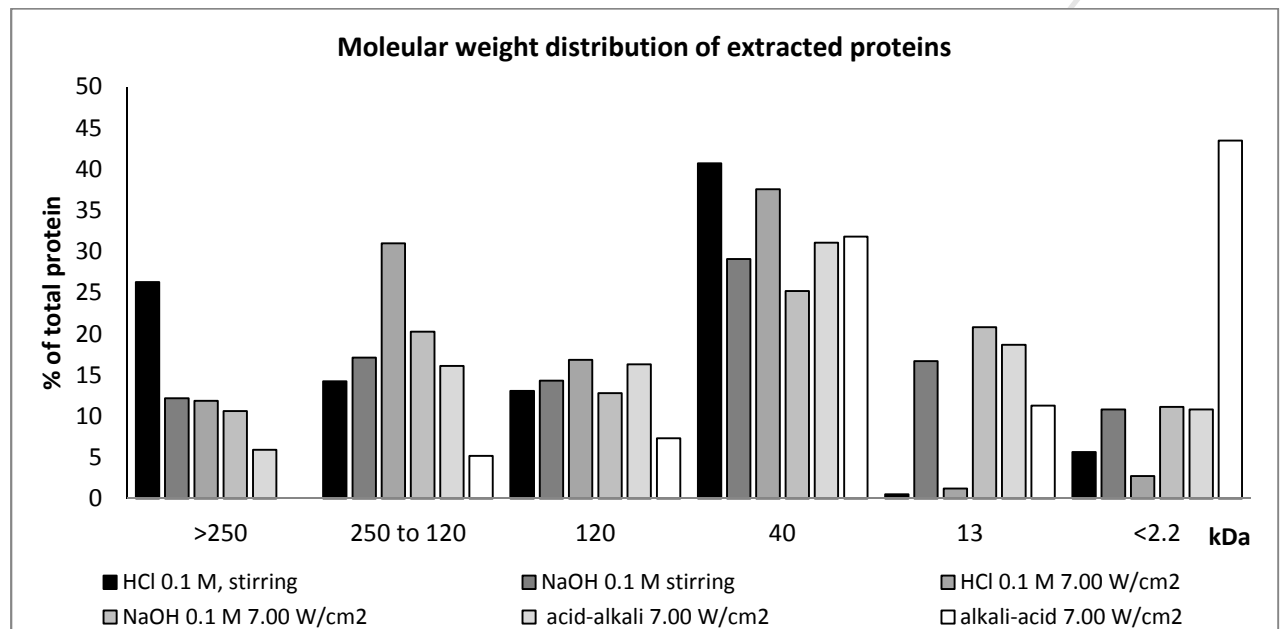**Highlights:**

- Isoelectric extraction method has been modified for fish protein processing
- Modified extraction method achieves higher yields than those of traditional method
- Ultrasound increased the amount of protein recovered using acid or alkali extraction
- Amino acid profile of extracted proteins was not modified by extraction methods