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Optimised protein recovery from mackerel whole fish by using sequential acid/alkaline isoelectric solubilization precipitation (ISP) extraction assisted by ultrasound

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Abstract

The growing fishery industry needs to find new green-processes in order to provide a solution to the huge amount of wastes and by-products that such industrial activity produces. Currently, around a 40% of the total weight of the mackerel is considered a by-product, because just the fillets are used in the food market. ISP method has been revealed as a useful tool for protein recovering, however the yield of this process is traditionally lower than enzymatic methods. In present work, the use of sequential acid/alkaline extraction and alkaline extraction assisted by ultrasound, have been implemented in order to increase the yield of the process. It has been demonstrated that (i) sequential extraction is able to recover practically 100% of total protein, and (ii) applying ultrasound to alkaline extraction is possible to recover more than 95% of total protein from mackerel by-products. Extracted proteins were characterized according to their size, and the amino acid profile of final product was determined.

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

Keywords:

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

1 Introduction

According to the Food and Agriculture Organization of the United Nations (FAO) statistics (http://www.fao.org/fishery/statistics/global-capture-production/en), in 2015 almost 200 million tons of fish were produced between captures (93.7 million tonnes) and aquaculture (106 million tonnes). One of the most challenging and important issues to handle is the supply of high quality proteins to all the growing population. United Nations and FAO claim that more than 10% of population lives in constant malnutrition. It is therefore necessary to unlock the potential of our marine bio resources as a source of protein. About 25% of the total production is considered inedible (i.e.by-products). However, the nutritional value of the by-products is similar to that of the edible parts. If not used, this biomass would be discarded either as waste or as low value by-products, which would generate additional waste disposal and environmental problems. The recovery of proteins from fishery by-products would therefore, be of great importance since it would not only alleviate the serious concerns related to the management of visceral waste but would also help produce novel low-cost proteins for industrial application (Simpson, Nayeri, Yaylayan, & Ashie, 1998).

The by-product of mackerel fish processing which represents around a 40% of fish total weight and has a protein content comparable to the fillet 14-16% (Ramakrishnan, Ghaly, Brooks, & Budge, 2013). Mackerel is one of the most discarded fish species, ranging from 16 to 37% of total mackerel catches between 2003 and 2012. In 2015, it was reported that more than ten thousand tonnes of mackerel were discarded in Spain and Germany (ICES, 2016). Since, 2015 onwards a landing obligation for European Union fisheries was introduced for small pelagic fish including mackerel (EC No 1393/2014). Therefore, it is expected and increase in the volumes of landed whole mackerel, not suitable for direct commercialisation, but which has the potential to be employed as source of proteins. Technological interventions can assist in recovery of protein from unmarketable whole fishes. Solid fish wastes have been utilized as a source of proteins and essential amino acids of high nutritional value during the last decade (Benhabiles, Abdi, Drouiche, Lounici, Pauss, Goosen, et al., 2012; Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; Ferraro, Carvalho, Piccirillo, Santos, Castro, & Pintado, 2013). Enzymes can be applied to recover proteins from mackerel wastes

yielding a good result, almost a 80% of total protein can be recovered in form of peptides and free amino acids (Ramakrishnan, Ghaly, Brooks, & Budge, 2013), but time employed to reach this recovery yield (4 hours) is quite high when compared with ISP technique that only needs 10 minutes to be completed. Additionally, due to final molecular weight of the peptides gelling and textural properties are practically lost.

Isoelectric solubilisation-precipitation (ISP) enables protein recovery from a variety of sources. Such processing allows selective, pH-induced water solubility of muscle proteins with concurrent separation of lipids and removal of materials not intended for human consumption such as bones, scales, skin, etc.(Tahergorabi, Beamer, Matak, & Jaczynski, 2012). This method is based on the fact that solubility of muscle proteins is reduced at the isoelectric point, usually around at pH 5.5, while becoming gradually more soluble as the pH is more acidic or basic than this value (Tahergorabi, Beamer, Matak, & Jaczynski, 2011; Taskaya, Chen, Beamer, Tou, & Jaczynski, 2009). Using low temperatures along the process (2 – 4 °C) avoids the protein degradation and enables recovery of proteins with good textural properties (Tahergorabi, Beamer, Matak, & Jaczynski, 2012), gelation ability (Liang & Hultin, 2005; Undeland, Kelleher, & Hultin, 2002), furthermore nutritional quality is maintained (Gehring, Gigliotti, Moritz, Tou, & Jaczynski, 2011). Comparisons between acidic and alkaline pH, regarding protein yield, have been made employing different raw materials. Kristinsson, Theodore, Demir, and Ingadottir (2005), compared the yields achieved from acid and alkaline solubilisation from a variety of fish species. Using acid solubilisation obtained protein yields of 71.5% from catfish, 73.6% from Spanish mackerel, 81.2% from croaker and 78.7% from mullet, when alkaline precipitation was used the recovered protein was 70.3, 69.3, 58.9 and 65.0% respectively. Yields reported in the literature vary in the range of 42% and 90% and generally alkaline solubilisation usually results in higher protein recoveries than acidic treatments (Gehring, Gigliotti, Moritz, Tou, & Jaczynski, 2011). Besides, it has been studied how ISP technique can be applied as mild non-thermal pasteurization (Lansdowne, Beamer, Jaczynski, & Matak, 2009).

ISP can be assisted by ultrasound to increase the yield of protein recovery, as it was reported for other valuable compounds (Rastogi, 2011). Ultrasound waves are mechanical waves which can be

propagated by rarefactions and compression through solid, gas and liquid media. Such expansion cause negative pressures in liquids, leading to the formation of vapour bubbles when the pressure exceeds the tensile strength of the liquid. Vapour bubbles undergo implosive collapse which is known as bubble cavitation (Luque-García & Luque de Castro, 2003). Cavitation generates macro-turbulence, high-velocity inter-particle collisions and micro-porous which improve the permeability of the matrix concluding in a best permeation of the solvent into cell the matrix, allowing target compounds to interact with solvent and making easier the extraction. (Both, Chemat, & Strube, 2014; Li, Pordesimo, & Weiss, 2004). This effect increases the efficiency of extraction by increasing mass transfer and internal diffusion mechanisms (Vilkhu, Manasseh, Mawson, & Ashokkumar, 2011).

This work demonstrates the efficiency of ultrasound treatment in increasing the extraction yield of protein and application of sequential ISP extraction to increase the protein from discarded whole mackerel compared to traditional ISP methods.

2 Material and methods

2.1 Isoelectric solubilization precipitation

Fish samples were supplied by the Cashelmara Company. The whole fresh fish were blended using a blender *(Robot Coupe R4 1500)*, vacuum packed and stored in -20^{0}C for further use. Twenty grams of homogenized fish were mixed with different acid (HCl) and alkali (NaOH) solutions at several concentrations (0.1M, 0.2M, 0.3M and 0.4M) at sample/solvent 1:10 ratio. Mixture was homogenized for 30 seconds using a laboratory homogenizer (T25 digital ULTRA-TURRAX®). Homogenized samples were immediately placed in the stirrer allocated in a cold room at 4^{0}C. After ten minutes of extraction the samples were then centrifuged using a laboratory batch centrifuge (Sigma 6K10 and Sigma 2-16 PK) at 9000g for 20 minutes. Proteins in supernatant were then precipitated by shifting the pH value to 5.5 by adding HCl or NaOH at 1M or 0.1M for fine adjustment. Precipitates obtained were then weighed and the protein content was determined using 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

particle size, 150 Å pore size) and Zorbax GF-450 (6 µm particle size and 300 Å pore size). Injection volume was of 20 µL. The result was monitored at 254 nm in a Photodiode Array Detector (Waters 2996) and the area of each peak was evaluated using the Empower Pro 2 software (Waters Corporation). A calibration curve was made using albumin (66kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa), angiotensin II acetate, (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; 1046 Da) and leucine enkephalin, (Tyr-Gly-Gly-Phe-Leu; 555 Da).

2.5 Amino acid profile

Proteins were hydrolyzed in 6M HCl at 110°C for 23 hours and the resulting hydrolysates analyzed on the amino acid as per free amino acids method (Hill, 1965). Samples were deproteinised by mixing equal volumes of 24% (w/v) tri-chloroacetic acid (TCA) and sample, these were allowed to stand for 10 minutes before centrifuging at 14400 x g (Microcentaur, MSE, UK) for 10 minutes. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2 to give approximately 250 nM of each amino acid residue. Samples were then diluted 1 in 2 with the internal standard, norleucine, to give a final concentration of 125 nm/mL. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column.

2.6 Statistical analysis

All experiments were carried out by duplicate and analyses were carried out on all samples. In order to see difference between the groups an ANOVA with multiple comparison of Games-Howell (no parametric test) was performed using SPSS version 17.0. Values were considered significant at $p<0.05$

3 Results and discussion

3.1 Protein recovery using ISP at different acid and alkali concentration

In order to determine which concentration of acid or alkali yields the best protein recovery, concentrations from 0.1M up to 0.4 M of HCl and NaOH were tested. The ratio employed of samples/solution was of 1:10, it has been demonstrated that best yields are achieved when this high solution to sample ratio since the ionic strength of the medium is lower(Torres, Chen, Rodrigo-Garcia, Jaczynski, & Shahidi, 2007). Although at the same alkali or acid concentration the solution has the same ionic strength, the higher the ratio of solution to sample the diluted the salts from the sample and therefore the ionic strength is lower. The results obtained are shown in Table 1. Protein yields obtained in this study were significantly higher with alkaline compared to acidic condition extraction.

Protein extraction was remarkably better when alkaline solution was employed compared to acid solubilisation; NaOH solution concentration of 0.1 to 0.3 M showed differences ($p<0.05$) between them, however the highest percentage of recovered protein (74%) was obtained using 0.4 M NaOH for extraction. Yields reported in the literature vary in the range of 42% and 90% and generally alkaline solubilisation usually results in higher protein recoveries than acidic treatments (Gehring, Gigliotti, Moritz, Tou, & Jaczynski, 2011). When HCl is employed as extraction solution, the highest yield was found at lowest acid concentrations (49.5%); on the other hand the lowest yield (19.3%) was founded when the highest acid (0.4 M) concentration was employed. This effect can be due to the different behaviour of Na^+ and Cl^- ions. As a result of increased ionic strength (IS) the myofibrillar

proteins precipitate at a lower pH; and therefore, pH must reflect the changing pI in order to achieve maximum protein precipitation (i.e., recovery efficiency) during continuous ISP processing. Increased IS causes a shift in the pI to a lower pH because the Cl^- binds positively charged amino acids (AA) to a greater extent than the Na^+ that binds negatively charged AA (Ockerman, 1996).The lower recovery yields with acidic solution are likely due to the pH of the isoelectric point being lowered and the pH of 5.5 used for precipitation of solubilized proteins no longer being the pH of minimum solubility of the soluble proteins . Because of this factor a negative effect can be seen when concentration of chloride ions is increased.

3.2 ISP assisted by ultrasonics

After assessing the influence of acid and alkaline on the extraction yield of mackerel proteins, ultrasound was employed to assist the extraction in order to achieve a highest recovery yield. A control experiment was carried employing distilled water as solution and testing different ultrasonic treatments: extraction by stirring, ultrasound bath, ultrasound probe at 20% of amplitude and ultrasound probe at 60% of amplitude. In all cases the pH was 6.0 with no significant changes in pH were detected after extraction process. Extraction times, centrifugation process and sample/solution ratio employed were the same as previous section. The results obtained are showed in Table 2.

At this pH, only 5.9% of total protein can be recovered as soluble protein; it has been reported that at this pH sarcoplasmic proteins are completely insoluble, meanwhile myofibrillar proteins are slightly soluble (Torres, Chen, Rodrigo-Garcia, Jaczynski, & Shahidi, 2007). However, a significant increase ($p>0.05$) in protein recovery was achieved following extraction with ultrasound. This fact confirms that, ultrasound leads to a better interaction between extraction solutions and proteins. As the ultrasonic intensity is increased the protein recovered is higher (13.6% at 60% of amplitude). In such case the amount of energy applied to the samples is higher and the cavitation processes leads to a higher level of matrix degradation (Ito, Tatsumi, Wakamatsu, Nishimura, & Hattori, 2003), allowing the extracting solution to be in contact with proteins thereby increasing the yield of the process(Kim, 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% ($p<0.05$).

As it was mentioned, sequential extraction (acid-alkaline) was able to recover almost the 100% protein contented in the fish sample, so it is not necessary to use ultrasound assisted extraction to improve yield. On the other hand, when alkaline extraction is followed by acid extraction the total yield achieved was 83.3%, extraction assisted by ultrasound increased the yield up to 92.5% and the 97.3% of the proteins when 20% and 60% of amplitude was employed. It is a significant improvement ($p<0.05$) when compared to traditional ISP. It implies that ultrasound is a green technology able to increase the recovery yield in ISP process, open new possibilities for protein recovery from fisheries by-products or developing new fish-based products. The possibility that solvent/sample ratio can be reduced, keeping the same high yield, is now open; that technology can lead to a more sustainable fish protein industry.

The fact that more proteins can be extracted in the second step of the process could be as a consequence of a higher degree of matrix degradation, due to a longer exposure to ultrasound. It was reported that ultrasound can lead to some degree of protein hydrolysis at mild pH (6.5) (Ito, Tatsumi, Wakamatsu, Nishimura, & Hattori, 2003; Kim, Kim, Kim, Park, & Lee, 2012), such hydrolysis could be more intense at extreme pH, as used in present work, this may make protein extraction easier as the structural proteins of the muscle (titin, nebulin, and collagen) are broken down.

3.4 Molecular weight of recovered proteins

Size exclusion chromatography was employed to evaluate variation of the protein profile after different extraction processes. Chromatograms are showed in Figure 2 and the weight distribution of the extracted proteins is shown in Figure 3.

Fish myosin, the major myofibrillar protein, is a 520 kDa hexamer consisting of two 220 kDa polypeptides referred to as myosin heavy chains (MHC). Each MHC is non-covalently attached to two 18–25 kDa light chains (LC)(Kristinsson & Hultin, 2003). It has been recently reported that titin (the largest protein known to date), nebulins (in the range of 600-900 kDa) and collagen β, α1 and α2 (250 and 120 kDa) can be extracted using ISP, however these protein remains practically unaltered and they can be detected using SDS-PAGE electrophoresis. However, when the same samples are

extracted using alkaline pH assisted by ultrasound, the bands corresponding to these very high molecular weight proteins were less evident (Tian, Wang, Zhu, Zeng, & Xin, 2014) due to a process of degradation.

When acidic or alkaline solutions are employed for extraction, high molecular weight proteins can be observed, those proteins corresponds to titin, nebulin and heavy chain of myosin, based on the peak corresponding to protein >250kDa acid extraction is more effective in solubilizing larger molecular weight proteins. However, the main peak observed is that corresponding to 35-41 kDa, that is composed by actin (41 kDa), and tropomyosin α and β (37 and 33 kDa). This peak shows that the 40% and 29% of total proteins extracted in acid or alkaline conditions respectively are in this range Furthermore , a small peak (13 and 14% of total area) which corresponds to collagen chains (120 kDa) can be detected. Such profile obtained is in agreement with results published by Tian et als, 2014. Even some traces of short peptides, in the range of 1-5 kDa could be detected, being higher (around 10% of total protein extracted) when ISP is conducted at alkaline pH. An unidentified peak in the size of 13 kDa, accounting for 16.7-20% of total extracted protein, is present in with alkaline extraction; however such peak was not detected after acidic extraction, with either traditional ISP or with ultrasound assisted process. This peak is an indication that the alkaline extraction process is more effective; probably an alkaline soluble protein is being extracted.

When protein size was analysed after ultrasonic assisted extraction, it was found that the area of the peaks corresponding to larger proteins (100 to more than 500 kDa) was slightly lower; meanwhile the area corresponding to those proteins within the range of 10 and 40 kDa was higher. This fact, together the increase in yield, suggests that the largest proteins are still extracted at the same time that some hydrolysis process is taking place. It has been reported how titin and collagen can be degraded after being exposed to ultrasonic treatments (Kim, Kim, Kim, Park, & Lee, 2012), and when these structural proteins are solubilized, the extraction of more actin and myosin can be achieved; which explains how ultrasound enhances the yield in protein recovery

The molecular weight of the proteins recovered in the second step of sequential acid/alkaline or alkaline/acid extraction was also determined. Those peaks corresponding to myosin, titin, nebulin and collagen are absent; indicating that these proteins were completely recovered in the first extraction step of the process, regardless the pH employed. However, small amounts of actin, collagen and peptides can be still recovered.

3.5 Amino acidic profile of recovered protein

High quality protein (i.e., complete protein) is determined based on the presence of all the nine essential amino acids (EAA) in adequate quantities to support human or animal health. To assess protein quality, it is important to determine the amount of EAA in proteins recovered with ISP, to this end the amino acid profile of recovered proteins was characterized. Regardless of the method employed for protein extraction, amino acid profile was constant and no significant changes were detected. In the case of sequential extraction both pellets were mixed before obtaining the profile. Table 4 shows the relative amount of each amino acid compared to total amount, results have been compared with US National Nutrient Database for Standard Reference (USDA, 2017) and other profiles reported in literature(Leu, Jhaveri, Karakoltsidis, & Constantinides, 1981). Main difference seen in the present work is a low recovery of tyrosine; its regular value is within the range of 3.2-3.5%, while in the present work values of 0.9% were detected. On the contrary, histidine content was found in levels twice as high as those reported in the compared references. It is remarkable how histidine yields the 42% of free amino acid in final product.

Data suggest that ISP-US assisted and sequential ISP extraction are feasible, fast and simple method to recover high quality protein that can be applied for multiple purposes. The quality of proteins recovered by ISP, although lower than that of egg protein, is comparable to or higher than that of soy protein and milk protein. The nutritional quality of proteins recovered from whole krill using ISP has been determined (Chen, Tou, & Jaczynski, 2007).

4 Conclusions

A new sequential extraction process, based on ISP technique has been developed; applying this new method it was possible to increase the recovery yield from 49% (using HCl 0.1M) or 64% (using NaOH 0.1M) to 100% (using 0.1M HCl/NaOH sequential extraction). Besides, the influence of ultrasound on the process yield was determined, it was found that 60% of amplitude for ten minutes in 0.1M NaOH solution was able to recover the 94% of total protein in a single extraction step. It was shown that lower amplitudes (20%) or ultrasonic bath increases the yield of the extraction when compared to traditional ISP. Alkaline extraction was shown to solubilize a broader range of proteins than acid extraction, including a non-identified 13 kDa protein which can explain the differences in yield between acid and alkaline extractions. From a nutritional point of view, the proteins recovered by the methods explored in this work, are suitable for food purposes due to its high content en essential and non-essential amino acids.

That implies that ISP-US assisted extraction is a promising tool in order to develop greener extraction process, since the amount of water and reagents can be decreased; however further research is needed in order to optimize the process.

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Figure captions:

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

Figure 2: SE-HPLC chromatograms of extracted proteins: (a) HCl 0.1M (stirring); (b) NaOH 0.1 M (stirring); (c) HCl 0.1 M (ultrasound assisted, 20% amplitude); (d) NaOH 0,1 M (ultrasound assisted, 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 amplitude10 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 amplitude10 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

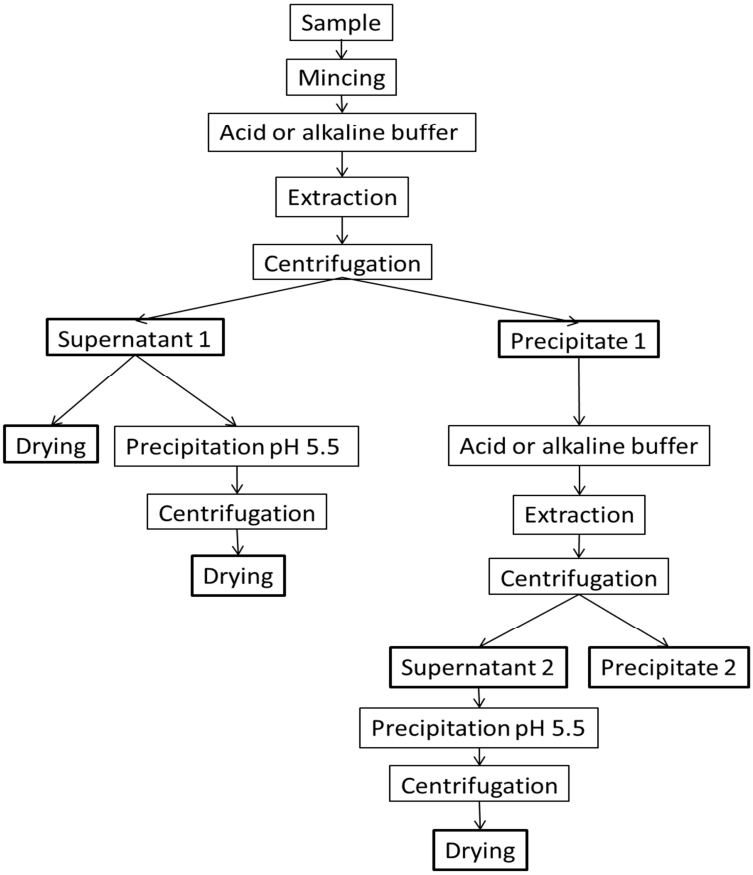
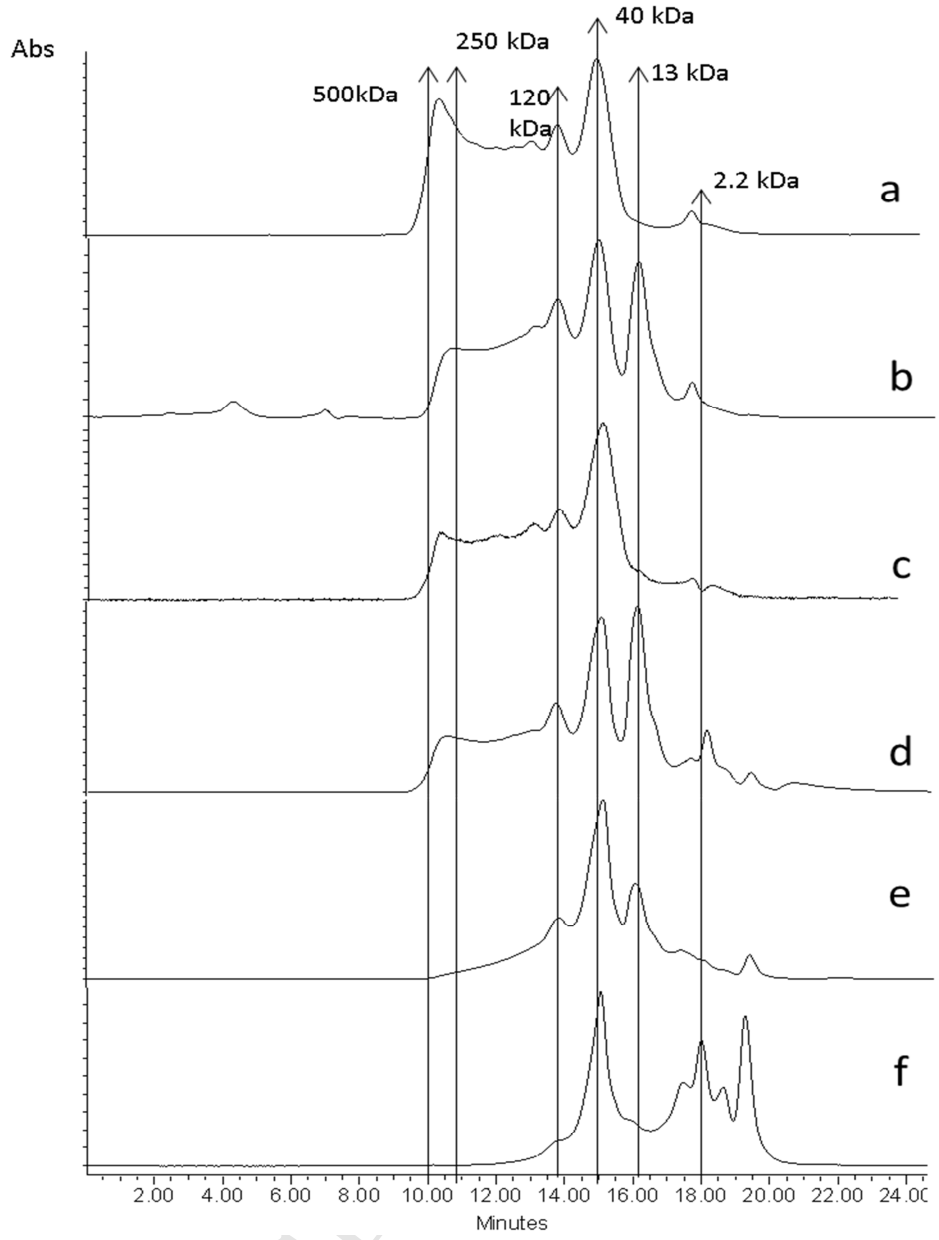


Figure 2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120 kDa
40 kDa
13 kDa
2.2 kDa
a
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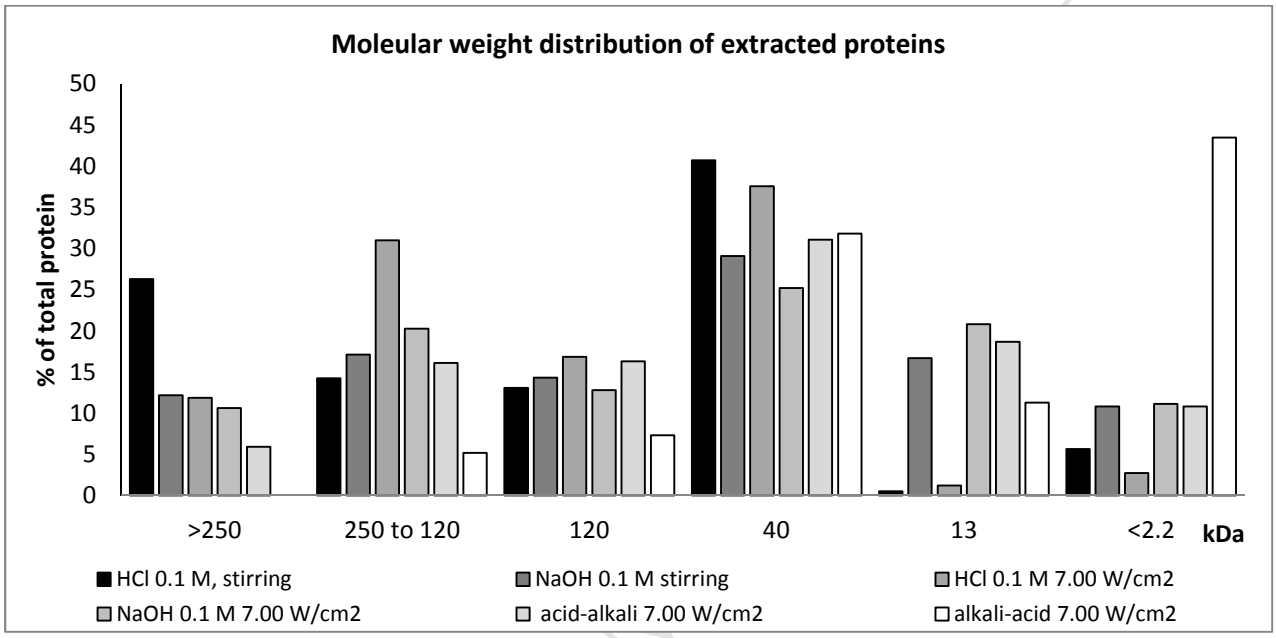
Minutes

Figure 3



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