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Proteins from fish wastewater: a valuable source

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1. Introduction

1.1 Contribution of fishery and aquaculture to waste production

Nowadays, in the worldwide agro-food sector it is unthinkable to neglect issues like food waste and food loss. Indeed, food waste became an actual problem to deal with since the word sustainability was defined. As the global population is growing up, both food demand and the awareness to manage food production in a different way are increasing. It is time to switch to a sustainable production. In general, along all the food production chain, every phase can contribute to achieve a more sustainable production: since the obtaining of the raw material to management of food waste. Food loss refers to food lost for environmental, aesthetic or safety concerns. Food waste "refers to the decrease in the quantity or quality of food resulting from decisions and actions by retailers, food service providers and consumers"(*The State of World Fisheries and Aquaculture 2020*, 2020). Food waste is also one among the several indicators of the SDS (sustainable development goals) of the Agenda 2030 by FAO. According to FAO (2020), food waste and food loss account for one third of the food produced thus highlighting the need for food waste and food loss reduction in the agro-food system and involve the entire food chain. The sector focused by FAO in a report of 2020 is the fisheries and aquaculture sector: it provides an overview on the state of fisheries and aquaculture. In this report apparent consumption per capita of fish was 20,5 Kg in 2018 and the total production reached 178,5 million tonnes. Production from aquaculture is increasing along the years because of the need of more fish and fish products supplies. As shown in figure 1 in 2018, it was responsible of 46% of the total production, but fisheries sector is today the principal source of fish and fish products yet, except in China where aquaculture is prevalent over fisheries.

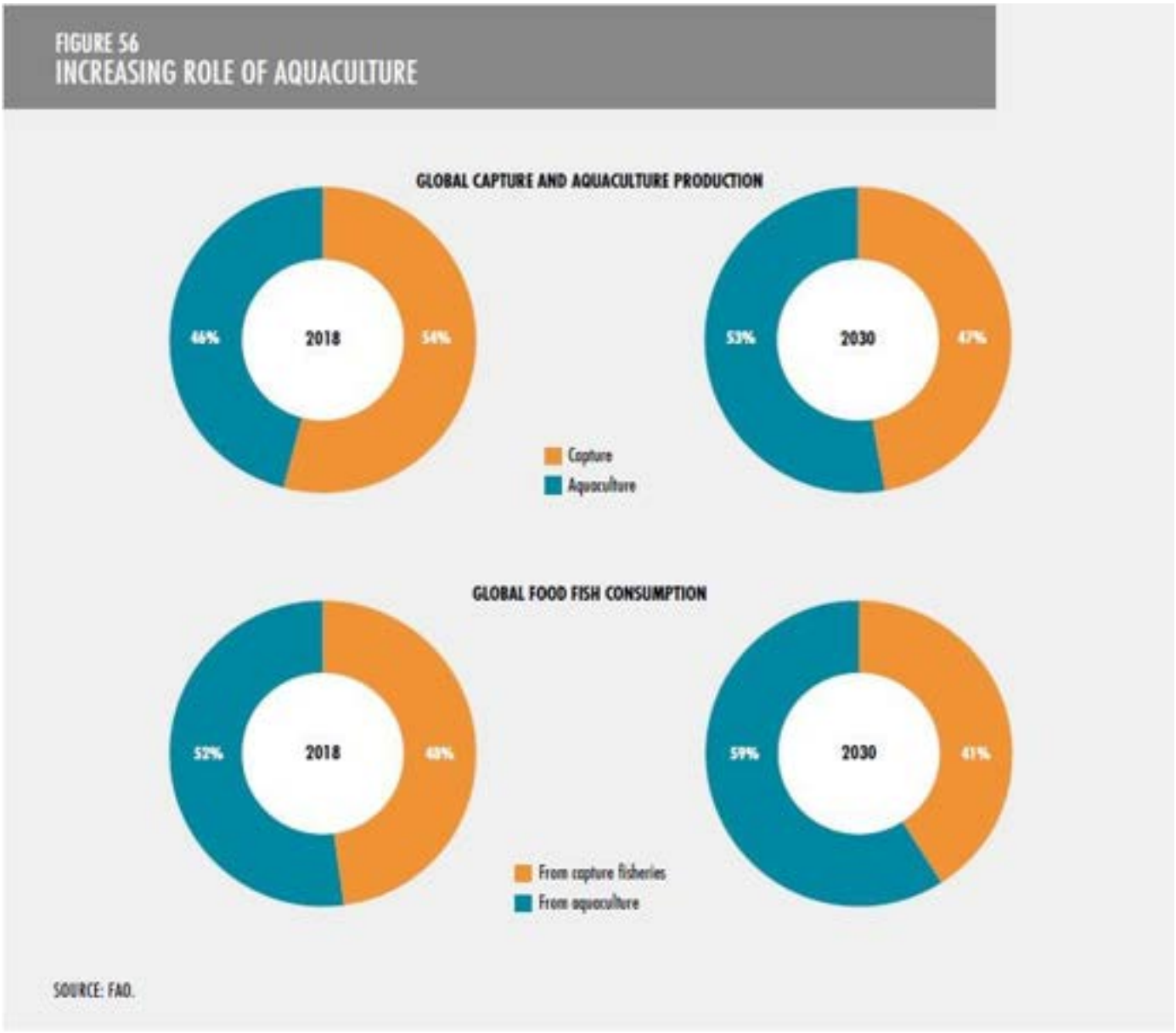

Fig. 1. Data from FAO (2020).

Fish is a perishable food that requires attention during capture, storage, handling and processing. Thus, big amount of products can be lost when it is not treated correctly. In addition, two-thirds of the total amount of fish is discarded as waste, creating massive economic and environmental concerns. Usually, this fish doesn't match weight or market requirement because it is smaller than the legal size, damaged or it is from low-value species. It is estimated that about the 25% of all the caught fish never reaches the market meaning that 27 million tons of unwanted fish are discarded into the sea. This because the current food system is based on a linear and globalized

production. Circular economy can help to exploit underused or discarded marine material for a sustainable development of high added value products derived from fishes. In 2015, EU-Common Fisheries Policy introduced the “landing obligation”, in force from 2019, which aims to eliminate fisheries discards by encouraging fishers to fish more selectively and to avoid unwanted catches.
Fish waste is partially destined for the creation of fertilizers, fishmeal, and fish oil with low economic profit or used as raw material for direct feeding in aquaculture , and partially thrown away. Consequently, a better fish-waste management is required to defeat environmental issues and for the totally use of biomass for goals of high-commercial value. In light of this, the growing current attention to valid alternative uses of fish by-products perform an important task in the economic growth and sustainable development. Many investigations have reported analysis regarding their possible uses, since they are a rich source of value-added compounds, including bioactive peptides, enzymes, and bio-polymers, with many possible uses in several fields.

Another issue concerning sustainability is fish by-products management. Fish processing generates a huge amount of side streams that can be managed in an intelligent way. More than 70% of fish catch is processed, and from 20 to 80% of the products result as waste, depending on the level of processing (filleting, descaling, gutting) and species, because every species has a peculiar composition, size and shape. Fish filleting, fish descaling, removing of the skin or also fish washing are operations that create by-products that are usually incinerated (FAO).

In general, the relative weight of the different constituents of fish by-products are:
-muscle-trimmings 15-20%;
-skin and fins 1-3%;
-bones 9-15%;
-heads 9-12%;
-viscera 12-180;%

-scales 5%.

On the other hand, the proximate composition of fish by-products are as follows (Kandyliari et al., 2020):

-proteins ≃ 49-58%;

-ash ≃22-30%;

-fats ≃7-19% .

Another side stream of fish processing are wastewaters, which is originated from washing food during different processing operations. Wastewaters can be rich in scales, blood, or skin residues. This water is a valuable sources of proteins that can be extracted and used in food industry or in pharmaceutical products, for this reason it is gaining increasing interest discovering new proteins from wastewater of fish processing in order to use them as food additives, flavourings or for the formulation of new food products.

In 2018 12% of the total amount of world fisheries and aquaculture production (around 22 million tons) was used for non-food purposes. About 18 million of tons were transformed to fishmeal and fish oil, the remaining 4 million tons were used for other purposes like pharmaceutical uses, bait, or pet food (FAO, 2020).

It is worth to mention that a fish based diet has a positive impact on human health because they contain a great number of biomolecules and bio active compounds: Notably, fish and fish products are a valuable source of high quality proteins with essential amino-acids, essential long chain fatty acids with omega 3, vitamins and a lot of minerals. For this reasons, the consumption of fish is recommended to the whole population, but, in particular, to women during pregnancy in order to promote the full growth of the fetus, also to support brain faculties and cardiovascular system. In fact, biomolecules from fishes have been used in medicine and also like dietary

supplements.

1.2 Fish by-products and uses in food industry

Since the generation of by-products is unavoidable, today there are several ways to manage them. By-products usually were thrown away or used for fishmeal and in general for feed. Since the composition of by-products shows the richness in biomolecules, it is possible to use them for the extraction of valuable compounds like proteins, fat, peptides, enzymes, chitin, chitosan, collagen, pigments, minerals and others. These compounds, if extracted, are suitable for pharmaceutical or biotechnological applications and also for food industry.

Many compounds extracted from fish by-products are used in food industry for their properties as additives or functional food ingredients as well as to enrich nutritional value of food. For instance, fish bones are very rich in calcium whereas, some peptides have shown different bio-active properties on human health and contribute to the prevention of diseases. Numerous peptides have shown, anti-oxidant or anti-carcinogenic properties. Pepsin can be used as a rennet substitute in cheese production(Helkar & Sahoo, 2016).

Finally, proteins and fats often are simply used for enrichment in nutrients.

Fish oil is a precious source of omega 3 polyunsaturated fatty acids like eicosapentaenoic acid (20:5 n3, EPA) and docosahexaenoic acid (22:6 n3, DHA), as reported by Sellami et al. (Sellami et al., 2018).

As shown in **figure 2**, these fatty acids are polyunsaturated and the last double bound is located in the third carbon starting from the aliphatic chain. These authors studied the composition of fish oil from three rays species and they noticed the dominance of unsaturated fatty acids and, in particular, the percentage of presence of EPA was 3.36–5.51%, while the percentage of presence of DHA was

9.07–30.50%. In addition, these polyunsaturated fatty acids showed anti-cancer activity, neuroprotective and immune system improvements.

As others authors affirm (Zárate et al., 2017) it is well known that a diet rich in fish and omega-3 can help in prevention of diseases of cardio circulatory system, like coronary heart diseases because its activity against the accumulation of triglycerides in the arteries (Schuchardt et al., 2014) and like coronary atherosclerosis (von Schacky et al., 1999).

For the extraction of fish oil enzymatic, chemical, or supercritical fluid extraction methods can be used. These methods can be applied in combination with other technologies such as microwave or PEF to enhance extraction yields. The selection of the extraction method depends on several factors and economic reasons. However, a higher extraction of fish oil would allow to reduce the intensive fishing of fatty fishes satisfying the market demand of fish oil. It is important to bear in mind that omega-3 fatty acids can be extracted also from zooplankton, fungi or some plants.

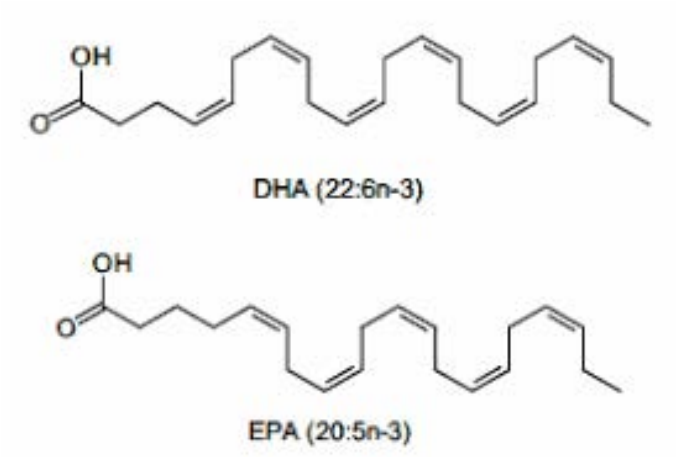

Fig.2: chemical structure of DHA and EPA.

Collagen is one of the principal structure protein, jointly with actin and myosin, and It is present in fish bones, skin, connective tissue, and cartilage (**fig.3**); it can

represent the 30 % of the total proteins. It gives mechanical properties to bones and skin. 28 types of collagen actually have been discovered. Denaturation of collagen can lead to the formation of gelatine which can be used in food industry, and as opposed to gelatine from bovine it has no risk of the transmission of spongiform encephalopathy (Caruso et al., 2020).

It can be used in cosmetic and pharmaceutical sectors for wound healing and cell and cartilage regeneration. In food industry it was used for edible film and coatings, also as a food additive in food and drinks for improving flavour and texture (Hashim et al., 2015)

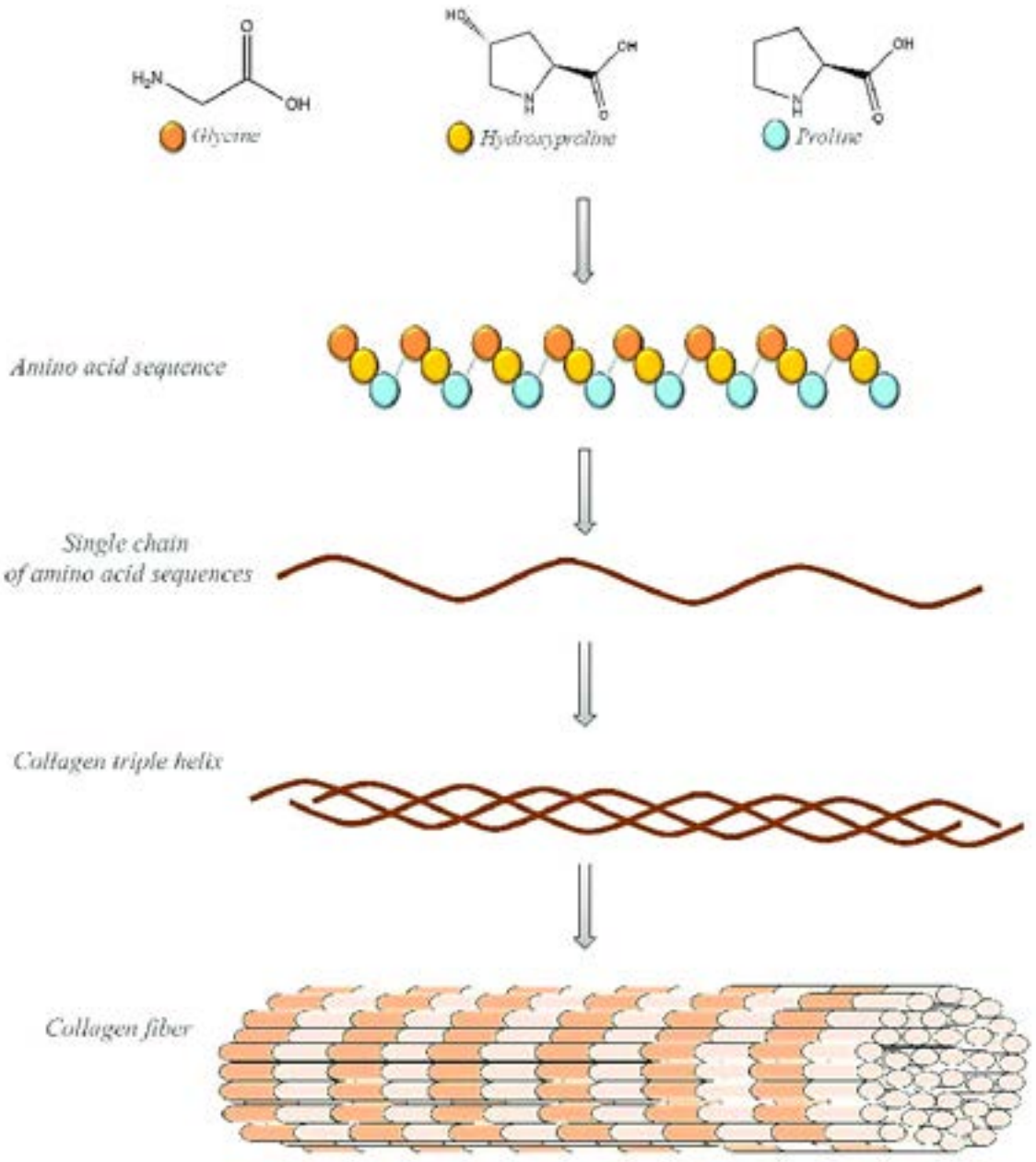

Fig.3: structure of collagen.

Chitin and Chitosan are both polysaccharides and used in pharmaceutical field for wound dressing(Jayakumar et al., 2011), due to their adhesive, antioxidant and antimicrobial properties. Chitin can be recovered from fish scales or crustaceans. It was also used for the treatment of underground water contaminated by iron(Irawan et al., 2018)

Chitosan showed high solubility in acid solvent and good properties in the creation of film with flexibility, resistance and gas barrier properties. Uranga et al.(Uranga et al., 2018) and Araújo et al.(Araújo et al., 2018) developed a bio-film using myofibrillar proteins incorporating also other compounds such as anthocyanin, as well as chitosan, extracted from fish waste, able to prevent lipid oxidation (Racioppo et al., 2021).

Enzymes Market volume was around USD 6.3 billion in 2017. The growing attention to food safety led to an increasing use of enzymes in food and beverage industry. Enzymes can be used in the formulation of detergents or in the production of paper. The internal organs, such as stomach, pancreas, and intestines, are the most important fish by-products concerning of number of enzymes discovered. Proteases constitute the biggest group of enzymes naturally found in fish.

Hydrolysis of peptide bonds is catalysed from these enzymes. Proteolytic enzymes from fish by-products belong to four different groups, depending on the substrate specificity, classified as aspartic proteases thiol or cysteine proteases (calpain, cathepsins B, H, L), (pepsin, cathepsin D), thiol or serine proteases (chymotrypsin, trypsin) and metalloproteases (collagenases) (Shahidi & Kamil, 2001). Enzymes from fish by-products do not require toxicological tests, because extracted from eatable animals (Venugopal, 2016)

Proteases approximately account the 60 % of the total enzymes in the market (Sawant & Nagendran, 2014). For instance, they can be used, for bakery purposes.

Fish Protein Hydrolysate

Hydrolysates are proteins that are chemically, both with acid or alkaline solutions, or enzymatically broken down into peptides of different sizes (D’avila et al., s.d.).

Hydrolysis through proteolytic enzymes can be carried out using endogenous enzymes from viscera and muscle of fishes or by adding enzymes from other sources as shown in **figure 4**.

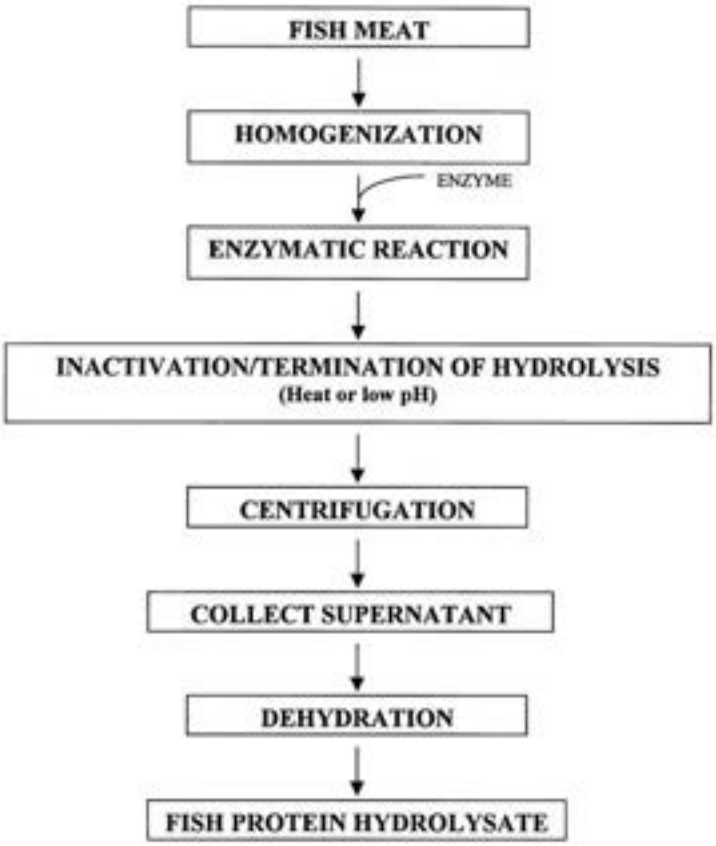

Fig.4: flow sheet of enzymatic hydrolysis of fish proteins to obtain fish protein hydrolysate. Image obtained from (Kristinsson & Rasco, 2000).

For instance, chemical hydrolysis is used in the USA for obtaining peptides from vegetable proteins that will be used as flavouring agents in processing of meat, crackers or soups.

Depending on the type of proteins, the chemical hydrolysis can led to reduce the nutritional qualities and functionality (Loffler, 1986; Webster et al., 1982).

According to Gildberg, an advantage of the hydrolysis of fish flesh, is the capability to modify and enhance the proteins, that can be used as food ingredients (Gildberg, 1993).

Mullalay et al. (MULLALLY et al., 1995) assert that enzymatic hydrolysis creates a mixture of free amino-acids and peptides of varying sizes. Furthermore, this process changes the functional properties and characteristics of the proteins improving the quality and their availability because it increases the number of polar groups and thus the solubility of the proteins.

Peptides from fish muscles have shown antimicrobial, anti-oxidative and immune modulatory properties (Caruso et al., 2020). Peptides can be extracted from whole fishes or from different body parts. However, to get the peptides various active extraction methods must be applied. Hydrolysis can be carried out also with fermentation performed by microorganisms. After the extraction, peptides can be purified and their amino acid sequence determined. They are used for treatment of human pathologies, for skin wrinkles and elasticity in cosmetic field (Coppola et al., 2021).

Real products example Many countries use fish protein hydrolysates as functional foods. Examples of products currently on the market are: **Vasotensin®**, a supplement based on peptides derived from fish bonito waste with effects on hypertension; **Seacure®**, a dietary supplement formulated from white fish filleting waste to promote gastrointestinal health; **Fortidium Liquamen®**, another dietary supplement constituted of protein hydrolysates from fish guts with anti-stress and antioxidant effects; **Peptydiss®** a supplement made of hydrolysed proteins from sardines with anti-stress and sleep-disrupting effects; **Stabilium®** 200, useful for helping intellectual faculties and memory (**Fig.5**); **Nutripeptin®**, a supplement derived from cod useful for people affected by diabetes (Racioppo et al., 2021).

Fig.5: Stabilium® 200.

S. Y. YU. and L. K. TAN. (YU & TAN, 1990) used hydrolysed fish proteins from *Oreochromis mossambicus* in fried crackers.

YU SWEE YEAN et al. (YEAN et al., 1994) used proteins from wastewater of fishball and they found out that using fish proteins for the crackers between 10 and 20% made the product acceptable from the panelists.

These crackers are a popular snack in maritime south-east Asia and associated to culinary traditions of Indonesia and in particular in Javanese cuisine. They usually are deep fried, grilled or hot sand fried. (Wikipedia)

Malaysian name is Keropok and fish proteins are used as food flavouring (**Fig.6**).

Fig.6: "Fish Keropok": popular snack of crackers flavoured with fish proteins.

Another fish-proteins based food is Surimi (**Fig.7**). It can be defined as a food formulation using fish proteins: the white-fleshed fishes are gutted and bones and skin are removed, then the flesh is minced, mixed with other ingredients and shaped as the traditional shape of sticks. Alaska pollock (*Theragra chalcogramma*) is one of the most valuable species used for Surimi because of its technological properties, such gelation, and because of its sensorial properties. However, in the report of FAO of 2020 (The state of world fisheries and aquaculture), Alaska pollock is the second fish in the rank of most caught fishes with 3.4 million of tons. This is clearly a red flag for food companies that produce Surimi: the attention should be focused on the exploitation of underutilized or less popular species or fish proteins recovered from fish waste or by-products in order to start to think and to act in a more sustainable way. Surimi is washed several time with water to remove impurities and unwanted compounds like fat, nitrogenous compounds, pigments and others. A.M. Martín-Sánchez et al. explain how the recovery of fish proteins from the surimi process wastewater can be re-used for another surimi production and so on in order to minimize

wastewater and creating a sort of circular economy inside the surimi processing formulation(Martín-Sánchez et al., 2009).

Fig.7: Surimi.

1.3 Aquaculture with special focus in Spain

Nowadays aquaculture production is almost equalizing fisheries production because of the increasing demand from the market and because of the need to find an alternative to the overexploited seas and oceans. Two of the most farmed species are Sea bream and Sea bass, that represent the 92% of the of the production regarding marine fish aquaculture in EU with a market value of almost 1 billion of € in 2019 (EUMOFA, based on elaboration of EUROSTAT data).

In Spain, in particular, the sole production of Sea bass (*Dicentrarchus labrax*) and Sea bream (*Sparus aurata*) amount to 88,8% of the total marine fishes aquaculture production (EUMOFA, based on elaboration of EUROSTAT data). It almost reached 38 thousands of tons in 2019 and these fishes are often intended to be processed in order to obtain fillets for the market. The edible part of Sea bass and Sea bream is between 50% and 60%.

Wastewaters from fish filleting are important because of their abundance and the presence of different compounds. Wastewaters result from the collection of the water used for rinsing and cleaning of fishes during different operations such de-scaling or filleting. Thus, this water may contain organic substances such as blood, scales, bones, and skin that can be recovered and exploited for several uses. Fish processing wastewaters are usually discharged and treated as waste for disposal. However, they can be regarded as a valuable source of protein and biomolecules. The recovery of proteins may contribute to reduce protein scarcity and improve food production sustainability. These proteins can be used in food industry as food ingredients with different techno-functional properties and including the formation and stabilization of emulsions and foams, water-holding and fat-binding capacities and ability to form gels.

1.4 Protein recovery

There are many methods for the extraction of fish proteins from fish by-products but some of them give products with a low functionality and nutritive value. Chemical hydrolysis, for instance, is used nowadays mainly for producing fertilizers, because it gives products with high solubility but also with bitterness, reduced nutritional qualities, and poor functionality which reduces their potential use as food ingredient (Chobert et al., 1996).

Enzymatic hydrolysis uses enzymes such as papain, ficin, trypsin, pancreatin, pronase, alcalase, and neutrase, but also endogenous enzymes can be used. If it is prolonged can cause the creation of short peptides and the loss of functionality. Ultrafiltration consists in the recovery of proteins and concentration of the recovered material and, in general preserving the quality proteins. The process depends on the membrane, temperature and pressure of extraction and liquid pre-treatment. Emerging methods like Subcritical water hydrolysis or Supercritical fluid extraction with CO_2 or Ohmic treatment seem to be very promising [28].

In addition Al Khawli et al. (Khawli et al., 2019) show that ultrasound assisted extraction can allow to extract up to ≃94% of total proteins of the raw material. Despite that, a simple, cheap and environmentally friendly method to recover proteins from fish processing wastewaters is the pH-shifting method or also referred as Isoelectric Solubilisation Precipitation (ISP). It typically involves the extraction of proteins using first a solubilisation of the proteins at extreme value of pH (<4 or >11), and then their recovery through precipitation at the isoelectric pH. The first step is aimed to solubilise all possible proteins using a low value pH (<4) or a high value pH (>11), then it is followed by a centrifugation to obtain the solution with proteins. After this, the pH of this solution is adjusted to 5,5 which is the usual isoelectric point (pI). At this pH, solubility power is zero and so they precipitate. Therefore, it is possible the recovery of precipitated proteins through a centrifugation. At the end the solid fraction can be dried, like in this case, to obtain powdered proteins. Different types of proteins can react in different way depending on the alkaline or acid treatment.

In the review of (Sanmartín et al., 2012), the authors explain that ISP process could also be applied to Surimi formulation replacing the conventional mechanical extraction of flesh, obtaining higher protein yields, probably for the recovery of sarcoplasmatic proteins, and also less fat presence in the product due to the centrifugation step. Furthermore, for ISP process, the raw material does not need to be extracted mechanically before the process. Chen and Jaczynski (Chen & Jaczynski, 2007) and Chen and others (Chen et al., 2007) demonstrated the high yield of the process (78 to 89%) and also the high quality of recovered materials for human food uses. Extraction yield of ISP method can vary depending on factors such as fish species or centrifugation force used.

Moreover, the combination of different techniques such as ISP and supercritical fluid extraction and ultrasound assisted extraction can increase the yield of the recovered proteins and avoid the presence of unwanted compounds.

1.5 Techno-functional properties of proteins

In order to employ proteins in a complete and efficient way it is essential to characterize them from chemical point of view to understand the effect of proteins composition and protein changes on their techno-functional properties. It is also important to compare the functional properties of recovered proteins from fish wastewater with typical commercial proteins with well-known techno-functional properties in order to highlight their properties as food ingredients.

Functional properties of proteins are the result of the intrinsic physico-chemical properties depending on the composition of the proteins, structure, bonds and interaction with other molecules (water, fats, other biomolecules), on the molecular weight, and number and polarity of amino-acids. For instance, the composition in amino-acids and their interactions with other molecules influence on the hydrophobicity, solubility, hydration or gelation properties of the proteins. Interactions with other molecules is enhanced by charged amino-acids, which can give compactness and stability to proteins. Source of the proteins, processing history or environmental factors can also affect the functionality of proteins.

Solubility

Protein solubility is an important feature for the valorisation of proteins as this determines many potential uses. The recovery of proteins from wastewater has been addressed in a limited number of studies. Therefore, the nature of these proteins is relatively unknown but we could think they come from blood and skin. Fish muscle proteins can be divided in sarcoplasmatic, myofibrillar and stroma proteins: depending on the solubility. For instance, beef plasma is used in several countries and is an outstanding binding gent and foaming agent.

Heating or freezing can affect the functionality of proteins from animal origin, in particular temperature and rate of freezing can be modified in order to obtain the best conditions of processing. Fish proteins seem to be more sensitive to freezing and about this, preservatives can be used in freezing conservation for long time of Surimi (Suzuki Taneko, 1981) and more, some amino-acids showed protective action to fish muscle proteins.

Solubility of fish muscle proteins can be "turned on" or "off", as described by (Gehring et al., 2011), in specific circumstances. Proteins side chains have a charge given by amino-acids which can be modified by adding acid or bases, responsible of protonation or deprotonation of the groups of amino-acids. Changing charges, proteins are able to create electrostatic interactions with water, thus increasing the water-solubility. So, solubility is mainly related to pH, which is capable to change the charges of the protein up to the isoelectric point, in which a protein loses all the charges and precipitate in the medium: it means that the solubility in this moment is zero (Zayas, 1997). The correlation between pH and solubility is show in the **figure 8**.

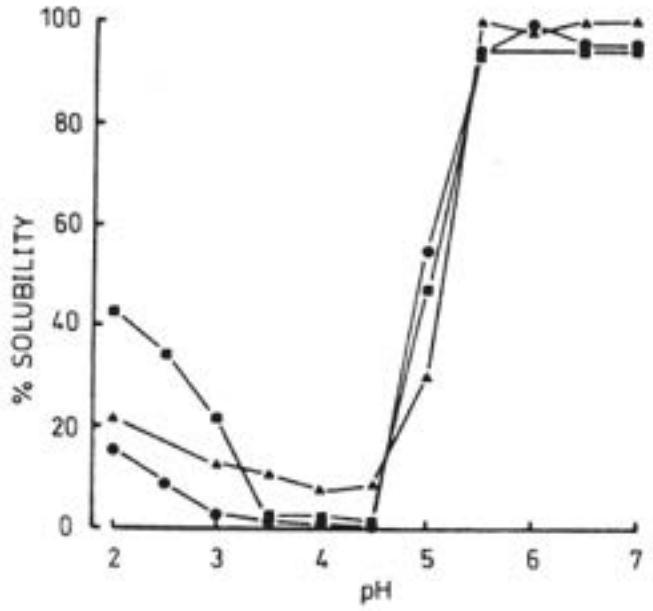

Figure 8. Effect of pH on solubility of sodium caseinate (●-●), α_s-/k-casein-enriched caseinate (▪ - ▪) and β-casein-enriched caseinate (▲-▲). Image obtained from (Murphy & Fox, 1991)

Emulsifying capacity

Emulsification properties are very important in food processing industry as they are necessary in great number of products such as mayonnaise, salad dressings, fish and meat products. An emulsion consists on a heterogeneous mixture of fat globules and solution. The droplet sizes are usually between 0.2 and 50 μm and there are two types of emulsions: oil in water (O/W) or water in oil (W/O) depending on the nature of the continuous and dispersed phase. However, a number of factors determine the physicochemical characteristics of the emulsions (stability, viscosity, colour, etc.). Proteins can participate in emulsification, their ability in emulsifying oil is studied as a functional characteristic which play an important role in developing new food products. Proteins, combined with carbohydrates and fats, are a very important stabilizers of food emulsions. Emulsifying activity is the ability of protein to participate and stabilize the created emulsion. Emulsion stability is the capacity of emulsion droplets to remain dispersed without separation by creaming, coalescing, and flocculation. Emulsifying capacity of proteins depends on a lot of factors such as the shape, hydrophobicity of the molecules, charge and neutrality of dipoles(Zayas, 1997).

Foaming capacity

Foaming capacity is the ability of proteins to create foam which is an important feature in the elaboration of many food products such as bakery, whipped creams, ice creams, mousses, etc. Foam can be defined as a two-phase structure made up of air cells separated by a continuous layer of liquid called lamellar phase. There is a uniform distribution of air bubbles which can give different attributes to food like body or lightness. Air bubbles of a food or drink facilitate the volatilization of flavours When a foam is generated by high speed blending or whipping treatment we can use

the term “whippability”.
Foaming formation process can be described in three phases:

1. In which the soluble proteins reduce surface tension.
2. In which proteins unfold and they orientate hydrophobic groups towards non-aqueous phase, and hydrophilic groups to aqueous phase.
3. In which a film is created by polypeptides around air bubbles, helpful to the formation and duration of foam.

The ability of proteins to unfold and change the orientation can be a useful property in case of small proteins, so that they can reduce tension very quickly (Abdollahi & Undeland, 2018).

In general, the fundamental function of proteins in foams is to lower interfacial tension, to strengthen viscous and elastic properties of the liquid phase and to form strong films. These continuous films are formed as an effect of polypeptides protein-protein interactions, and associations through electrostatic and hydrophobic interactions and hydrogen bonds.
The surface tension is affected by temperature, the higher is the temperature, the lower will be the surface tension (Purdon et al., 1980).

Foaming properties of proteins are influenced by source of protein, parameters of processing (mixing time, pH, temperature, method of foaming creation), equipment and method of agitation.

In food industry, protein foaming agents can be used ad ingredients such as casein or other milk proteins, gluten, soy proteins or egg white. As also inhibition agents can be employed (water-insoluble substances).
As said about the emulsifying capacity, also regarding foaming capacity, flexibility of proteins is important in order to quickly act towards the surface film. Factors that can affect foaming capacity are: proteins solubility, pH, hydrophobicity of

proteins molecules, concentration of proteins (**Fig.9**). Correlation between flexibility of proteins and foaming capacity of some proteins was studied by Townsend and Nakai(TOWNSEND & NAKAI, 1983).

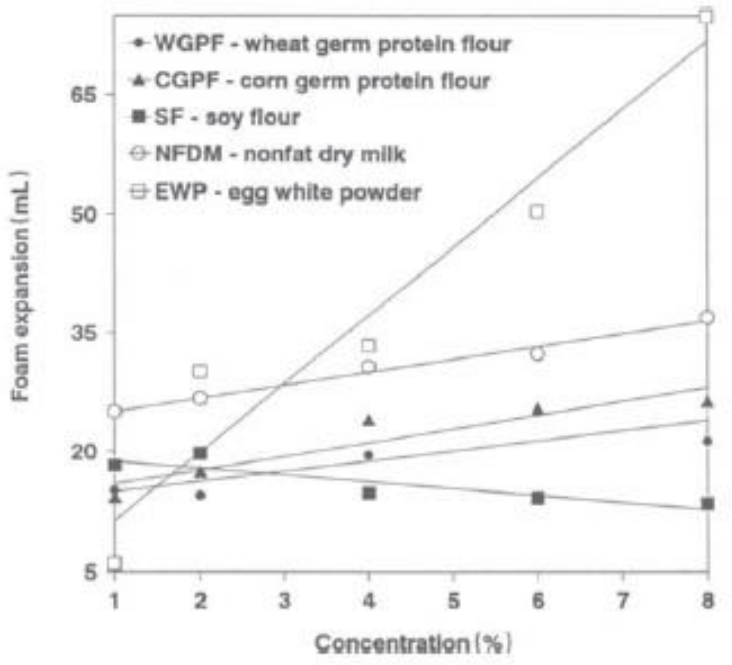

Figure 9. Effect of protein concentration on foam expansion. Image obtained from (Zayas, 1997)

Another important concept is Foaming stability. Since foam decreases with time, it is a parameter which needs to be studied. It is the result of all the properties of foam regarding the strength of the film (viscous, elastic, continuous and air-impermeable) and the concentration of proteins. It also depends on the Marangoni effect, which explains that proteins move from a region with low surface tension to a region with higher surface tension. Stability is also influenced by pH, temperature, proteins' interactions and film thickness (**Fig. 10**).

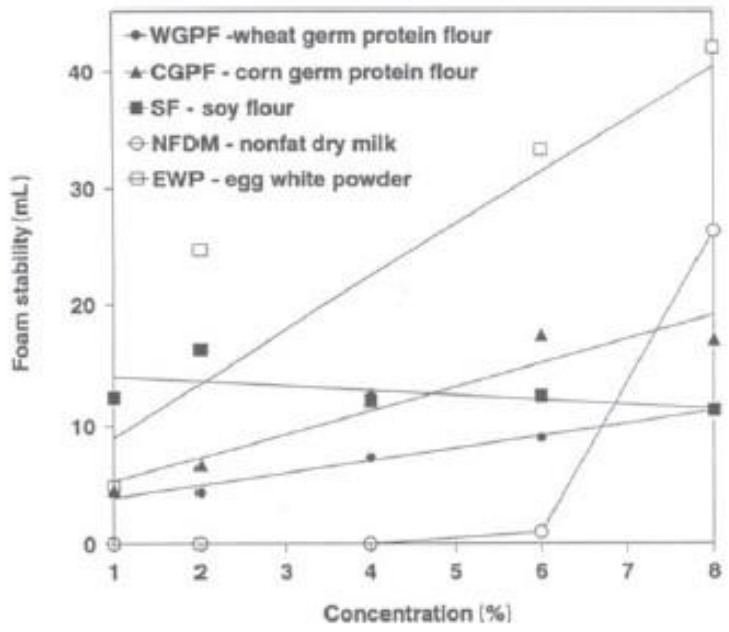

Figure 10. Effect of protein concentration on foam stability; I h incubation. Image obtained from (Zayas, 1997)

Thiol content

Thiol content is expressed by µmol of –SH groups in one gram of protein. Sulfhydryl functional group or thiol group (-SH) is the characteristic group of cysteine or methionine, responsible of the formation of the disulphide bridges, strong bonds between two amino-acids that link proteins so that proteins can fold and create tertiary and quaternary structures. Therefore, sulphide bonds have an important role in some functional properties of proteins described above. The different results of thiol groups reflect the different composition in amino-acids of the recovered proteins, also relating to dissimilar part of the fish from whom the proteins are recovered.

Hydrophobicity

Kato and Nakai (Kato & Nakai, 1980) demonstrated the relationship between protein structure and functional properties such as emulsifying capacity and foaming capacity, in particular the strong connection between emulsifying capacity and hydrophobicity of proteins. An increased hydrophobicity influences positively on the emulsifying activity.

Hydrophobicity is related to the presence and the characteristics of hydrophobic amino-acids showed from the molecule of protein, which is affected by protein unfolding in the course of denaturation. The distribution of the hydrophobic groups in the protein can vary emulsifying abilities. Hydrophobic amino-acids will show their groups to the hydrophobic molecules in the solution (oil) in order to cover them and facilitate their presence in the solution. This ability is called fat-binding capacity which is also an important techno-functional characteristic.

When present in an emulsion, proteins are able to lower the interfacial tension between water and droplets of oil they cover, forming a film and slowing down the coalescence of fat droplets. Insoluble and hydrophobic proteins have an elevated oil binding capacity.

Therefore, proteins can fold, unfold, absorb and create a layer at the interface between oil and water(Macritchie, 1978). To be able to fold and unfold, proteins need to be flexible, this will influence the interfacial film between water and oil. Emulsifying properties are influenced by pH, ionic strength, heating, protein concentration among other factors.

It has seen that proteins with massive molecular weight and residual tertiary structure create more stable emulsions than proteins with lower molecular weight(Phillips, 1981).

2. Aim of the work

Until now most of the by-products exploited for recovering bio active compounds and biomolecules, were solid material, only few studies focused on wastewater coming from fish processing. As stated previously, wastewaters can be used for the recovery of proteins which come from different parts of the fish and may contain different compounds such as proteins, peptides, amino-acids and fats.

The present experimental work was carried out during my Erasmus for traineeship in Girona, Spain, at the IRTA (Institute of Agrifood Reasearch and Technology), in the laboratory of the department of Food safety and functionality, that is experienced in industrial research in fishery processing sector.

The research belongs to the HORIZON-2020 project Newtechaqua (**Figure11**) that has, among one of its objectives, the valorisation of fish by-products and wastewater. In particular, my research focused on the analysis of fish processing wastewater obtained from processing line of Sea bream and Sea bass of two different batches. However, the main goal of the NewTechAqua project is to expand and diversify European aquaculture production of finfish, molluscs and microalgae by developing and validating technologically-advances, resilient and sustainable applications. Concerning sustainable ad circular aquaculture, wastewater and by-products can be used for the formulation of new diets.

Figure 11. NewTechAqua logo.

The aim of this study was to obtain and evaluate the physicochemical and structural properties of the proteins recovered from fish processing wastewaters using isoelectric solubilisation/precipitation process and examine the techno-functional properties of protein isolates to assess their potential uses in the food industry. Furthermore, the recovered proteins will be compared to other commercial techno-functional proteins namely soy proteins isolate, sodium caseinate and egg albumin. The recovery of proteins may contribute to reduce protein scarcity and improve food production sustainability.

These proteins can be used in food industry as food ingredients with different techno-functional properties and including the formation and stabilization of emulsions and foams, water-holding and fat-binding capacities and ability to form gels [33].

3. Materials and methods

Chemicals

Tris HCl was provided by Amersham Biosciences (Amersham, United Kingdom). DTNB, Glycine, EDTA, SDS, B- mercaptoethanol, ANS, TCA, BSA kit, CuSO4, Urea, $NaH_3PO_4 \cdot H_2O$, and Na_2HPO_4 were provided by Sigma-Aldrich (St. Louis, Missouri, USA).

Sample preparation

Three types of wastewater fractions have been studied. These wastewaters come from mechanical fish filleting of seabream and seabass after collecting the effluents at three different points classified as follows:

-scale wastewaters (AS), collected at the beginning of the mechanical filleting of seabream and in the presence of scales.

-packaging waters (AE), collected at the end of the mechanical filleting of seabream before being packaged.

The samples of wastewater were provided by a local aquaculture company from the region of Murcia (Spain) and collected during processing line and then frozen in plastic bins. The samples were thawed for 72 hours at 4°C. Water was filtered from scales and subjected to the ISP process as follows: the pH was adjusted to 11 with 1M NaOH, it was centrifuged for 20 minutes at 8500 rpm at 4°C. The liquid was recovered and the pH was adjusted to 5,5, with 1M HCl in order to precipitate proteins and then stirred for 20 minutes. It was centrifuged to recover solids and the pH of solid fraction was adjusted to 7. Lastly, the precipitate was freeze-dried.
In this work 4 samples were analysed:
-AS: water from filleting operations containing scales, dried as described above;
-AE: water from packaging washing operations, dried as described above;
-initial water, only filtered (from scales) and homogenized of AS and AE batch.

For each samples, each analysis was carried on almost 3 replicates or more.

Proteins content and solubility

For the determination of the proteins content the bicinchoninc (BCA) method was used. This method is based on the formation of a Cu2+ protein complex under alkaline condition, followed by a Cu2+ reduction to Cu+. The amount of copper reduced is proportional to the proteins concentration. The quantification is achieved by means of

the reaction of BCA with Cu+ giving a purplish colour to the solution which is measured by means of spectrophotometer (562 nm). The proteins content can be determined using a BSA as a standard in the calibration line.

The lyophilized proteins (6.5 mg of AS or 25 mg of AE) were suspended in 5 ml of water and thoroughly homogenized using a Tissueruptor (Qiagen Company). Suspensions were centrifuged at 10000 rpm, for 10 min at 4 °C and the protein content of the supernatant was analyzed. Dilutions of the supernatant were prepared and to 100 µl of each dilution were added 2 ml of working reagent (BCA:CuSO4, 50:1) and incubated 15 min. at 37 °C. After cooling down, 100 µl of sample were analyzed using a microplate reader (Thermo Fisher Scientific Varioskan; Waltham, Massachusetts, USA) at 562 nm. Protein content was calculated comparing to a BSA calibration line (0 – 1000 µg/ml).

For the determination of protein solubility, proteins content was also analysed before centrifuging the suspension to roughly calculate the total proteins (proteins after centrifuge/proteins before centrifuge*100).

Foaming capacity

These properties were determined according similarly as described elsewhere (Abdollahi & Undeland, 2018). Foam was generated by stirring 20 ml of sample (0.4 g/L protein for AS and 0.1 g/L protein content for AE) in a 50 ml capacity Falcon tube for 10 min, 17500 rpm using the UltraTurrax T25 model disperser. From foam height and falcon diameter was calculated the volume of generated foam in ml ($V\ foam = \pi \cdot r^2 \cdot h$). Foaming ability (F0) was calculated as the initial volume of foam per g of protein. Foam stability was determined from the foam height right after formation and compared to that after 30 and 60 min of storage.

Foaming capacity (%)=(V_1- V_initial)/V_initial x 100

Foaming stability (%)=(V_60- V_(initial))/V_(initial) x 100

Emulsifying capacity

These properties were determined according to the method of Pearce and Kinsella(Pearce & Kinsella, 1978) with minor modifications. Oil in water emulsions were prepared by mixing 2 ml of sunflower oil and 8 ml of sample (0.4 g/L protein for AS and 0.1 g/L protein content for AE). The mixtures were prepared in 50 ml tubes and blended for 1 min at 13,500 rpm with an UltraTurrax T25 model disperser. Then, a 20 µl aliquot of emulsion (taken from the bottom) was poured to 1.5 ml of 0.1 % sodium dodecylsulphate (SDS) and the absorbance at 500 nm (A500) was measured at 0 min in a Shimadzu model UV-1800 spectrophotometer (Shimadzu, Japan). Emulsifying Activity Index (EAI) expressed in m2/g protein was calculated according to the equation:

$$EAI = \frac{2 \times 2.303 \times A500 \times DF}{C \times \varphi \times \theta \times 1000}$$

DF is the dilution factor (1520 µl / 20 µl), φ is the optical path (1 cm) and θ is the volume fraction of oil in the emulsion. After 10 minutes, 20 µl were taken and measured as described before. Emulsion stability (ESI) was calculated as the percentage of maintained absorbance after 10 min.

Sulphide content

The methodology was similar as described elsewhere with minor modifications(Abdollahi & Undeland, 2018; Gong et al., 2016). 100 mg of lyophilized samples were suspended in 10 ml of Tris-Glycine buffer (0.086 M Tris, 0.09 M Glycine, 4 mM EDTA disodium salt, pH 8) and 9.61 g of Urea were added (8 M). The mixture was homogenized (TissueRuptor, Qiagen Company), at the lowest speed, and stirred for 30 minutes with orbital stirrer. Then, the solution was centrifuged at 10000 rpm for 10 min and 4 °C.

To determine active thiol groups content, 1 ml of supernatant were added 40 µl of 5,5′-dithiobis (2- nitrobenzoic acid) (DTNB, 4 mg/ml) and incubated for 15 min at 25 °C. Thereafter, the absorbance was measured at 412 nm against a blank consisting in the buffer in 8 mM urea plus DTNB. The active thiol content (µmols/g protein) can be calculated as follows:

$$\text{Active SH} = \frac{73.53\ x\ Abs412}{Conc.}$$

Where 73.53 comes from extinction coefficient of 5-thio-2-nitrobenzoic acid formed upon DTNB reduction, Abs412 is the absorbance at 412 nm and Conc. is the protein concentration in mg/ml.

To determine total sulphide content, 2 ml of supernatant were added 4 µl of ß-mercaptoethanol to reduce S-S bonds to thiol functional groups (S-H). The solution was gently shaken at 25 °C for 2 hours. Then, 5 ml of trichloroacetic acid (12 %) were added and stored for 1 h at 25 °C, to precipitate the proteins. Next, mixture was centrifuged 10 min at 10000 g and 4 °C. Supernatant was removed and the pellet was washed twice with 5 ml of TCA. After that, pellet was re-suspended in 3 ml of Tris-Gly buffer and 120 µl of DTNB (4 mg/ml) were added to 2 ml of filtered sample and subsequently kept for 15 min at 25 °C. Then the yellow solution was centrifuged at 10000 g for 10 min at 4 °C. Total disulphide content can be calculated using the same equation used for active thiol content. Disulphide content can be calculated from difference between total sulphide content and active thiol groups.

Surface Hydrophobicity (H_0)

Surface hydrophobicity of samples was measured similarly as described by other authors(Abdollahi & Undeland, 2018).In brief, 20 mg of AS or 30 mg of AE were suspended in 5 ml of buffer (0.1 M sodium phosphate, pH = 7) by using a high-speed homogenizer (TissueRuptor, Qiagen Company). Dispersions were then centrifuged at 7800 rpm, 15 min at 4 °C to remove insoluble matter. The protein content of supernatant was determined and various serial dilutions were prepared. Then, two ml of each dilution was mixed with 10 µl of 8 mM 1- anilino-8-naphthalenesulfonic acid ammonium salt (ANS). The mixtures were shaken for 15 min while being protected from light and thereafter 200 µl of each dilution were analyzed by fluorescence in the microplate reader using excitation and emission wavelengths of 390 nm and 470 nm, respectively after heating at 25 °C for 2 min. Each sample was also analyzed without the addition of the ANS probe and its fluorescence value subtracted from the result of sample with ANS. The slope from fluorescence intensity vs. protein concentration plot was used as H_0.

Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS – PAGE)

Separation and identification of the proteins were carried out by SDS-PAGE on a discontinuous buffered system according to Laemmli(Laemmli, 1970) using a separation gel (12% polyacrylamide) and a stacking gel (4% polyacrylamide). A volume of 10 µL (1 mg/mL of protein) of recovered proteins was mixed with 19 µL of Laemmli buffer (Bio-Rad, Hercules, CA, USA) and 1 µL of 2-mercaptoethanol (Merck, Darmstadt, Germany). A protein molecular weight standard (Bio-Rad, Hercules, CA, USA) composed by myosin (211 kDa), β-galactosidase (119 kDa), bovine serum albumin (79 kDa), ovoalbumin (53 kDa), carbonic anhydrase (37 kDa), tyrosine inhibitor (29 kDa), lysozyme (18 kDa) and aprotinin (6 kDa) was run in parallel for protein identification. Electrophoresis was performed in a Mini-Protean Tetra system (Bio-Rad, Hercules, CA, USA) and was conducted at 220 V until the front

reached the gel baseline. Gels were fixed for 15 min in a methanol/water/acetic acid (50:43:7) solution, stained with 0.05% (w/v) Coomassie brilliant blue R-250 (Panreac, Barcelona, Spain) dissolved in a methanol/acetic acid/water (45:10:45) solution for 2 h, and destained overnight in a methanol/ethanol/acetic acid/water (20:10:5:65) solution.

Colour

A colorimeter (Minolta CM 600d, Konica Minolta Sensing Americas, Inc., Chiyoda-ku, Tokyo, Japan) was used to measure the colour in the CIE-Lab space (lightness (L*), redness (a*) and yellowness (b*)) of powdered samples. The illuminant used was D65 with 10° observer angle.

4. Results and discussion

This study belongs to a research project and was directed toward a first exploration of the powerful of aquaculture wastewater as source of bioactive compounds useful in the food sector and was an original study carried on by the research group that is partner of the project. The obtained results represent preliminary data useful to explore general properties and address more studies aimed to deeply characterize the products. Among these, as stated in the introduction, protein fraction is among the most important nutritional constituents of the studied by-products and for this reason both technical, physical and biochemical parameters were evaluated.

Colour

The visual appearance is critical for the acceptability of food products. In this regard, protein isolates are preferred with neutral characteristics meaning that a white colour is desired. **Table 1** shows the instrumental colour analysis of the different samples. It can be observed that minor differences were found when comparing the results obtained in the dark or in the presence of light. This finding suggests that the characterization of the colour in the powdered ingredients is unaffected by light in our measurement conditions. The slightly higher L* values of AS indicate a more whitish than AE. Conversely AE is more reddish (a*) than AS which may be due to the presence of haem proteins in the sample. Yellowness (b*) is also higher in AE samples. This parameter is known to be influenced by fat oxidation and heme compounds(del Olmo et al., 2013). CIE-Lab values can be used to calculate whiteness.

Abdollahi and Undeland (Abdollahi & Undeland, 2018)also studied fish protein powders obtained from fish by-products. These authors found that L* values depend on the studied fish species (salmon, cod, herring). Herring showed the lowest value (57.59) which is close to AS. Abdollahi et al (Abdollahi et al., 2020), found that L* values of herring by-products obtained with alkaline solubilisation was 47.2 whereas in acid solubilisation was 51.6. The authors also reported that the addition of other compounds during the process (brown seaweed, shrimp shells and lingonberry) may enhance the colour indicating that the colour of the ingredient may be improved by means of different strategies. Whiteness is a serious quality feature for restructured fish products such as surimi-based seafood products (crab-flavoured seafood)(Gehring et al., 2011).

As it can be observed the AS samples are whiter than the AE samples which makes AS more interesting as protein for the development of fish products. However, in case these proteins are used at low levels the final impact in the product can be minimal.

Table 1. Instrumental colour analysis of the studied samples analysed in the dark and in the presence of light.

Sample	Analysis conditions	L*	a*	b*	Whiteness[a]	RGB[b]	Colour
AS lyophilised	Light	55.37	1.25	13.55	53.34	142 131 109	
	Dark	55.44	1.26	13.56	53.40	142 132 109	
AE lyophilised	Light	46.80	3.68	18.93	43.41	125 108 79	
	Dark	45.67	3.83	18.83	42.37	123 105 77	

[a]Whiteness = $100-\sqrt{(100-L)2+a2+b2}$; (Abdollahi & Undeland, 2018)

[b]Font: https://www.nixsensor.com/free-color-converter/ (0 to 250)

Proteins content and solubility

As shown in **table 2** the proteins content between AS and AE is considerably different. According to previously literature protein recovery yields for ISP range between 42 and 90% (Chen et al., 2009; Chen & Jaczynski, 2007; Kristinsson & Liang, 2006; Nolsøe & Undeland, 2009; Taskaya et al., 2009). Differences of yields can be attributed to fish species, centrifugation forces used during ISP and relative concentrations of water-soluble sarcoplasmic proteins among other factors. Soluble sarcoplasmic

proteins are more likely to be present in wastewaters which seem to be only partly recovered with ISP. The lower proteins concentration in wastewaters from packaging (AE) was expected since AS belongs to washing wastewaters of fish. Proteins solubility can affect characteristics of many proteins-based products, such rheological and surface activity properties, and it depend mainly on surface hydrophobicity. AS showed a good solubility value of 66 % ± 6 at pH =7 probably at extreme value of pH the solubility could decrease due to precipitation of high molecular-weight proteins (Vázquez-Sánchez et al., 2021).
In their study with minced tilapia wash-water, Vazquez-Sanchez et al. (2021) acquired very high value of protein solubility, ranging from to 65% to 94% depending on the pH and protein concentration. It is important to note that, the nature of the species is different (freshwater and saltwater). In addition, other factors such as the nature of the fish-by products, the fact that are not subjected to ISP recovery and the likely different body composition (blood, scale, skin) may help to explain the different solubility between studied. This fact is reinforced when comparing the two wastewaters obtained by means of the same procedure.
Despite that, AS presents a relatively good solubility which makes it suitable for certain food applications.

Table 2. Protein solubility, initial protein content and protein recovery from the different samples.

Sample	Protein content (mg/g)	Protein content after centrifugation (mg/g)	Protein solubility (%)	Initial protein content (%)	Protein Recovery (%)
AS	752 ± 34	495 ± 46	66 ± 6	0.087 ± 0.012	43
AE	374 ± 73	63± 3	17 ± 3	0.010 ± 0.002	37

Protein recovery = (weight of lyophilised solid · protein content) / (initial sample weight · initial protein content) · 100).

Foaming capacity

AS sample showed the highest foaming capacity expressed per g of protein when compared to the three commercial references (**Table 3**). However, the stability of the foam seems to have intermediate values. At the same time, AE sample didn't create any measurable foam like Soy isolate that showed minimal foaming capacity. This poor foam capacity from AE sample may be attributed to the low proteins concentration. Foaming capacity can also be affected by the degree of integrity of proteins after the ISP process.
Foaming stability after one hour decreased of around three fourth. Proteins isolated from skipjack tuna roe showed a foaming stability of 68,9% after 60 minutes. The source of protein is different and also the concentration is enormously different: 71,4%(Cha et al., 2020). This shows how concentration of proteins can affect foaming stability.

Sodium caseinate (SC) is a widely used food additive employed in food industry in order to thicken, stabilize and texturize food products(Sarode et al., 2016). AS sample showed higher foam stability after 30 and 60 minutes' checks (**Fig.12**).

Values of foaming capacity (ml of foam/g of protein) demonstrated much higher than the ones showed by Pires et al. (Pires et al., 2012) despite the original by-product is different.

It is important to underline that the comparison among studies should be taken with caution as there are many methods not fully comparable. For instance, the foam formation inside the sample by air flow, can be affected by the management of the equipment (in this case Ultraturrax homogeneiser (IKA)). During the different analysis the volume, dimensions of the container and the depth of the rotating arm should be defined and maintained the same way because this can influence the entrance of the air inside the centrifuge tube used (in this case 50 ml).

Table 3. Foaming properties evaluated on the analysed samples.

Sample	Foam volume (ml)	Foaming capacity (ml foam/ml sample)	Foaming capacity (ml foam/g protein)	Foam stability 30 min (%)	Foam stability 60 min(%)
Sodium caseinate (0.1 %)	19.7 ± 0.6	0.99 ± 0.03	985 ± 31	23.9 ± 1.2	18.8 ± 0.6
Soy Isolate (0.1%)	< 0.6	--	--	--	--
Egg Albumin (0.1 %)	11.0 ± 2.2	0.55 ± 0.11	550 ± 109	85 ± 5	77 ± 9
AS (0.04 %)	13.1 ± 1.1	0.65 ± 0.05	1636 ± 131	44 ± 6	27 ± 3
AE (0.01 %)	--	--	--	--	--

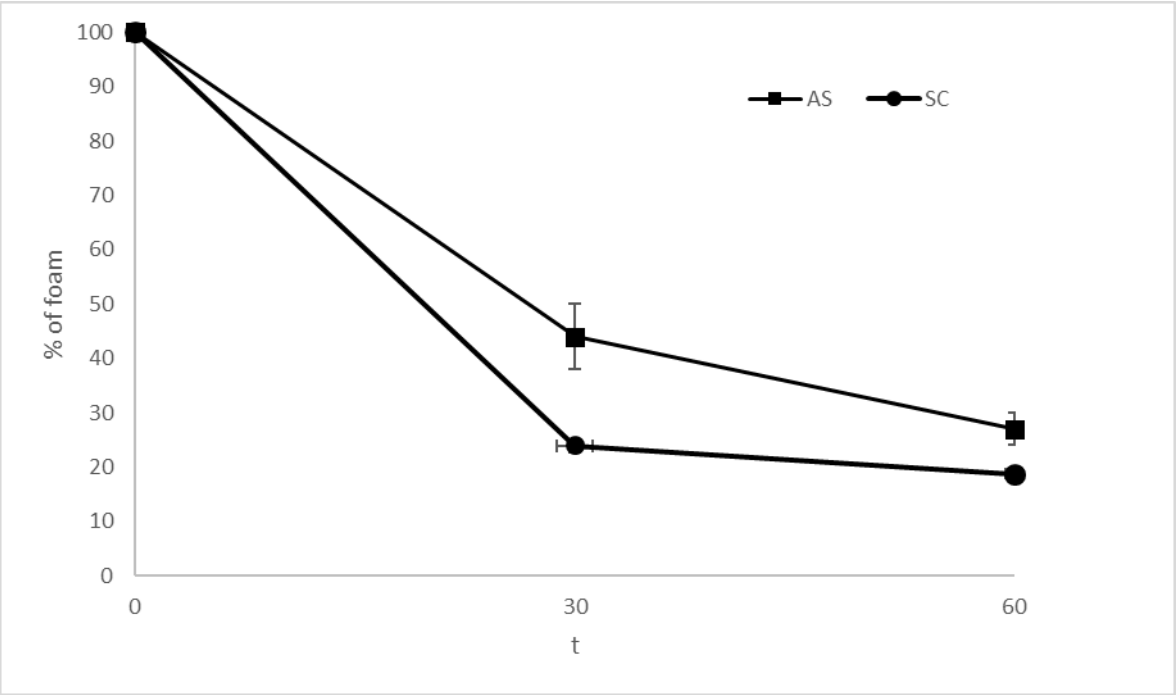

Figure 12. Percentage of foam of AS after 30 and 60 minutes compared to SC (sodium caseinate).

Emulsifying capacity

Table 4 shows the emulsifying properties of the wastewaters samples and commercial samples for comparison. EAI of AS showed intermediate value, while the stability expressed in % and minutes was low related to the three references used, and also as demonstrated by Vazquez- Sanchez et al. (Vázquez-Sánchez et al., 2021), (to take into account that Vazquez-Sanchez et al. (2021) used fish proteins powder obtained from different fish species).

It is worth noting that the emulsifying properties are similar to soy isolate. Despite its concentration was much lower than AS, AE showed a remarkable EAI and ESI which makes this protein source of particular interest with regards to this technological property. In agreement with these results, Huidrobo et al. (Huidobro et al., 1998)

reported similar ESI results in washwater from minced fish resulting from Surimi manufacture.

Table 4. Emulsifying properties of the studied samples.

Sample	Emulsifying Activity Index (m^2/g)	Emulsifying Stability Index (%)	Emulsifying Stability Index (min)
Sodium caseinate (0.1 %)	112 ± 13	44 ± 3	8 ± 1
Soy Isolate (0.1%)	86 ± 6	91 ± 2	103 ± 27
Egg Albumin (0.1 %)	70 ± 5	58 ± 7	14 ±4
AS (0.04 %)	86 ± 2	32 ± 8	5.9 ± 0.1
AE (0.01 %)	689 ± 51	78 ± 4	37 ± 9

$$ESI\ (\%) = 100 - (Absi - Absf)/Absi \cdot 100$$

$$ESI\ (min) = (Absi \cdot t) / (Absi - Absf)$$

Thiol content

Sulphide bonds have an important role in some functional properties of proteins. The different results of thiol groups reflect the different composition in amino-acids of the recovered proteins, also relating to dissimilar part of the fish from whom the proteins are recovered. β-mercaptoethanol is responsible for the breaking of disulphide bridges between amino-acids and in this sense is used to obtain reactive –SH groups. In this way, it is possible to determine the total thiol content and compare with the determination without the addition of the reductant which reflects the content of active thiols in the sample.

Table 5. Total and active thiols content of the studied samples.

Sample	Active thiols (µmol/g protein)	Total thiols (µmol/g protein)	Ratio Active / Total (%)
Sodium caseinate	0.9 ± 0.1	38 ± 3	2.4
Soy Isolate	4.4 ± 0.0	53 ± 3	8.3
Egg Albumin	32.4 ± 0.8	170 ± 12	11.8
AS	20 ± 1	150 ± 4	13.3
AE	96 ± 1	610 ± 77	15.7
AS initial waters	27 ± 1	112 ± 3	24.1
AE initial waters	46 ± 2	210 ± 45*	21.9

* Data from four replicates from different days, high variability due to difficulty to separate the liquid from solid

Relating to the three commercial references used , the two samples AS and AE seem to have a high number of thiol groups, in particular AE (Table 5). pH-shift process can change the conformation of proteins because severe pH values during the process can cause unfolding of proteins(Abdollahi & Undeland, 2018), this could be the reason why AE shows a higher number of active and total thiols than the initial waters. Unfolding of proteins structure can led to expose more sulfhydryl than the normal ones it would show at normal conditions. A higher content in thiols may be related to a more flexible state of the proteins. This may allow the formation of intermolecular disulfide bonds and stabilize emulsions(McCLEMENTS et al., 1993; Young Lee & Hirose, 1992). A higher flexibility and/or the formation of more stable emulsions due to disulfide bonds may explain the observed higher emulsion capacity of AE (**Table 4**)

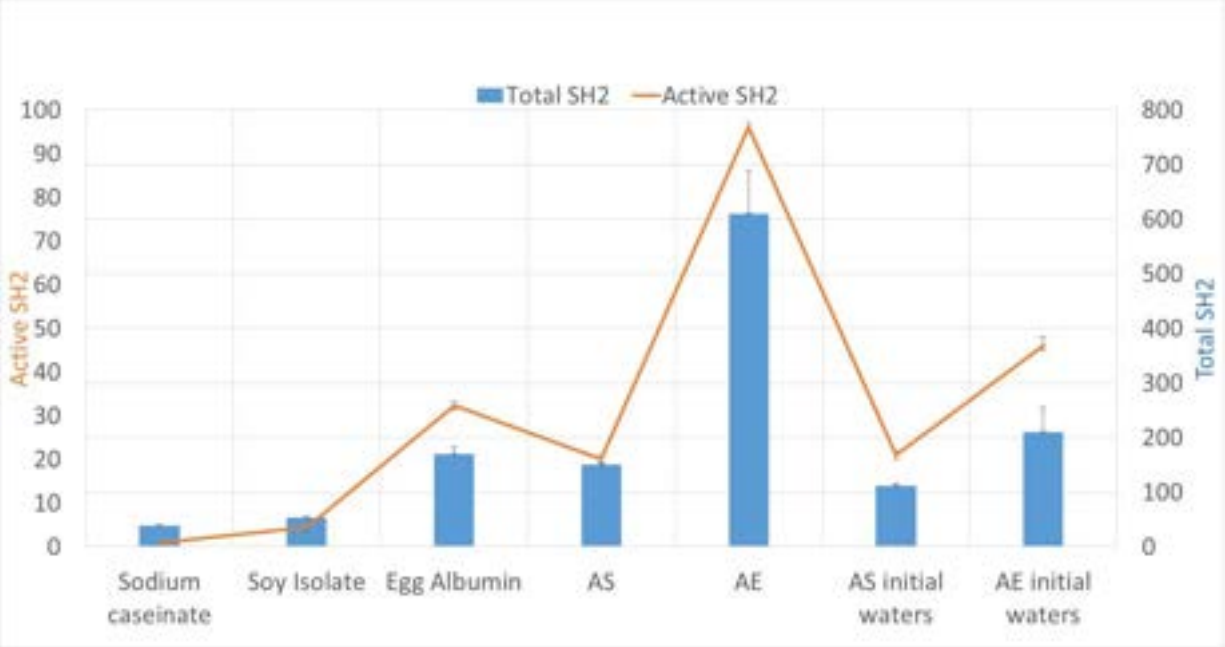

Figure 13. Comparison among active and total thiols content in the analysed samples.

Surface Hydrophobicity

Table 6. Hydrophobicity value of the samples.

Sample	Ho
Sodium caseinate	349 ± 26
Soy Isolate	227 ± 22
Egg Albumin	107 ± 6
AS	161 ± 7
AE	127 ± 9
AS initial waters	225 ± 7
AE initial waters	350 ± 29

Hydrophobicity different values are a consequence of the different arrangement of the proteins' structure after pH-shift process. Operating in acid or basic conditions may affect the conformation of the protein and contribute to a different exposition of hydrophobic groups. In general, the higher exposure of hydrophobic groups is the lower solubility exhibits.

To obtain the results of H_o a plot was created, putting on the x axis the protein concentrations and on the y axis the value of absorbance read from the microplate reader (Varioskan, model, Thermofisher). The initial slope of the plot was considered to be an index of proteins hydrophobicity.

Hydrophobicity of AS and AE recorded low values, lower than sodium caseinate and soy isolate but higher than egg albumin (**Table 6**) (**Fig. 14**), while the initial waters showed higher values of H_o demonstrating that during process to recover proteins they undergo into conformational changes: in particular in this case the hydrophobicity decreased, the contrary to what affirmed by (Gehring et al., 2011) when studying fish muscle proteins. Since the samples studied don't belong to fish muscles protein it could be that the composition of proteins recovered from wash-water, in this case, is not the same of fish muscle proteins or other by-products of the references, and thus explain this different behaviour. An increased hydrophobicity may lead to lower solubility but also to an increased emulsifying capacity or oil absorbing capacity. From the observed data it seems that the observed emulsifying properties of AE may be not directly attributed to Ho but to other factors such thiol groups and the likely stabilization of emulsions due to protein conformation and the formation of disulfide bonds.

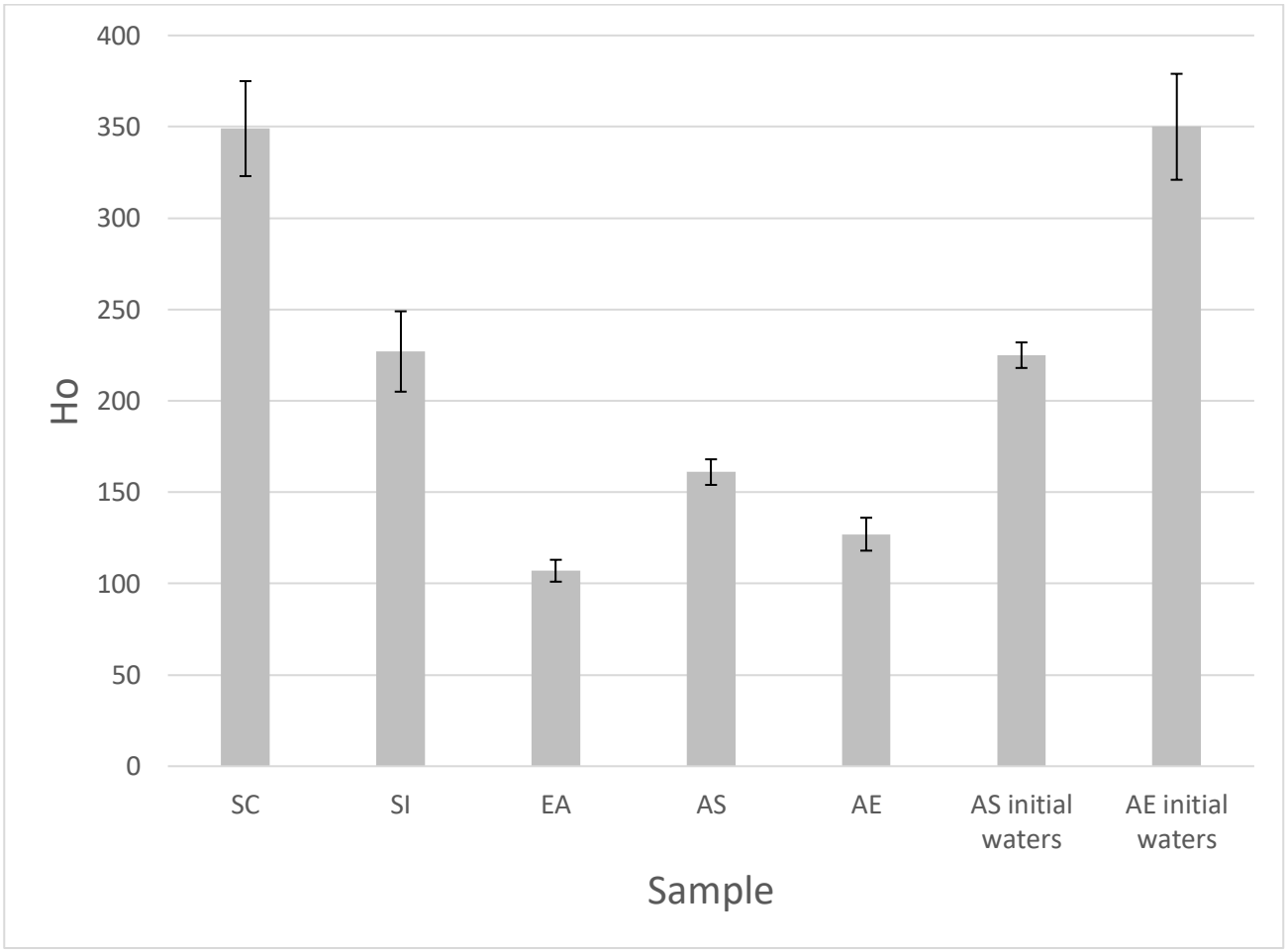

Figure 14. Comparison of Ho values of the samples: sodium caseinate (SC), soy isolate (SI), egg albumin (EA), AS, AE, AS initial water and AE initial water.

Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS – PAGE)

The qualitative analysis of total extracted proteins by SDS-PAGE, showed not so much different composition between AS and AE. Electrophoresis is a rough and qualitative characterization of the proteins.

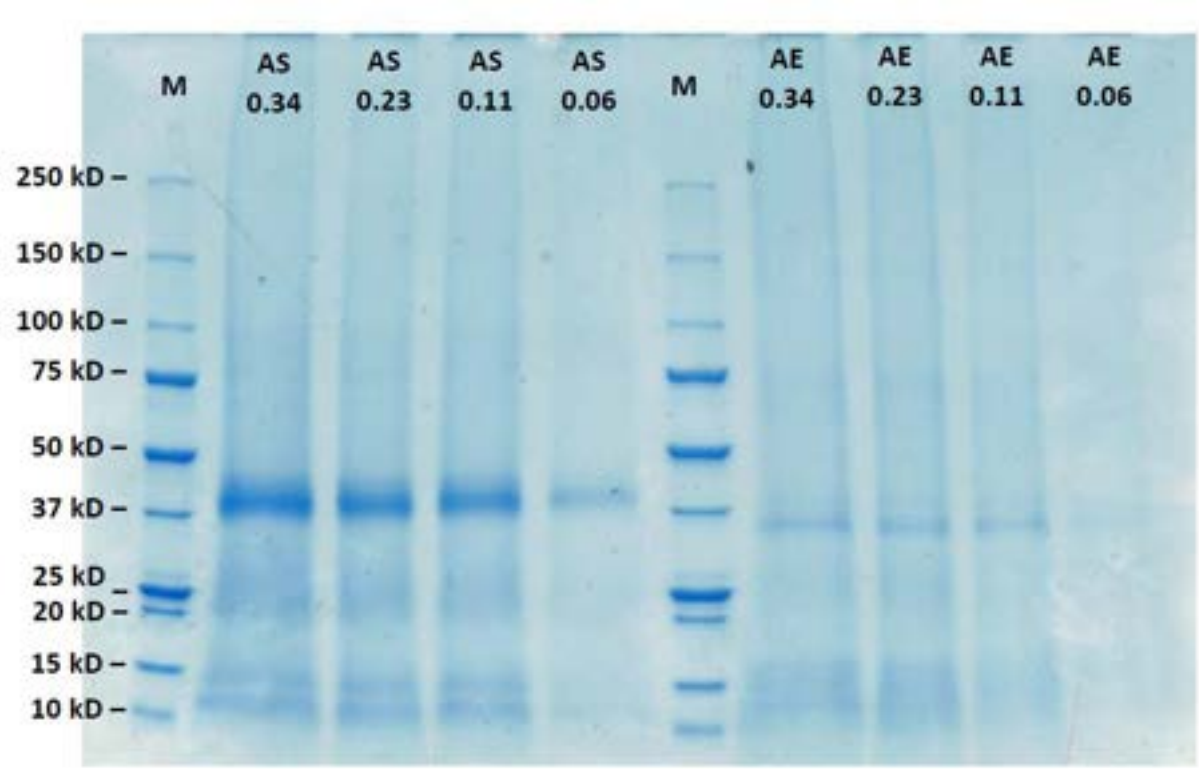

Figure 15. SDS-PAGE patterns of AS and AE at different concentrations.

AS showed in the figures, the more intense bands are in correspondence of proteins ranging from 37 kDa to 50 kDa, while lighter bands between 10 and 15 kDa and an unclear band around 25 kDa.

For AE, the analysis showed some bands around 15 kDa and some thinner ones around 35 kDa. (**Fig.15**)

Proteins with lower molecular weight can be more soluble and affect more techno-functional properties.

There is no red colour which could be attributed to haemoglobin (dimers and monomers between 32-15 kDa) and neither visible bands around 220 kDa that could be representative of fish myosin: this protein is a 520 kDa examer composed of two polypeptides of 220 kDa bonded to lighter chains of 15-20 kDa(Kristinsson & Hultin, 2003) , in both cases there are no traces, so we could affirm that fish myosin is not for sure present in the samples.
The same bands around 15 kDa and between 37 and 50 kDa of AS were also founded in minced Tilapia (Oreochromis niloticus) (Vázquez-Sánchez et al., 2021), and in hake by-products (Pires et al., 2012) with bands around 37-50 kDa and less than 20 kDa.

The band around 42 kDa can be representative of fish actin as stated by Pires et al (2012) that found bands between 70 and 220 kDa, not present in AS and AE samples: this can be due to proteins solubility or conformational changes during proteins recovering process. The limited existing literature on fish waste waters does not allow a deeper discussion of the protein bands.

5. Conclusions

It is important to highlight the crucial role of aquaculture in providing fish and fish products in our diets. However, this activity also results in the production of remarkable amounts of fish by-products leading to important environmental issues. Therefore, it is of great interest the concept and actualization of a circular economy in order to move production to a sustainable plan creating new profits for the companies by using fish by-products and reducing the environmental impact of the side-streams.
This study was aimed to valorise fish proteins from washing wastetwaters by obtaining techno functional ingredients that can be used in the development of different foods. The results obtained from the analysis of the study suggest that their re-utilization is possible showing interesting properties such as the interesting emulsifying activity of

AE. A number of other possibilities can be discovered considering the huge variability of characteristics of fish proteins. It is noticeable that the extraction method and many parameters can affect the variability and the final structure of proteins. Wastewater proteins have not been studied in so many investigations and the results obtained, comparing the proteins to commercial references already used in food industry, showed the huge potential of this by-products. More studies are necessary if considering the numbers of fish species handled during processing and the growth phase of aquaculture in the food system.

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