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BY-PRODUCTS OF THE WHELK PROCESSING INDUSTRY AS VALUABLE SOURCE OF ANTIOXIDANT PEPTIDES

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Abstract: The fish and shellfish industry processes 851,984 tonnes of fish per year worldwide. However, only 43% of that is consumed, and valuable proteins are processed as waste. Protein hydrolysates are widely used in food technology for their nutritional and functional properties. The goal of this project is to extract protein from whelk by-products derived from the shellfish processing industry and create protein hydrolysates that have marketable value. The by-products were divided into two types: raw (R) and cooked by-product (C). The proteins were extracted using the pH shift method and quantified using the Bradford assay. It was possible to extract a maximum of 455 mg/g at a neutral pH, for which R had the highest protein yield. Proteins were also qualified using reverse phase high-performance liquid chromatography (RP-HPLC) that showed that R has more hydrophilic proteins while the C extracted protein showed more peaks in the hydrophobic phase. The Fourier-transform infrared spectroscopy (FTIR) indicated the presence of glutamine, tyrosine, and serine in the extracted proteins. Extracted proteins were then hydrolyzed using Alcalase and α-Chymotrypsin. It was possible to obtain higher degrees of hydrolysis (DH) using Alcalase. The hydrolysates were tested for antioxidant activity using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical antioxidant assay. Alcalase hydrolysates showed to have overall lower IC50 for stabilization of the DPPH radical than α-Chymotrypsin, the lowest one being 13.92±1.57 µg/mL for the Alcalase hydrolyzed neutral proteins. The IC50 results obtained are significantly lower than the ones described in other studies using the same enzymes or other marine species. This can indicate that more heterogenous mixtures of by-product can originate extracted proteins that when hydrolyzed lead to higher radical scavenging activity, thus making shellfish industry by-product a sustainable and valuable source of antioxidant peptides.

Keywords: Shellfish; Bioactive peptides; Protein extraction; Protein hydrolysates, Waste management, Nutraceuticals

Introduction

Marine creatures compose about half of the earth's biodiversity and store multiple bioactive compounds (Kim & Wijesekara, 2010). Moreover, all these marine organisms are composed of high protein percentages, making them the ideal study material for protein derived bioactive peptides (Harnedy & FitzGerald, 2012).

The marine and seafood industry are of a great deal of importance to remote coastal regions mainly in Europe, providing a lot of job positions in diverse areas (Garcia-Garcia *et al*., 2019). The amount of seafood matter that is considered waste has been decreasing over the years due to its utilization for other products (Hynes & Hennessy, 2012). A significant volume of marine waste, (considered

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Category 3 by the EU regulation 1069 of 2009), are supplied as bait, sent for feeding furred animals, processed as animal or pet food, used in composting, and rendering processes as it has low economic value. Moreover, most of these by-products are disposed as waste by incineration adding costs to the marine processing industry (Mack & Huntington, 2004). Among these types of marine by-products there are shellfish shells, undersized specimens, or a combination of these with meat and water arising from the washing of the shellfish, most by-product material is composed of whelk (B. Lorcan, personal communication, October 2019). These materials may be an excellent, suitable, and economical source of proteins and other biologically relevant molecules.

Whelk processing waste is used in the Republic of Ireland and in the UK as fishing bait in the potting sector. The use of whelk waste for this purpose has been legally permitted and is a common practice, this is more profitable than incineration but only a small percentage of the waste is qualified to be used in this manner (Reeve *et al*., 2010). Whelk meat possesses mainly glutamic acid (13.11g/100g) and aspartic acid (8.98g/100g), it also has lower but still significant quantities of lysine, leucine, arginine, and isoleucine. Whelk's overall composition is structural proteins (such as collagen and elastin) that provide the mollusc with high elasticity along with other proteins that provide its elastic mechanical range like keratin (Corbett, 2006).

Nowadays the correlation between food and health consciousness has increased in western population (Hoque *et al.*, 2018). There seems to be a growing trend in the increase of consumption of fortified foods in health-conscious people (Lau *et al.*, 2013). Proteins, especially collagen, possess high nutritious value by themselves, but they have been proven to have other benefits when hydrolysed. Collagen hydrolysates were shown to have anti-oxidization and anti-fatigue properties *in vivo* (Ding *et al*., 2011). Keratin hydrolysed peptides also demonstrated to have antioxidant activity (Wan *et al.*, 2016) and are also used in the cosmetic industry in several hair products (Villa *et al*., 2013). The incorporation of these peptides in food products, would be a more profitable and sustainable use of the whelk waste by-products, while contributing to a circular economy.

While there have been studies done on bioactive peptides from protein extracted from different types of shellfish and fish (Bordbar *et al.*, 2018; He *et al.*, 2013; Ngo *et al.*, 2010; Huang *et al*., 2011; Jumeri & Kim, 2011) there has not been studies on the subject in relation to industry by-products that have more heterogeneous mixtures of product (several shellfish/fish species) as opposed to homogeneous mixtures (one species of shellfish/fish).

Oxygen is an essential molecule for living organisms, despite this its nature is extremely reactive and produces reactive oxygen species (ROS). Life forms have developed a complex network of metabolites and antioxidant enzymes that work together to prevent oxidative damage in cellular components such as DNA, proteins, and lipids. Antioxidant systems generally prevent the formation of ROS species or eliminate them before they can damage vital cell components. (Vertuani *et al*., 2004). Cellular ROS are comprised of free radicals such as the hydroxyl radical (· -OH) and the superoxide anion (O_2^-), hypochlorous acid and hydrogen peroxide (H_2O_2) (Valko *et al*., 2007).

Oxidative stress is thought to contribute to various diseases, including many neurodegenerative diseases such as: Alzheimer's disease (Christen, 2000; Nunomura *et al*., 2006), Parkinson's disease and motor neuron diseases (Barber *et al*., 2006; Wood-Kaczmar *et al.*, 2006), as well as pathologies caused by diabetes (Ramakrishna & Jailkhani, 2007) and rheumatoid arthritis (Wruck *et al*., 2011).

Shellfish processing by-products could be a valuable source of antioxidant peptides, this would result in the creation of inexpensive and sustainable ingredient source for use as an antioxidant in a fortified food product. The goal of this research is to extract the protein from these by-products and create antioxidant protein hydrolysates that have marketable value.

Materials & Methods

The shellfish by-products/waste from which the proteins were extracted was kindly provided by Sofrimar Ltd. Co Wexford. The by-product obtained is mainly composed of whelk processing by-products. The solutions in the methods section were made from stock solution of Hydrochloric acid reagent grade, 37% (Sigma-Aldrich, USA) and Sodium hydroxide ACS reagent, ≥97.0%, pellets (Sigma-Aldrich, USA). For the protein quantification Bradford's reagent, Biotechnology Grade (VWR, USA); and Bovine Serum Albumin (BSA), lyophilized powder (Sigma-Aldrich, USA) were used. For the Reverse Phase High-Performance Liquid Chromatography (RP-HPLC), Acetonitrile (VWR, USA), Trifluoroacetic acid (TFA) (Sigma-Aldrich, USA) and ultra-pure water were used. Alcalase and α-Chymotrypsin were ordered in lyophilised powder form (VWR, USA). The proteins were hydrolysed by Alcalase from Bacillus licheniformis (VWR, USA) and Bovine pancreas α-Chymotrypsin (VWR, USA). For the TNBS assay a Reaction Buffer (RB) 0.1M sodium bicarbonate, pH 8.5 was prepared, 1% TNBS (Sigma-Aldrich, USA) was diluted to a 0.1% TNBS solution in Reaction Buffer, the 10% SDS solution was also prepared.

Protein Extraction and Quantification

By-product preparation

The shellfish by-products that were provided arrived separated into two categories: Cooked (C) and raw (R). The C by product was cooked in the factory during its processing to be sold as cooked shellfish. The R by-products consisted of a solid (meat) phase and a liquid (water) phase.

Protein extraction

The protein extraction procedure was based on the protocol described in Neves *et al.* (2016). The marine by-product was weighted, and water was added at a 1:5 proportion at room temperature. The mixture was homogenized with a plastic jug blender (Russell Hobbs 24610, UK) at setting 2 of the blender for 30s. After homogenization, the mixture's pH was adjusted with 1M NaOH to be within the pH range of 10.0 – 12.0. The solution was then stirred at maximum velocity at room temperature for about 15 minutes (WiseStir MSH-A Witeg, Germany). This was then centrifuged for 15 min at 316987g (ROTANTA 460, Hettich, Germany), the pellet was discarded and the supernatant containing the proteins was stored at -20ºC until use.

Isoelectric Precipitation of proteins

The supernatant obtained from protein extraction went under isoelectric precipitation. The supernatant was divided into five different 50 mL Falcon conical tubes, each solution was adjusted to a different pH (2 and 5 - acid, 7 - neutral, 9 and 12 - basic) using 1M NaOH and 1M HCl solutions respectively. Each solution was then centrifuged for 5 min at 316987g (ROTANTA 460, Hettich). The pellet was

discarded, and the supernatant was stored separately in the freezer (-20ºC) until protein quantification. Basic pH extracts and acids pH extract were further combined and are referred as basic soluble and acid soluble proteins.

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)

To determine the type of molecules present in the samples a high-performance liquid chromatography was performed (SPD-20A Shimadzu, USA). The protein samples were filtered using a Syringe filter - Pore size: 0,2 µm (VWR, USA). The chromatographic separation was performed on a reversed-phase HPLC column (4.6 × 100 mm, 5 µm particle size, Dikma Technologies, Beijing, China) with the mobile phase being solvent B, 80% acetonitrile (VWR, USA) and 0.1% Trifluoroacetic acid (TFA) (Sigma-Aldrich, USA), and solvent A, composed of ultra-pure distilled water at a flow rate of 1.0 mL/min.

The analysis was performed at both 218nm and 280nm wavelengths, each sample injection volume was 1mL and had a 30-minute run time.

From this part on the raw by-products, consisting of the solid (meat) phase and a liquid (water) phase were homogenized into the raw by-product category (R).

Protein quantification

The protein samples were quantified using the Bradford assay (Bradford & Marion, 1976).

The samples were divided into acid soluble proteins (AP), pH=2 and pH=5, neutral soluble proteins (NP), pH=7 and basic soluble proteins (BP), pH=9 and pH=12. Three replicates were measured for each sample. A standard BSA stock solution (100 µg/µL) solution was serially diluted and used for the calibration curve readings. The absorption reading was performed at a 595nm wavelength using a UV/VIS Spectrophotometer (UV 1800 Shimadzu, USA).

Protein qualification

The cooked extracted protein solution started to become a gelatine consistency overtime and it was hard to solubilize and analyse in the lab without ruining equipment, it might be because the extracted proteins were denatured from the cooking process making it not an ideal source (Yu *et al.*, 2017), also the RP-HPLC results indicate the presence of hydrophobic protein that could have possibly been precipitating in the aqueous solution. Considering this and the fact that the raw by-product showed to have a higher yield of protein extraction over the cooked one, only the raw by-product was used in the continuation of this study.

Fourier-transform infrared spectroscopy (FTIR)

To discern the type of amino acids that make up our proteins, FTIR was performed on lyophilized protein samples. The IR spectra were acquired with the instrument (PerkinElmer, model SPECTRA 65) in the 650–4000 cm^{-1} wave number range.

Protein Hydrolysis - pH-Stat Method

Hydrolysis with Alcalase was performed at a 1:50 ratio and at a 1:100 ratio with α-Chymotrypsin.

The hydrolysis assays were performed in a jacketed beaker water bath maintained at 60ºC for Alcalase hydrolysis, and 47ºC for α-Chymotrypsin hydrolysis, by a stirring hotplate (WiseStir MSH-A Witeg, Germany). The initial pH for hydrolysis was 7.5 and 8.3 for Alcalase and α-Chymotrypsin respectively.

The proteins were hydrolysed for 90 minutes, and the final hydrolysates were stored at -20 ºC for later bioactivity testing.

Protein Hydrolysis Quantification - 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) assay

The degree of hydrolysis (DH) was extrapolated using the TNBS assay, following the R.B. Sashidhar *et al*. (1994) protocol.

During the hydrolysis assays, samples were collected from different time points throughout hydrolysis (t=0; t=5; t=30; t=60; t=90). For a standard curve, a 0.1mg/ml α-isoleucine solution was prepared in reaction buffer, and subsequently serially diluted. For each sample 0.25mL of 0.01% (w/v) solution of TNBS was added, after which they were incubated in a dark cabinet at room temperature for two hours. After incubation 0.25mL of 10% SDS and 0.125mL of 1M HCl were added to each sample in order to stop and stabilize the reaction. The absorbance of each sample was measured at a 335nm wavelength (UV 1800 Shimadzu, USA).

Bioactivity assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay

For this assay, a 0.2mM solution of DPPH (Sigma-Aldrich, USA) was prepared in MeOH (VWR, USA). The DPPH solution was serially diluted vertically in a row of a 96 well microplate to extrapolate a standard curve and in a different row 0.1mL of DPPH was added to each well to be used as a control. Each hydrolysis sample (Alcalase – AP, NP, and BP samples and α-Chymotrypsin – AP, NP, and BP samples) was vertically serially diluted in two rows, ones to use as a blank sample and the other where DPPH is added.

After DPPH is added to the respective wells the microplate is wrapped in foil and incubated in a cool dark place for 30 minutes. Subsequently the absorbance was measured at 517nm wavelength (VersaMax ELISA, USA).

Statistical Analysis

The results from the protein quantification and qualification based on pH solubility were tested using one-way ANOVA and the Tukey test using Excel 2019 (Microsoft office, Microsoft, USA).

Results and Discussion

Extraction and Quantification of Proteins

The protein extraction of both by-product types led to the results presented in the following graph.

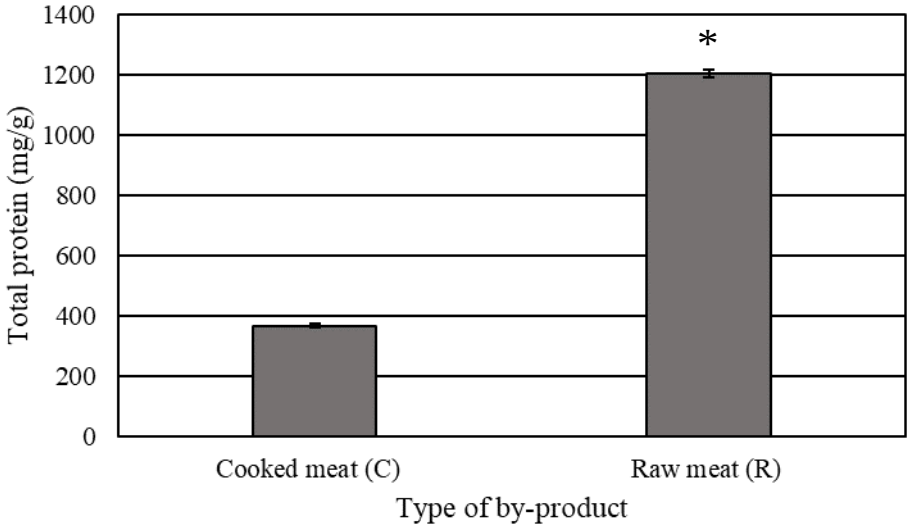


*Figure 1 - Protein concentration in the extracts from cooked meat (C) and raw meat (R) by-products from the whelk processing industry. Values represent averages of n=3 ± sd and, * represents significantly different values.*

Overall, the by-product type with the largest amount of protein is the raw meat (R), consequently the cooked meat (C) has the lowest amount of protein. Considering these results and the fact that the cooked by-product is significantly harder to manage and handle throughout the processes of extraction, the water and raw meat by-product were homogenized into one sample and were the only types of by-product studied. Overall, the raw meat and water by-products yielded a protein extraction of 1202.8±18.1 mg/g and the cooked meat yielded 368.1±18.15 mg/g.

The concentration of proteins extracted from these by-products is higher than that obtained from other by-products from the shellfish processing industry. Neves *et al*. (2016) reported obtaining 141.25±12.41 mg/g from the by-product of the mussel processing industry when extracted with similar conditions comparing with the 1200.00±12.90 mg/g. To the best of our knowledge no other study reported the extraction of proteins from by-products from the whelk processing industry.

There is a large difference in yield between this extraction and the one described in Neves *et al*. (2016) which can be due the high lipid content of the mussel by-product used. The nature of the of the by-product provided by the shellfish processing industry (Sofrimar Ltd) is more heterogenic (the product received was a mixture of meats from different shellfish) and diverse than the mussel by-product (the only meat present in this by-product was mussel meat).

Biochemical Analysis

Isoelectric precipitation

The isoelectric precipitation of both by-product types was performed in acid, neutral and basic conditions. The yield for each pH category relative to each different by-product type led to the results presented in the graph below.

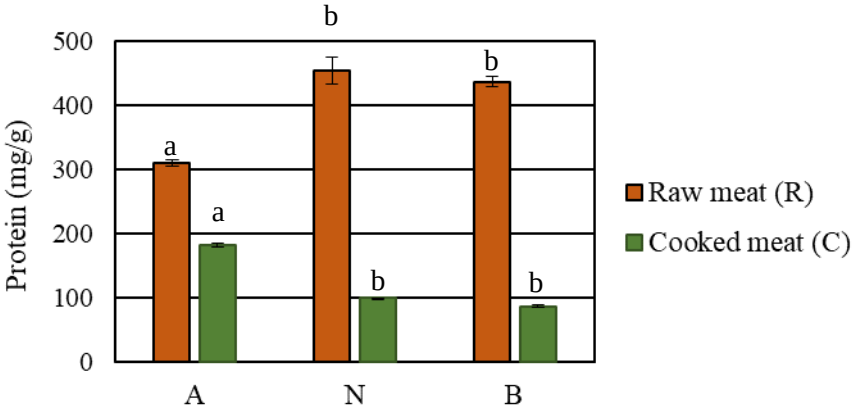


Figure 2 – Protein concentrations (mg/g) in different by-product types (R=Raw; C=Cooked); A (acid soluble protein); N (neutral soluble protein); and B (basic soluble protein). Values represent averages of n=3. For each by-product type different superscript letters are significantly different (P<0.05)

The R by-product has similar concentrations of basic and neutral soluble proteins these two showing the largest amounts, closely followed by the acid soluble protein. The C by-product, like R, has similar quantities of basic and neutral soluble protein, but unlike in R by-product N and B are of lower concentration, than the AP, which, has the highest concentration.

In the meat cooking process, the high temperatures cause the denaturing of proteins as well as the reduction of the capacity of water retention of the meat (Lawrie & Ledward, 2006). Within the two by-product types (C and R) the neutral and basic protein quantity is not significantly different from one another. This is opposite to A and N quantities, and A and B quantities which are different from each other in all by-product types.

Other studies that used a similar protein extraction method using mussel meat reported protein recoveries of 310 mg/g of protein with acid extraction and 480 mg/g for alkaline extraction (Vareltzis & Undeland, 2012). Although protein extraction of pure mussel meat has a higher yield, the proportion difference between the A and B yields are similar.

Extracting proteins at neutral or close to neutral pH, has been reported as more beneficial to the food processing industry since the process does not need the addition of any chemicals reducing the costs (Neves *et al*., 2016).

Protein Qualification

HPLC

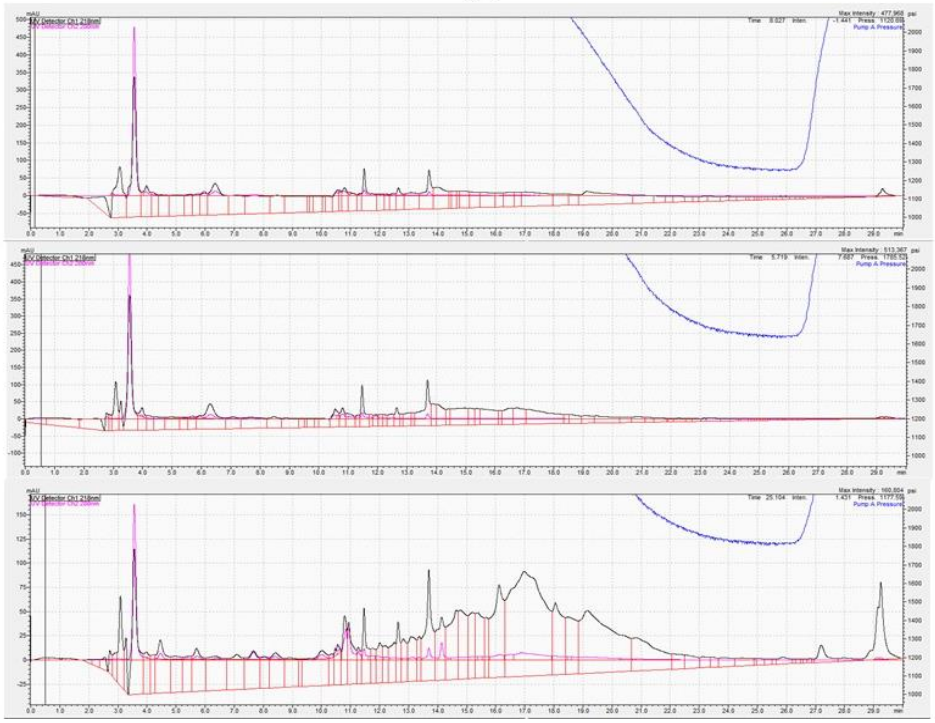
The HPLC analysis was performed with a hydrophilic phase of ultra-pure water and a hydrophobic phase of 70% Acetonitrile. The following results were obtained for each sample.

(C)

(A)

(N)

(B)

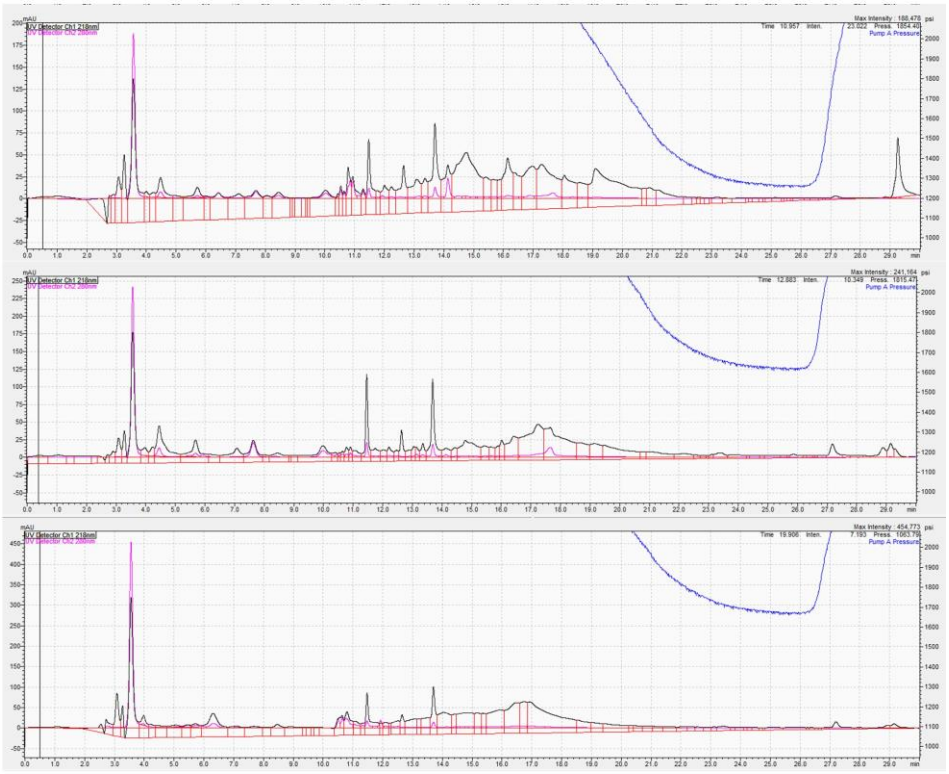


(R)

(A)

(N)

(B)



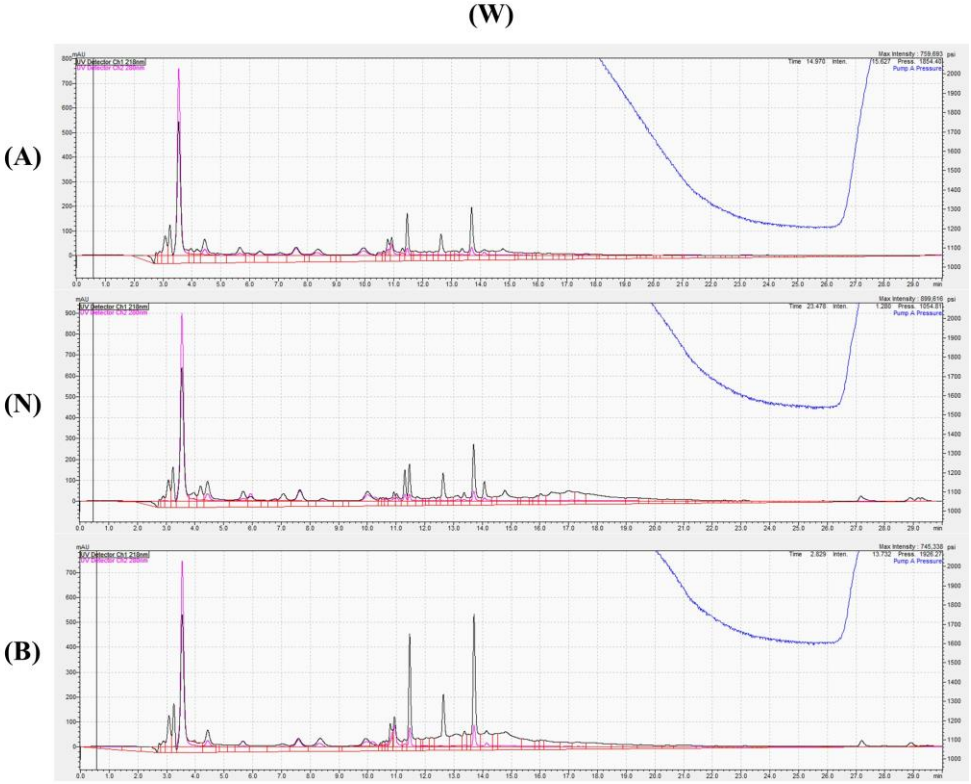


Figure 3 – HPLC graphical results of the cooked (C), raw (R) and water (W) by-products for the acid (A), neutral (N) and basic (B) protein. The black line represents absorption at 218nm and the pink at 280nm.

The HPLC results of all samples show similar peaks in the hydrophilic part of the spectrum both at 218 and 280nm. For the cooked by-product protein extraction samples all three (A, N and B) show similar peaks in the hydrophilic part of the spectrum both at 218 and 280nm. All three protein samples also show peaks in the middle part of the graph (intermediate hydrophilicity), although the ones for B protein are much more intense, and both A and B protein show peaks in the most hydrophobic part of the spectrum at 218nm being that B protein ones are more intense.

For the raw by-product extracted protein similar to the cooked all three protein samples show similar peaks in the hydrophilic part both at 218 and 280 nm. All three samples also show various peaks in the middle part of the graph at 218 nm but for the R by-product they are more intense for the A protein and least intense for the B protein. The N and B protein have very faint peaks in the hydrophobic part of the spectrum at 218 nm while A sample has a higher intensity peak comparable to the B sample of the C by-product.

For the W by-product like the other by-products shows similar peaks in the hydrophilic part both at 218 and 280 nm for all three protein types, and peaks in the intermediate part of the spectrum, these are similar for the A and N protein and more intense for the B protein, overall, these peaks are less intense than the ones for the C and W by-products. Peaks in the hydrophobic part of the spectrum are practically nonexistent which is expected for proteins dissolved in water.

Research indicates that cooked meat shows increased hydrophobicity in its myofibrillar proteins and its connective tissue, and also a significant increase in protein oxidation (Yu *et al.*, 2017), therefore

the presence of majority hydrophobic protein in the cooked by-product extracted protein is to be expected.

FTIR

Raw meat acid, neutral and basic samples were shown to have the following IR spectra.

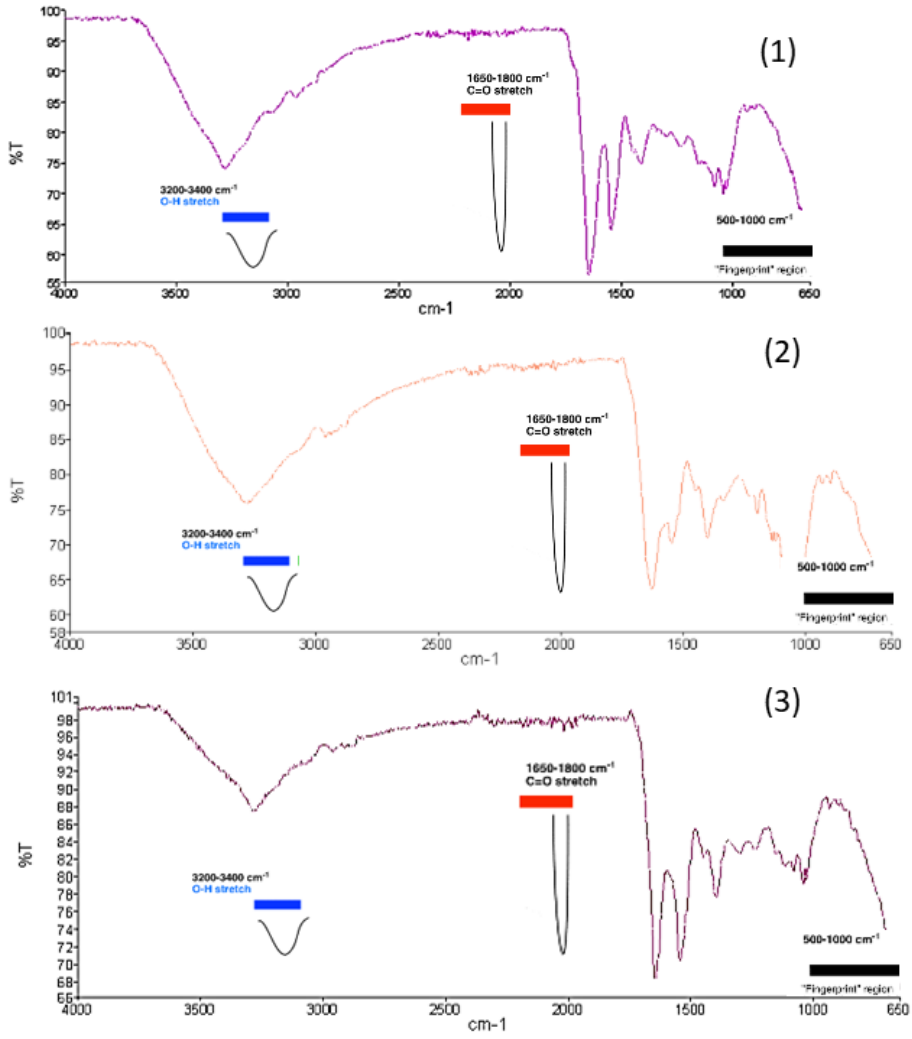


Figure 4 – Fourier-transform infrared spectroscopy (FTIR) results for lyophilized raw meat protein for all three types of protein AP (1), NP (2) and BP (3).

All three protein samples have similar FTIR spectrum profiles, except NP shows less strong peaks in the 1000-1800 cm-1 range. In relation to protein FTIR spectrums the presence of peaks in the OH and C=O bonds are to be expected since these are bonds present in all types of amino acids.

All proteins present a peak around 1668-1687 cm-1 range, considering infrared (IR) vibrational modes characteristic to proteins, this is characteristic of the C=O bond of glutamine and alanine. Another peak also present in all three samples is in the range of 1235-1270 cm-1 which indicates the present of tyrosine (C-O) same as the 1025-1030 cm-1 range that indicates the presence of serine (C-O) (Barth, 2007).

Studies on the influence of temperature (60ºC to 100ºC) on meat during the cooking process have revealed that heat can alter the structure of amino acid residues (Yu *et al.,* 2017). Glutamine is derived from glutamic acid (Krebs, 1935) and it is reported that alanine and glutamic acid are primarily responsible for the taste of cooked seafood. Alanine contributes to the sweet taste and glutamic acid to the savoury taste of shellfish (Venugopal, 2018). Therefore, the presence of these amino acids seems to align with the amino acid composition of most shellfish species.

Protein Hydrolysis

Hydrolysis with Alcalase and α-Chymotrypsin were shown to have degrees of hydrolysis presented in the following graph.

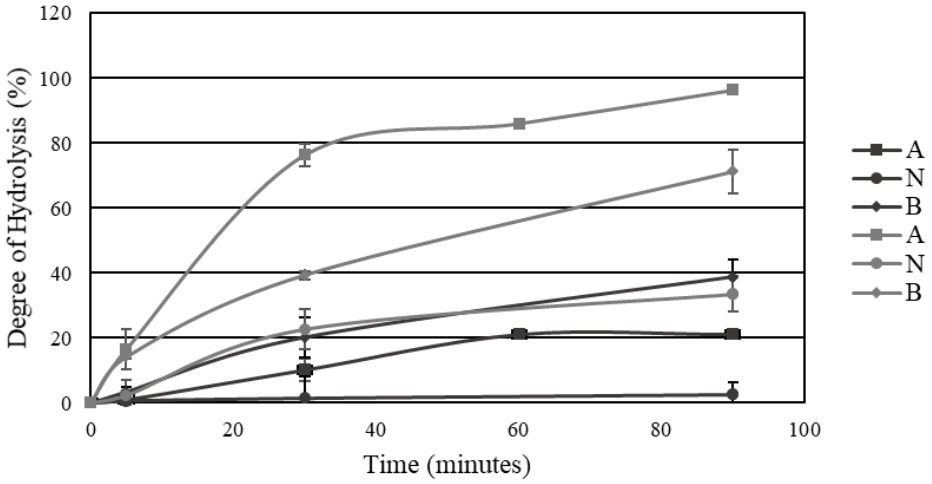


Figure 5 – Degree of hydrolysis of the extracted protein with alcalase (grey) and α-Chymotrypsin (black), in relation to the more hydrolyzed sample (acid alcalase hydrolysates).

During hydrolysis α-Chymotrypsin caused a higher pH shift than Alcalase but after performing the TNBS assay, hydrolysis using alcalase produced a higher concentration of released amino groups and therefore a higher degree of hydrolysis (DH). This was expected due to alcalase's broad peptide cleaving spectrum (Sbroggio *et al*., 2016), conversely, α-Chymotrypsin only hydrolyses the C-terminal side of aromatic amino acids like phenylalanine, tryptophan, and tyrosine (Kumar & Venkatesu, 2012).

These results are similar to what was described in other studies where shrimp by-product extracted protein was hydrolysed with alcalase the DH was within a 59-60% range (Dey and Dora, 2014). Studies where blood cockle and green mussel extracted protein was alcalase hydrolysed the degree of hydrolysis was 34.00% (Amizah and Masitah, 2016) and 43.81% (Ismail and Hasni, 2014), respectively when hydrolysis was performed at optimal conditions.

α-Chymotrypsin is not as studied as alcalase in the scope of producing bio active peptides due to its very selective range of cleavage. Regardless, it still as the potential to produce peptides with relevant bio activities.

Antioxidant Activity

The IC50 for DPPH scavenging activity of each sample is presented in the following graph.

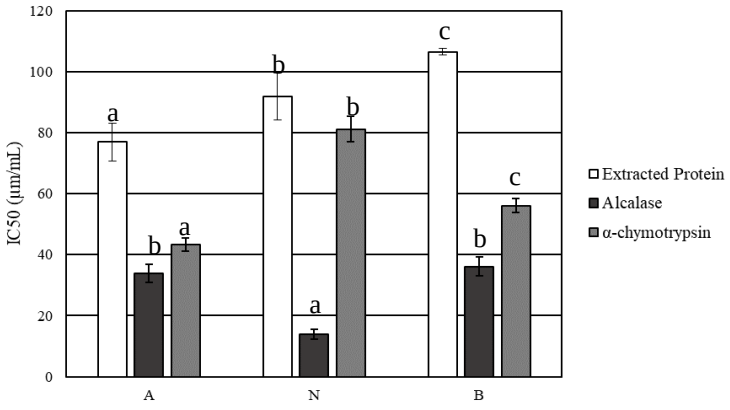


Figure 6 – IC50 for antioxidant activity of non-processed protein (Extracted Protein) and alcalase and α-Chymotrypsin hydrolyzed protein. Values represent averages of n=3. For each category (extracted protein, alcalase and α-chymotrypsin) different superscript letters are significantly different (P<0.05)

The A, N and B non-hydrolyzed protein showed an IC50 for DPPH of 76.91± 6.30 µg/mL, 91.82±7.66 µg/mL and 106.51±1.04 µg/mL respectively.

Alcalase hydrolysates showed to have an IC50 for the DPPH assay of 33.79±2.96 µg/mL, 13.92±1.57 µg/mL and 36.09±3.04 µg/mL for A, N and B protein, respectively, and α-Chymotrypsin hydrolysates 43.24±2.11 µg/mL, 81.18±4.07 µg/mL and 56.02±2.29 µg/mL for A, N and B protein, respectively.

In other studies DPPH assays performed with alcalase hydrolysates from marine species such as tunicate (*Styela clava*) was shown to have an IC50 value of 370.9 µg/mL (Jumeri & Kim, 2011), others like stone fish (*Actinopyga lecanora*) presented an IC50 of 500 µg/mL (Bordbar *et al*., 2018), along with shrimp processing byproducts with an IC50 of 500 µg/mL (Huang *et al.*, 2011).

Alcalase hydrolysis of mung bean protein (*Vigna Radiata*) was described in Murillo, 2017 to show an IC50 for the DPPH assay of 152.52±1.42 µg/mL, and alcalase hydrolyzed Nile tilapia proteins showed an IC50 of 660 ± 0.40 µg/mL (Ngo *et al.*, 2010).

In other studies DPPH assays performed with α-Chymotrypsin hydrolysates other protein sources such as Pistachio Nuts (*Pistacia vera L*.) was shown to have an IC50 value of 451±1.047 µg/mL (Dumandan *et al.*, 2014)

Marine species extracted proteins hydrolyzed with trypsin generated DPPH IC50 values of 30 µg/mL for *Paratapes undulatus* a species of saltwater clam (He, *et al.* 2013), pepsin generated hydrolysates for tunicate (*Styela clava*) protein showed an IC50 value of 750.0 µg/mL (Jumeri & Kim, 2011).

Both alcalase and α-Chymotrypsin showed significantly lower IC50s from those reported from different protein sources. Alcalase results are comparable to the trypsin hydrolysates described in He *et al.*, 2013.

Conclusions

It was possible to extract proteins from shellfish processing industry by-products using the pH shift method, overall, for all three protein types (A, N and B). Raw meat yielded a higher quantity of protein per gram of by-product and had similar chromatographic profile to the water portion of the by-products. Taking that into account, the water and raw meat were homogenised and only those were used for the rest of the research. This shows that the raw by-product stream can be used for the porpoise described in this research without separation between water and meat, which is valuable for the shellfish processing industry.

The protein characterization results showed that proteins of different degrees of solubility (hydrophobic and hydrophilic) are present in the extract as well as different sizes. Protein extraction with pH shift, also show different protein solubility, although a higher quantity of neutral and basic protein extracted.

Hydrolysis with Alcalase and α-Chymotrypsin let to different degrease of hydrolysis, showing once more the heterogeneity of the proteins extracted and their potential for the generation of different peptides with different biological activities.

For the antioxidant test preformed, the IC50 values of intact protein versus protein hydrolysates significantly drop, showing hydrolysis with both enzymes increases the extracted protein antioxidant potential. Moreover, non-hydrolyzed protein also has a significantly low IC50, which means that extracted protein can potentially act as an antioxidant without further processing.

The IC50 results obtained are significantly lower than the ones described in other studies using the same enzymes or other marine species. This can indicate that more heterogenous mixtures of by-product can give origin to extracted proteins that when hydrolyzed possess higher free radical scavenging activity.

These by-products show potential to be a new generation of bio active peptides that can be valuable to the industry as novel and sustainable ingredients for the food industry as supplement/fortified foods and nutraceuticals. Further studies need to be completed to assess the antioxidant capacity of these hydrolysates using other *in vitro* antioxidant assays, as well as *in vivo* studies to assess the bio availability of these peptides. Furthermore, there is potential for other bio activities linked with the hydrolysates generated in the current study.

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