



# Fish Processing Industry Residues: A Review of Valuable Products Extraction and Characterization Methods

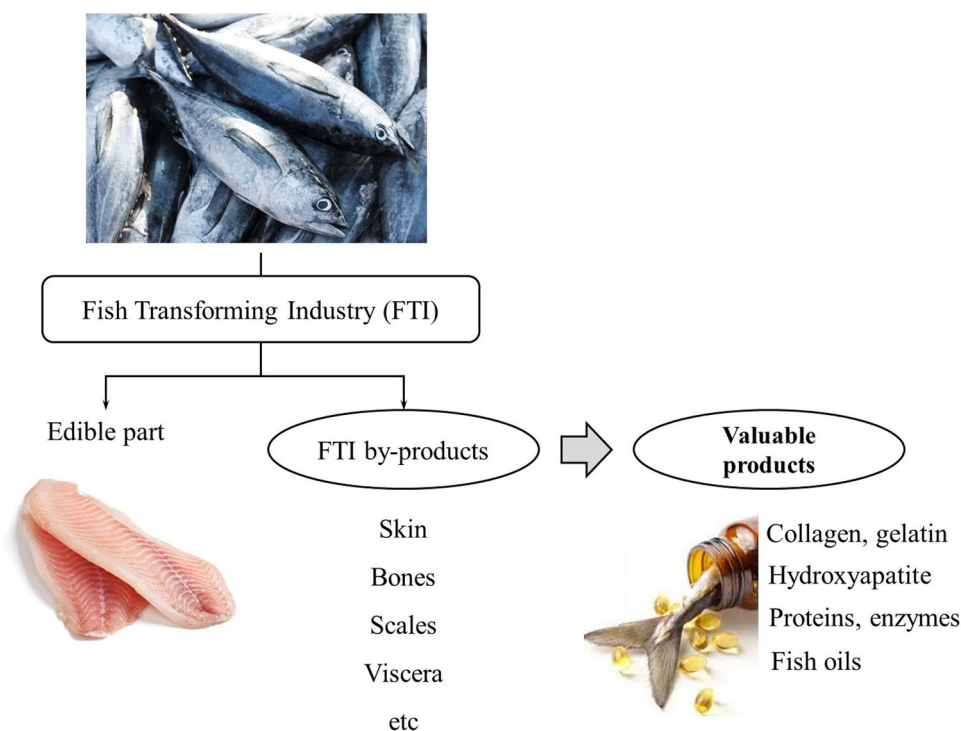
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## Abstract

Fish processing industry has experienced significant growth, playing an important role in the world economy. The increased exploration of marine resources contributes to the generation of considerable amounts of biowaste, which ends up as discards. In the face of the resultant disposal and environmental problems, many efforts have been made to deal with the fishery waste in more efficient ways. Nowadays, these by-products are regarded as important sources of high added value compounds, such as hydroxyapatite, collagen, gelatin, lipids, enzymes, hydrolysates and bioactive peptides, with great potential for human health applications. The present paper aims to review the current methods of extraction and characterization of added value products from fish by-products, as well as their actual and potential applications.

## Graphic Abstract



**Keywords** Fish processing industry · Marine resources · Waste · By-products · High added value compounds

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## Statement of Novelty

The increased exploration of marine resources contributes to the generation of considerable amounts of biowaste. Marine processing by-products have brought more value out of what is until recently regarded as biowaste. These residues, commonly discarded, have been considered for their potential to generate high added value compounds, such as hydroxyapatite, collagen, enzymes, proteins and fish oils, etc. Previous studies have shown the efforts made so far to maximize these marine sources by exploring different variables (e.g. fish species, extraction procedures, etc.). Our review intends to provide an overview of the research being carried out for the extraction of high added value compounds from fish by-products. In this review, we covered the most important methods of extraction and characterization available for these compounds. The updated information and critical analysis on this issue will largely benefit the research dedicated to this topic.

## Introduction

According to the Food and Agriculture Organization (FAO), fish production is continuously growing at an average rate of 3.2% each year, reaching 158 million tons of fish captured in 2012 [1].

Fish processing industry (FPI) is responsible for the production of significant amounts of seafood and marine products. Only 40% of the catch is used for human consumption while more than 60% is discarded. As a result, considerable amounts of waste are generated [2, 3], causing severe environmental problems that have been addressed to the fact that these discards have no direct application. The dual problem arising from fish processing industries which comprise environmental issues and the less profitable applications for by-products and waste have emphasised the need to obtain valorisation from fish processing waste and, at the same time, contribute to a more sustainable fish industry.

Aware of these serious problems, fish industries have made some efforts to deal efficiently with fish waste, including the application as animal feed, plant fertilizers, fish oil and fish meals [4]. However, in the context of biowaste valorization, these products are not economically attractive to fish industries [3, 5].

The paradigm is changing and the processing of by-products can transform a product with low value into a product that is able to cover all the costs of processing and disposal, with consequent higher added value and reduced environmental damage [6].

These fish by-products comprise carcass, head, skin, scales, bones and viscera. In this sense, researchers have

reported the finding of high value products that could be extracted from fish by-products, including proteins (proteolytic enzymes, protein hydrolysates, bioactive peptides, collagen and gelatin), fish oils and hydroxyapatite [7–11]. In fact, the extraction of these compounds seems to be economically promising for their recognised positive impact on human health. Regarded as high added value compounds (HAVC) these products show great potential for a variety of applications, from human foodstuffs and food ingredients to pharmaceuticals, medical and cosmetic products [3, 12–14]. Therefore, the search for new methodologies for extraction and purification of high-value products from fish waste may represent an excellent opportunity for the fish processing industries to bring more value to what is so far regarded as waste.

This review intends to analyse and discuss the methods available for the extraction of selected high added-value compounds from fish processing waste as well as their characterization. Also, it explores the potential applications of extracted components. Table 1 summarizes the information covered throughout the review, namely in terms of extraction and characterization of the different valuable products.

## Proteolytic Enzymes

The fish internal organs (or viscera) constitute about 5% of fish weight and are one of the most important by-products from the FPI. Along the fish gastrointestinal system, different types of enzymes are secreted, making viscera a rich natural and cheap source of enzymes [12]. Different factors, such as life cycle, age, distribution, feeding and season, can lead to natural variations in the amount of proteolytic enzymes produced by many fish species [15]. An efficient and economic process for isolation of the enzymes from viscera would be preferable for the fish industry [16]. Enzyme-based processes have an important role in the fish industry, including fish and sea food processing (deskinning, descaling, peeling), production and/or recovery of by-products (fish protein hydrolysates, fish curing and fermentation, collagen and pigments extraction, etc.) and enhancement of shelf-life and product quality [17]. Applications in the other areas of the food and textile industries, clinical and diagnosis are also documented [4].

Among naturally available enzymes from marine sources, proteases (including pepsin, trypsin and chymotrypsin, collagenase, etc.) constitute the most important group with commercial expression [4]. This group refer to all enzymes which catalyse the hydrolysis of proteins into polypeptides [18]. Pepsin and trypsin are presented in this review as two representative enzymes of acidic and alkaline proteases, respectively.

**Table 1** Summary of the methods for extraction and characterization of different valuable products from the fish processing industry (FPI) by-products

Source	Extraction and purification	Characterization	References
<b>Pepsin</b>			
Viscera (stomach)	<i>Extraction:</i> Preparation of crude extract with buffer solution <i>Purification:</i> Ammonia sulfate fractionation (ASF) Ion-exchange chromatography (IEC) Gel filtration chromatography (GFC) Aqueous two phase system (ATPS)	Protein concentration Determination of enzyme activity Purity, homogeneity and molecular mass Structure determination (SDS-PAGE) Isoelectric focusing Enzyme kinetic studies Effect of inhibitors and activators	[4, 16, 19–28, 30–41]
<b>Trypsin</b>			
Viscera ( <i>pyloric caeca</i> , intestine and spleen)	<i>Extraction:</i> Extraction with buffers (i.e. Tris–HCl) <i>Purification:</i> Ammonia sulfate fractionation (ASF) Dialysis Gel filtration chromatography (GFC)		
<b>Proteins and protein hydrolysates</b>			
Fish by-products, in general	Enzymatic hydrolysis Autolysis Thermal hydrolysis Bacteria fermentation	Protein concentration Fourier transform infrared spectroscopy (FTIR) X-Ray diffraction (XRD) Molecular weight (SDS-PAGE)	[2, 45–51, 53, 54, 58–64]
<b>Bioactive peptides</b>			
Fish by-products, in general	Isolation from fish protein fractions: Nanofiltration Ion-exchange membranes Reverse-phase HPLC	Evaluation of functional properties (solubility, water and oil absorption) and surface active properties (emulsifying and foaming properties) Bioactive properties (i.e. antioxidant and antibacterial activity)	
<b>Collagen</b>			
Mainly skin and scales	<i>Pretreatment:</i> Alkaline hydrolysis, demineralization and degreasing <i>Extraction:</i> Acid treatment (ASC, acid-soluble collagen) Extraction with pepsin (PSC, pepsin-soluble collagen) Isolation: Salting-out and dialysis	Fourier transform infrared spectroscopy (FTIR) X-Ray diffraction (XRD) Circular dichroism (CD) Denaturation temperature (DSC, differential scanning calorimetry) Viscosity measurement Molecular weight (SDS-PAGE) Morphology (SEM) UV–Visible spectrometry Effect of pH and NaCl on solubility Determination of isoelectric point	[6, 73, 74, 79, 86–89, 91–96, 100, 106–108]
<b>Gelatin</b>			
Mainly skin and scales	<i>Preparation by partial denaturation of collagen:</i> Acid conditions (type A gelatin) Alkaline conditions (type B gelatin) <i>Extraction from fish residues:</i> Non-collagenous proteins and pigment removal, pretreatment (chemical or enzymatic) and thermolysis		

**Table 1** (continued)

Source	Extraction and purification	Characterization	References
Hydroxyapatite			
Scales and bones	Thermal calcination Alkaline hydrolysis Enzymatic hydrolysis Ionic liquid pretreatment	Thermal gravimetric analysis (TGA) Fourier transform infrared spectroscopy (FTIR) X-Ray diffraction (XRD) Chemical analysis Morphology and particle size (SEM and TEM) <i>In vitro</i> cytotoxicity and proliferation studies	[10, 110, 111, 115–125]
Fish oils			
Muscles and perivisceral organs (liver and intestine)	Organic solvents Soxhlet extraction Supercritical fluid extraction	Thin layer chromatography (TLC) Quantitative and qualitative characterization (GC-FID and GC-MS) Fourier transform infrared spectroscopy (FTIR) Nuclear magnetic resonance (NMR)	[4, 126, 127, 129–142, 146]

Enzymes, such as pepsin and trypsin are extracted from viscera, hydroxyapatite from bones and scales and collagen and gelatin mainly from skin and scales. Fish oils are isolated from muscles and perivisceral organs, such as liver and intestine, and proteins can be obtained from fish by-products in general

## Extraction and Purification

The first step of the extraction of enzymes from fish viscera involves the preparation of a crude extract with an appropriate buffer. Temperature, composition and pH of extraction buffers should be controlled in order to allow stabilization of the enzymes during the process. The most commonly used buffers are the sodium phosphate buffer and Tris–HCl buffer, containing  $\text{CaCl}_2$  and/or NaCl. The recovery of enzymes depends on several factors, such as the amount and quality of raw materials and methodology used for extraction [4].

Purification of extracted enzyme can be achieved by different procedures, such as ammonia sulfate fractionation (ASF), ion-exchange chromatography (IEC) and gel filtration chromatography (GFC) [16]. Figure 1 summarizes the procedure for extraction and purification of proteolytic enzymes from fish viscera. Ammonium sulfate precipitation is widely used because is a simple, fast and low cost procedure when compared with other protocols [19]. The raw extract is then dialyzed, centrifuged and the precipitate is discarded. Some authors apply a heat treatment step (incubation at 45 °C) before or after ASF [19–22]. Although the heat treatment does not improve the purification performance, its inclusion has proven to improve the performance in the subsequent stages since it promotes the hydrolysis of the thermostable contaminating proteins [20, 21]. Higher temperatures are not recommended, since enzymes most likely undergo denaturation and loss of their activity [23].

Some authors apply GFC to the crude enzyme extracts, using different types of stationary phases (e.g. Sephadex, DEAE-Sephacel, Sephacryl, Sepharose, etc.) [21, 23–25].

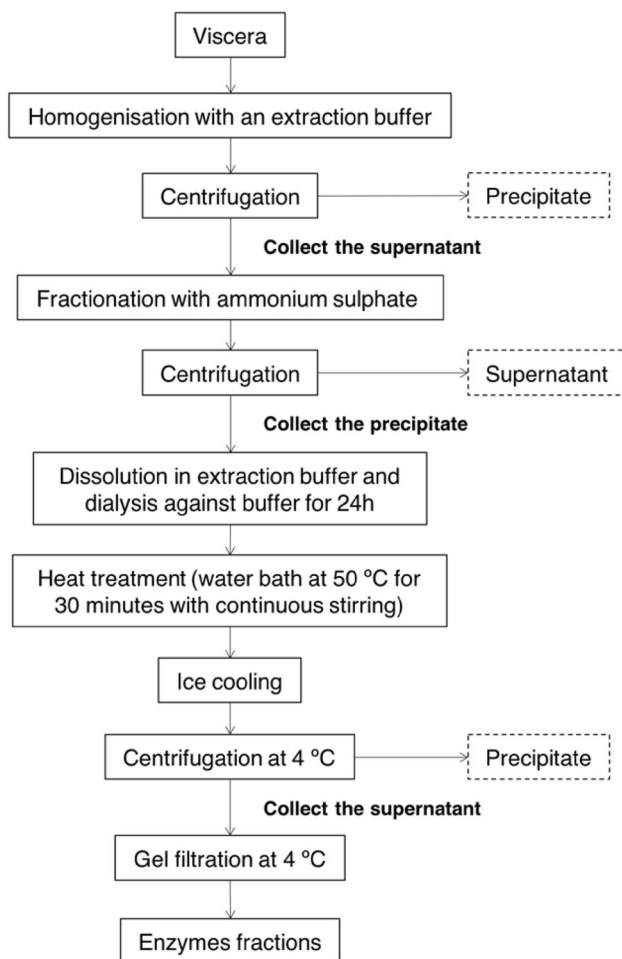
In addition to conventional methods of purification, aqueous two-phase system (ATPS) is also used, which is achieved by adding a salt aqueous solution to a polymer solution (commonly polyethylene glycol), resulting in a mixture of two immiscible solutions. Due to hydrophobic groups on their surface, proteins tend to migrate to the polymer phase [26–28]. Despite its advantages in terms of simplicity and duration, the purification factor, obtained by ATPS is typically lower than for conventional methods. Also, an additional precipitation step to separate the proteins from the polymeric phase is needed before further analysis/characterization.

In order to obtain the enzymatic extract in its purest state, it is advisable to use more than one purification method sequentially (e.g. ASF followed by IEC or GFC) to avoid interferences from matrix (when using spectrophotometric analysis) and to improve the specific activity. However, when the goal is to use the extracted enzymes in other processes, such as fish waste processing, this exhaustive purification methods do not reveal mandatory [29].

## Characterization of Proteolytic Enzymes

Colorimetric assays are commonly used to evaluate the enzymes activity, using specific substrates. Enzyme activity of pepsin is frequently performed using hemoglobin [30–35].

Different substrates for determination of trypsin activity are described in literature.  $\text{N}\alpha$ -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), a specific trypsin substrate, is the most common used [19, 20, 22, 23, 36]. However, when



**Fig. 1** Flowchart for the extraction of proteolytic enzymes (pepsin and trypsin) from fish viscera. Adapted from Shahidi and Janak Kamil [18]

BAPNA was used, the catalytic activity of the enzyme was not significantly reduced with the increase in pH value [22]. Casein [21, 25], and  $N\alpha$ -*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) [24, 37, 38] are used in less extent. A fluorimetric assay to measure trypsin activity was reported by Marcushi et al. [22] using carbobenzoxy-Phe-Arg-7-amido-4-methylcoumarin (z-FRMCa). For evaluation of the total proteolytic alkaline activity casein or azo-casein (non-specific protease substrates) are applied [19, 36]. Enzyme kinetics studies include the determination of optimum pH, pH stability, optimum temperature, thermostability, as well as kinetic parameters (Michaelis constant,  $K_M$ ; maximal velocity,  $V_{max}$ ) [21, 32, 35, 39–41]. The effect of inhibitors (phenylmethylsulfonyl fluoride, PMSF; ethylenediaminetetraacetic acid, EDTA; pepstatin; soybean trypsin inhibitor (SBTI) and iodoacetic acid), as well as activators ( $CaCl_2$  and  $NaCl$ ) should be addressed [20, 22, 23, 30, 38]. Some authors [19–21, 25] also studied the effect of metal ions (e.g. Al, Ba, Ca, Cd, Cu, Fe, Hg, K, Li, Mn, Pb, and

Zn). The evaluation of these parameters is important since genetic variation within species together with adaptation to different environmental conditions have resulted in differences in the kinetic properties of fish proteases.

Protein concentration is usually determined by the Lowry [22, 30, 32, 34, 37] or Bradford methods [21–23, 36] using bovine serum albumin (BSA) as standard. These colorimetric assays have some disadvantages related to interferences, accuracy and poor sensitivity, however, they provide a fast and cheap determination of protein content. Purity, homogeneity and molecular mass of the extracted enzymes are studied by electrophoresis in polyacrylamide gel and zymography [19, 22–24, 30, 32, 34]. Isoelectric focusing (or electrofocusing) is used to separate proteins according to their isoelectric point (pI) [30, 42, 43]. Superior characterization is achieved with the analysis of N-terminal amino acid sequence, using protein sequencers coupled to high performance liquid chromatography (HPLC) equipment [21, 25, 30, 32, 37]. Mass spectrometry methods are now the most widely used for protein sequencing and identification, but Edman degradation remains a valuable tool for characterizing a protein's N-terminus [21, 22].

## Fish Protein Hydrolysates

Fish discards resulting from traditional fish processing have been progressively recognized as a source of underutilized protein. In recent years, researchers have gathered efforts to maximize fish wastes by producing fish protein hydrolysates (FPH), which show high potential for applications such as food products or even pharmaceutical [4, 13, 44].

### Extraction of Fish Protein Hydrolysates

Most studies rely on the application of proteolytic enzymes to break down fish protein and obtain hydrolysates (Table 2). In fact, this is considered a fast and controllable method and it has gained more attention among researchers due to the ability to produce hydrolysates, retaining their nutritional value. Usually, during the enzymatic process, commercial enzymes (e.g. alcalase, flavourzyme, neutrase) are added to the protein samples [45–47]. In general, the study of the optimum conditions is based on the evaluation of the degree of hydrolysis (DH) [48].

Protein hydrolysates can be obtained by other methods than those using commercial enzymes. For example, autolysis which was reported by Samaranayaka and Li-Chan [43] prepared hydrolysates from Pacific hake fish used the high endogenous proteolytic activity from a parasitic infection. In parallel, FPH were produced by the addition of commercial enzymes (Validase® and Flavourzyme®) which resulted in higher DH in comparison to the autolysis process. It is

**Table 2** Examples of methods and optimized conditions for the production of fish protein hydrolysates

Fish species	Organ	Extraction	Optimized conditions				Yield (%)	DH (%)	References
			[E/S]	PH	T (h)	T (°C)			
Yellowfin tuna ( <i>Thunnus albacares</i> )	Stomach	Enzymatic hydrolysis (Alcalase)	3.0%	8.0	5.5	50	–	23	[45]
Tuna ( <i>Thunnus</i> )	Fish soluble concentrate	Enzymatic hydrolysis (Flavourzyme)	5.0%	6.0	6	45	68 <sup>a</sup>	62	[147]
Cod ( <i>Gadus morhua</i> )	Viscera and Backbone	Enzymatic hydrolysis (Flavourzyme)	0.1%	–	1	50	–	19.5–33.7	[46]
Pacific hake ( <i>Merluccius productus</i> )	Fillets	Autolysis	–	5.5	1	52	47.7 <sup>a</sup>	–	[148]
Herring ( <i>Pellona ditchelee</i> )	Fillets	Autolysis	–	5.0	1.5	50	–	40.2	[149]
Nile tilapia ( <i>Oreochromis niloticus</i> )	Skin	Thermal hydrolysis	–	4.0	3	121	13	–	[50]
		Enzymatic hydrolysis (894 alkaline protease + papain)	300 U/g	10 + 6	0.25 + 0.25	50	14	–	
Sardinelle ( <i>Sardinella aurita</i> )	Muscle	Bacterial fermentation	200–400 U/mL	8	24	37	–	–	[60]
Zebra blenny ( <i>Salaria basilisca</i> )									
Goby ( <i>Zosterisessor ophiocephalus</i> )									
Ray ( <i>Dasyatis pastinaca</i> )									
Carps ( <i>Labeo rohita</i> , <i>Gibelion catla</i> )									
Small-spotted catshark ( <i>Scyliorhinus canicular</i> )	Muscle	Enzymatic hydrolysis (Alcalase/Esperase)	0.5%	9.40/8.90	6	64.6/60.8	–	26.3/30.7	[47]

DH degree of hydrolysis, [E/S] enzyme to substrate ratio

<sup>a</sup>Yield under optimal conditions

noteworthy that autolysis is regarded as an economical and simple process to obtain FPH. However, the studies based on this method are still scarce due to the impossibility to control factors like the type and amount of enzymes [49].

Wang et al. [50] applied thermal hydrolysis for the hydrolysis of tilapia skin. In this study, no significant differences for the yield of the resultant hydrolysates were observed between thermal and enzymatic hydrolysis. Despite this, the enzymatic process was recommended since it needs less time and milder conditions to obtain hydrolysates.

More recently, bacterial fermentation has been proposed for the generation of fish hydrolysates. Three different

proteolytic lactic acid bacteria (*Enterococcus faecium*, *Pediococcus acidilactici* and *Pediococcus acidilactici*) were reported for the generation of protein hydrolysates from fish heads [51]. After the fermentation process, it was found that the DH ranged between 29.0 and 38.4% for the considered lactic acid bacteria (LAB). Furthermore, the hydrolysates obtained by this process showed promising bioactive properties (antioxidant and antimicrobial) turning them suitable to be used in food industry.

Nowadays, research on FPH is mainly focused on bioactive peptides due to their reported biological functions with potential applications in the food industry and benefits to



human health [44, 52]. Their potential bioactivity is strongly affected by their molecular weight and amino acid sequence [53]. After producing the FPH, bioactive peptides can be obtained through purification procedures. Within the available processes, ultra and nanofiltration, ion-exchange membranes and reverse-phase HPLC have been applied for the purification of protein hydrolysates [54]. Nevertheless, it is worthy to mention that FPH could themselves be used as bioactive compounds (bioactive peptides) although it has been proven that biological activity is enhanced after the purification of the hydrolysates [55].

### Characterization of Fish Protein Hydrolysates

Protein hydrolysates generated from marine sources show a variety of potential applications based on their unique properties. Table 3 resumes the functional properties typically evaluated for FPH such as solubility, water and oil absorption, and surface-active properties (like emulsifying and foaming properties).

Fish protein hydrolysates show a remarkable solubility (> 40%) which is comparatively higher than native proteins. In fact, the degradation of proteins leads to smaller peptides presenting an improved solubility [56].

Regarded as surface properties, foam expansion or capacity and foam stability give a better understanding about the transportation, penetration and rearrangement of molecules at the air–water interface [57]. An overview of the foaming properties revealed that foam expansion can be found at values from 17 to 200%, while foam stability is ranged from 4 to 100% [58–64].

Proteins are known for being excellent emulsifiers due to an amphipathic structure which promotes their absorption at the oil–water interface [65]. Jemil et al. [60] showed that the emulsifying activity of protein hydrolysate prepared from sardinelle decreased from 47.6 to 8.2 m<sup>2</sup>/g after increasing the protein concentration from 0.5 to 2% (w/v). Higher concentrations of proteins may lead to problems such as aggregation, precipitation and gelation. Besides this, an increased viscosity affects the interactions between proteins to form the interfacial membrane around oil droplets [61].

Hydrodynamic properties such as water and oil holding capacities (WHC and OHC, respectively) are also typically considered for the characterisation of protein hydrolysates. In general, fish protein hydrolysates reveal excellent WHC justified by the increased concentration of polar groups such as COOH and NH<sub>2</sub> resulting from hydrolysis [66]. Taheri et al. [63] reported a WHC of 5.1 mL/g for FPH which was significantly higher than hydrolysates resultant from poultry protein hydrolysates (PPH), 2.8 mL/g. The amino acid composition of both protein hydrolysate sources revealed also that FPH contained more glutamic and aspartic acids than PPH, indicating the presence of

more hydrophilic groups, which favours water absorption. OHC reflects the capacity of proteins to absorb and retain oil [67]. OHC is usually related to emulsifying capacity and it can be influenced by bulk density and DH [68].

Despite the diversity of the bioactive functions, this part of the review will be focused on the antioxidative and antimicrobial activity exhibited by the bioactive peptides derived from FPH.

For the evaluation of antioxidant activity some chemical methods could be considered. The most common antioxidative tests are 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging, hydroxyl radical scavenging, superoxide anion radicals scavenging, and inhibition of lipid peroxidation [54, 69]. Ktari and colleagues [61] evaluated the antioxidant properties of zebra blenny protein hydrolysates prepared with different crude protease extracts. Scavenging activity was measured, revealing high antioxidant activity against DPPH. The results showed that scavenging activity increased with the hydrolysate concentration until a maximum of 76.6% at 6 mg/mL. Significant differences between radical-scavenging activities were observed for each of the hydrolysates under analysis. According to the authors, this may be attributed to the amino acid composition of peptides within protein hydrolysates. In fact, hydrolysates that contained a higher concentration of hydrophobic amino acids were associated with the higher antioxidant activity. These results were in line with the work presented by other authors [58], who reported a scavenging activity against DPPH of 89% at 6 mg/mL for sardinelle protein hydrolysates. The antioxidant capacity of the hydrolysate could also be improved by the presence of aromatic amino acids, such as histidine. It was concluded that histidine-containing peptides show the ability to react with free radicals leading to the obtainment of more stable products [64].

Despite these findings, the above mentioned bioactive peptides have revealed a lower antioxidant capacity in comparison to butylated hydroxyanisole (BHA) which is one of the most commonly used synthetic antioxidants. As so, the use of these bioactive compounds derived from fish sources, as an alternative to BHA, without compromising the effectiveness of the antioxidant properties remains a challenge to researchers.

In addition, fish protein hydrolysates have also been regarded as promising natural sources for antimicrobial agents. Still, the number of studies on the antimicrobial activity of these hydrolysates are scarce. Jemil et al. [60] evaluated the antibacterial activity of protein hydrolysates prepared from different fish species towards some Gram-positive and Gram-negative bacteria. Among the FPH tested, hydrolysates obtained from sardinelle were the ones with stronger inhibitory activity assessed by the evaluation of the inhibition zone. Moreover, these hydrolysates were

**Table 3** Examples of functional and bioactive properties of fish protein hydrolysates

Fish species	Solubility (%)	Foam expansion	Foam stability	Emulsifying activity index	Emulsifying stability index	Water holding capacity	Oil holding capacity	Antioxidant activity	Antibacterial activity	Refs.
Bluewing searobin ( <i>Prionotus punctatus</i> )	40.5	–	–	54.4–70.8 m <sup>2</sup> /g	0.03–0.14%	2.4–3.7 g/g	4.5–5.5 g/g	–	–	[150]
Striped snakehead ( <i>Channa striatus</i> )/Bariramundi ( <i>Lates calcarifer</i> )	55.0	67.0/52.0 mL	62.0/36.0 mL	56.0/12.0 mL/g	–	315.0/198.0%	214.0/172.0%	DPPH: 56.7/13.2%	–	[151]
Zebra blenny ( <i>Sal-aria basilisca</i> )	>85.0	65.2–83.2%	4.0–58.3%	21.3–37.2 m <sup>2</sup> /g	23.0–46.0 min	–	–	DPPH: 61.2–76.6%	–	[61]
Striped catfish ( <i>Pangasianodon hypophthalmus</i> )	>65.0	17.3–23.3%	–	382.5–427.0 mL/g	–	–	1.0–1.4 mL/g	DPPH: ≈ 90.0%	–	[64]
Rainbow trout ( <i>Onchorhynchus mykiss</i> )	96.0	>200.0%	≈ 100%	≈ 80.0 m <sup>2</sup> /g	≈ 125.0%	5.1 mL/g	3.1 mL/g	–	–	[63]
Sardinelle ( <i>Sardinella aurita</i> )	>70.0	64.7–76.6%	43.1–46.9%	21.31–47.58 m <sup>2</sup> /g	22.6–47.8 min	3.6–7.5 g/g	1.2–6.0 g/g	DPPH: 65.0–75.0%	Significant anti-bacterial activity against Gram positive bacteria	[60]
Zebra blenny ( <i>Sal-aria basilisca</i> )										
Goby ( <i>Zosterisessor ophiocephalus</i> )										
Ray ( <i>Dasyatis pastinaca</i> )										
Silver Carp ( <i>H. molitrix</i> )	>65.0	48.2–58.1%	87.5–97.5%	75.6–89.7 m <sup>2</sup> /g	20.8–24.0 min	–	–	–	–	[62]
Sardinelle ( <i>Sardinella aurita</i> )	>65.0	71.1–85.1%	68.1–80.2%	76.1–86.6 m <sup>2</sup> /g	30.0–44.4 min	–	–	DPPH: 66.0–89.0%	–	[58]
Eels ( <i>Monopterus albus</i> )	85.0	64.7%	–	–	22.6 min	–	–	–	–	[59]
Zebra blenny ( <i>Sal-aria basilisca</i> )	–	–	–	–	–	–	–	–	Antibacterial activity against several Gram-positive and Gram-negative bacteria, MIC: 77–311 µg/mL	[152]



**Table 3** (continued)

Fish species	Solubility (%)	Foam expansion	Foam stability	Emulsifying activity index	Emulsifying stability index	Water holding capacity	Oil holding capacity	Antioxidant activity	Antibacterial activity	Refs.
Rainbow trout ( <i>Oncorhynchus mykiss</i> )	92.0	-	-	-	-	-	-	-	Antibacterial activity against fish farming bacteria, MIC: 2–5 mg/mL	[153]
Blue whiting ( <i>Micromesistius poussou</i> )	>80.0	-	-	37.8–70.3 m <sup>2</sup> /g	4.8–7.6 min	-	1.4–1.5 g/g	DPPH: 20–100%	-	[154]

more effective against the Gram-positive bacteria rather than Gram-negative which are known for being more resistant.

Antimicrobial activity of peptides appears to be affected by some factors like hydrophobicity, molecular weight and the proportion of polar groups. Regarding the fish hydrolysates, the biological activity of the peptides released is considerably influenced by the DH. This influence was studied by Tanuja and colleagues [64] that evaluated the inhibitory effect on 14 different bacteria, by hydrolysates prepared from trout with DH of 10, 20, 25 and 30%. From the results, it was demonstrated that antimicrobial effect was more pronounced for hydrolysates with higher DH (30%). Also, the resultant peptides showed a higher percentage of hydrophobic amino acids, which may improve the interaction with the bacterial membrane and therefore enable the antibacterial activity of the hydrolysate [70].

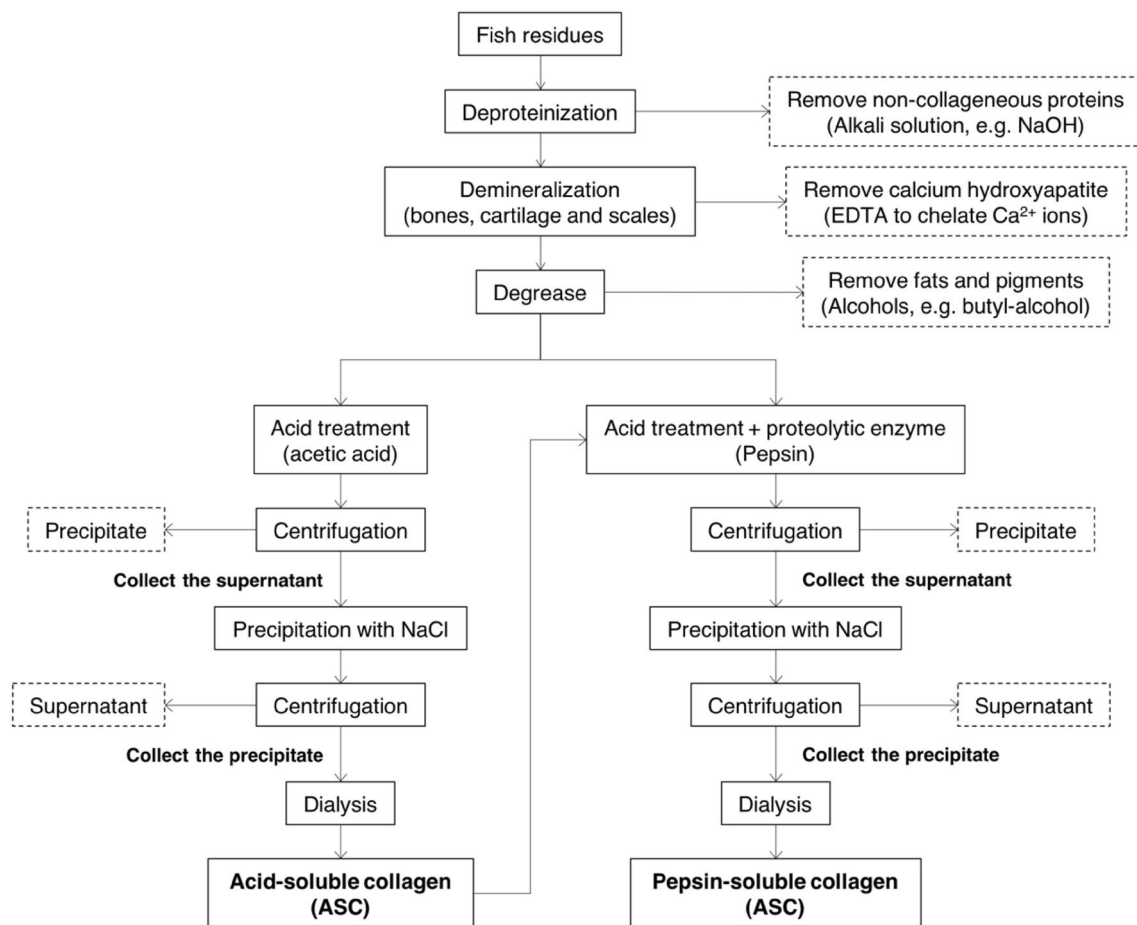
FPH have highlighted potential applications in food systems either as natural additive or emulsifying agent, but also in pharmaceutical and cosmetic industry [59–61, 64]. The incorporation of FPH into different food systems like cereals, fish, meat and dessert products has been reported in a previous review [2]. Still, despite the potential applications that have been attributed to FPH, there is insufficient information about the performance and stability of these hydrolysates in the final products. It also deserves to be mentioned that most of the investigation made so far on FPH bioactivity have been carried out in vitro. Therefore it is recommended that the outcomes showed so far should be confirmed by further in vivo studies to ensure health sustaining products derived from fish hydrolysates.

## Collagen and Gelatin

Collagen is the dominant structural protein of extracellular matrix found in animal body [71]. Its structure consists in three helical polypeptide chains with a repetitive tripeptide unit, Glycine-X-Y, where X and Y positions are generally occupied by proline (Pro) and hydroxyproline (Hyp), respectively [72]. Glycine represents about one-third of the total residues except in the N- and C-terminal regions, called telopeptides, that are composed by lysine and hydroxylysine residues [73]. Curiously, collagen is the only mammalian protein characterized by large amounts of hydroxyproline and hydroxylysine, and high content of the total imino acid<sup>1</sup> (Pro and Hyp) [74].

Until now, at least 29 different types of collagen from animal tissues, with a specific amino acid sequence, protein structure and function have been reported. Among these

<sup>1</sup> Is an obsolete term (IUPAC, 1997 “imino acids,”). In this review we will save the imino acid designation, for standardizing purposes.



**Fig. 2** A flowchart for the main procedure for the isolation of collagen from different morphological parts of fish residues. The extraction procedure comprises the chemical pre-treatment and acid-soluble collagen (ASC) method and pepsin-soluble collagen (PSC) method

various types, type-I collagen is the most common form and it can be found in connective tissue such as skin, tendons and bones [6].

Gelatin belongs to a class of protein fractions that are derived from collagen, by thermal hydrolysis which involve the breakage of hydrogen bonds between polypeptide chains of collagen molecules. As so, collagen and gelatin are two different forms of the same macromolecule [74, 75].

Traditionally, collagen and gelatin have been obtained from mammalian animals (e.g. pig, cow and poultry). However, due to its potential risks for viral and prion contaminations, these sources have been avoided [71].

In search for new sources to obtain collagen and gelatin, researchers have drawn their attention to fish by-products, namely skin, scales, bones, swim bladders and fins [76–80]. These materials contain different variants of collagen. Type-I collagen is the most common variant and can be found in the connective tissues such as skin and bones [81].

Collagen extracted from fish by-products has been considered attractive for cosmeceuticals [71], biomedical applications, as a scaffolding material and tissue engineering [82,

83], and for agriculture purpose [84]. Due to its characteristics, gelatin has most significant application in the food industry field [75, 85], pharmaceutical and cosmetics industries [73, 75].

## Extraction Methods

The extraction of collagen from fish by-products consists of three main stages: pretreatment of the raw material, isolation of the product of interest and purification (see Fig. 2) [74]. The pretreatment stage can include procedures such as separation of animal parts, size reduction, and chemical pretreatment—deproteinization, demineralization and degreasing. For example, in the case of scales and bones, a demineralization step is usually required to remove Ca or other inorganic materials from the raw material [6, 86, 87].

After pretreatment the extraction of collagen is usually carried out in acidic solutions in a procedure referred to acid soluble collagen (ASC). Due to its crosslinks, collagen has lower solubilization in acidic conditions and consequently lower yields under these conditions [73]. To improve the

collagen extraction, an enzymatic hydrolysis step was proposed. Pepsin is usually added to cleave some peptide bonds, resulting in a higher efficacy in collagen extraction (pepsin soluble collagen method, PSC) [88, 89].

As for the gelatin extraction two methods are commonly used: acid or alkaline process, which results in type A and type B gelatin, respectively. The acid process involves the pretreatment of raw materials with an acid solution followed by an extraction in acid medium. As for the alkaline process, the pretreatment step is carried out with an alkaline solution, in most cases followed by neutralization, so the extraction may be carried out in different medium (alkaline, neutral or acid) [89]. Moreover, the acidic treatment is most suitable for the less covalently crosslinked collagens while the alkaline process is recommended for the more complex collagens [74].

Following the extraction of collagen/gelatin a centrifugation step is performed. In the end the supernatant is collected and precipitated with NaCl being redissolved in acetic acid. Finally, samples are submitted to dialysis in order to purify the final product.

In Table 4 are listed some of the most recent studies regarding the extraction of collagen and gelatin from fish by-products, with special emphasis to the extraction conditions and characterization of these macromolecules.

In the extraction of collagen, organic acids (like acetic, citric and lactic) are commonly used, being more efficient than inorganic ones due to the ability to enhance the solubility of collagen during extraction [6].

As seen in Table 4, the yield of the final product can vary significantly with the process conditions, but also with raw materials considered for extraction. Sinthusamran et al. [90] described the extraction of collagen from two by-products of seabass, skin and swim bladder, following the ASC procedure. The authors verified that the swim bladder gave a higher yield compared to the skin (85.3% for swim bladder and 58.1% for skin). According to the authors, the swim bladder exhibits less cross-linked collagen, which facilitated the extraction process. In another study [86], a higher extraction yield of type I collagen was obtained from rohu (2.7% w/w) compared to the one from Carp catla (1.7% w/w).

The PSC procedure leads to significantly higher yields in collagen extraction (Table 4). Pepsin is responsible for the specific cleavage at the telopeptide region of collagen allowing a better control of the process which implies a considerable reduction of the waste [79, 91, 92]. Despite the improvement in the use of pepsin, the cost of enzyme may represent a disadvantage for the application of this process. Kaewdang et al. [79] have proposed the application of endogenous enzymes from crude stomach extract (mainly pepsin) for the release of collagen from swim bladders of yellow-fish tuna. The comparison of ASC and PSC procedures resulted in yields of 1.07 and 12.10%, respectively.

Endogenous enzymes from stomach were successfully used for the extraction of collagen from fish by-products, demonstrating the enormous potential of marine discards.

Other approaches have also been considered for the extraction of collagen. Ali et al. [93] studied the extraction efficiency of collagen from the skin of golden carp by using ultrasound treatment in conjugation with acid and pepsin soluble collagen procedures. The use of ultrasound in conjugation with acidic or enzymatic methods resulted in a marked increase in collagen yields. This effect was more pronounced in the ASC procedure (up to 1.57-fold). The cavitation effect promoted by the ultrasounds helps to loosen the fish matrix, improving mass transfer, thus enhancing the penetration of solvent and the extraction yield. However, ultrasound extraction has limited industrial applications due to its expensive scale-up.

A combination of physical methods like ultrasounds and vigorous mechanical mixing with conventional procedures (named IASC) was applied for collagen extraction from jellyfish tissue [94]. By comparison, the IASC process resulted in seven times higher collagen extraction than ASC and two times higher than PSC, contributing to a significant reduction in the time of extraction. Despite these results the use of physical methods may be disadvantageous for industrial purposes due to the high energetic costs.

As mentioned before, gelatin extraction protocols rely on the use of alkaline or acid treatment followed by extraction with distilled water at relatively high temperature (45–80 °C). The use of enzymes can also be included to achieve higher gelatin extraction yields [95, 96]. Zhang et al. [97] described the extraction of gelatin from the skin of spiny dogfish using a pretreatment with alcalase. According to the authors enzymes are effective under mild reaction conditions promoting the formation of high-quality gelatin in comparison to chemical hydrolysis.

Ultrasounds methodology has also been investigated for the extraction of gelatin from marine sources. Gelatin was obtained from the skin of golden carp according to different procedures with and without acid pretreatment and ultrasonication [92]. The authors were able to observe that the highest yield (62.12%) derived from the treatment with sulphuric and acetic acids followed by ultrasounds extraction.

## Characterization Methods

After extraction, it is important to study and evaluate the characteristics of collagen and gelatin to determine further applications [72].

Fourier-transform infrared (FT-IR) spectroscopy is a simple method to investigate the functional groups and to discuss the collagen triple helical structure [72]. Over the years, this technique has been widely used to examine the secondary structure of proteins including collagen

**Table 4** Examples of extraction and characterization methods for collagen and gelatin from fish by-products

Fish species	Part used	Product	Extraction	Yield (%)	Characterization	Refs.
Seabass ( <i>Lates calcarifer</i> )	Swim bladder and skin	Collagen	ASC—0.5 M acetic acid; 48 h	Swim bladder—58.1% (dwb) Skin—85.3% (dwb)	Amino acid SDS-PAGE FTIR DSC	[90]
Yellowfin tuna ( <i>Thunnus albacares</i> )	Swim bladders	Collagen	ASC—0.5 M acetic acid; 48 h PSC—0.5 M acetic acid + crude stomach extract; 48 h	ASC: 1.07% (dwb) PSC: 12.10% (dwb)	Amino acid SDS-PAGE FTIR DSC	[79]
Nothern Pike ( <i>Esox lucius</i> )	Scales	Collagen	ASC—0.5 M acetic acid; 48 h PSC—0.5 M acetic acid + pepsin 1% (w/w); 48 h	—	SEM FTIR Amino acid SDS-PAGE DSC Mechanical properties	[106]
Barbel ( <i>Barbus caliensis</i> )	Skin	Gelatin	Alkaline process—0.05 M NaOH + crude acid protease extract; 48 h	8.36% (wwb)	Amino acid SDS-PAGE Functional properties	[95]
Squid ( <i>Loligo vulgaris</i> )	Skin	Gelatin	Alkaline process—0.05 M NaOH + pepsin; 48 h	6.82% (wwb)	Amino acid FTIR Functional properties	[96]
Carp ( <i>Cyprinus carpio</i> )	Scales	Collagen	ASC—0.5 M acetic acid; 48 h	9.79% (wwb)	FTIR SDS-PAGE	[84]
Tilapia ( <i>Oreochromis niloticus</i> ) and channel catfish ( <i>Ictalurus punctatus</i> )	Skin	Collagen and Gelatin	ASC—lactic acid 60% (v/v); 46 h PSC—lactic acid 50% (v/v) + pepsin; 17 h Acid process—0.2% sulphuric acid + 0.2% citric acid; water at 45 °C, 12 h	ASC: 25.6–25.9% (dwb) PSC: 21.8–28.8% (dwb) Acid process: 80.6–93.1% (dwb)	FTIR SDS-PAGE SEM	[100]
Salmon ( <i>salmo salar</i> )	Skin	Gelatin	Acid process—0.05 M acetic acid at 60 °C	—	Amino acid SDS-PAGE X-ray diffraction	[103]
Snakehead ( <i>Channa Micropeltes</i> )	Scales	Collagen	ASC—0.5 M acetic acid; 48 h	—	SEM FTIR NMR	[87]
Antarctic ( <i>Kondakovia longimana</i> ) and Sub-Antarctic ( <i>Illex argentinus</i> ) squid	Skin	Collagen	ASC—0.5 M acetic acid; 72 h PSC—0.5 M acetic acid + pepsin 3.3 mg; 72 h	ASC: 0.81–1.05% (wwb) PSC: 0.88–3.26% (wwb)	FTIR DSC Amino acid SDS-PAGE SEM Mechanical properties	[91]
Carp ( <i>Catla catla</i> )	Scales	Collagen	ASC—0.5 M acetic acid; 72 h PSC—0.5 M acetic acid + pepsin 0.5% (w/v); 48 h	ASC: 1.7–2.7% (dwb) PSC: 1.48–2.26% (dwb)	SDS-PAGE Amino acid FTIR SEM DSC	[86]
Kumakuma ( <i>Brachyplatystoma filamentosum</i> )	Skin	Gelatin	Alkaline process—0.3 M NaOH; water at 58 °C, 6 h	19.7% (wwb)	Amino acid SEM Functional properties	[108]

**Table 4** (continued)

Fish species	Part used	Product	Extraction	Yield (%)	Characterization	Refs.
Tilapia ( <i>Oreochromis niloticus</i> )	Skin	Collagen	ASC—0.5 M acetic acid PSC—0.5 M acetic acid + pepsin 0.5% (w/w)	—	Amino acid CD X-ray diffraction FTIR SDS-PAGE SEM	[104]
Tilapia ( <i>Oreochromis niloticus</i> )	Skin	Collagen	ASC—0.5 M acetic acid; 48 h PSC—0.5 M acetic acid + pepsin 0.1% (w/v); 48 h	ASC: 19.80% (dwb) PSC: 20.03% (dwb)	Amino acid SDS-PAGE FTIR SEM	[107]
Carp ( <i>Probarbus jullieni</i> )	Skin	Collagen	ASC—0.5 M acetic acid; 48 h PSC—0.5 M acetic acid + pepsin 1% (w/v); 48 h UASC—ultra-sonication (30 min) + ASC UPSC—ultrasonication (30 min) + PSC	ASC: 51.90% (dwb) PSC: 79.27% (dwb) UASC: 81.53% (dwb) UPSC: 94.88% (dwb)	SDS-PAGE Amino acid FTIR DSC CD	[92]
Jellyfish ( <i>Acromitus hardenbergi</i> )	Tissue	Collagen	ASC—0.5 M acetic acid; 72 h PSC—0.5 M acetic acid + pepsin 10% (w/v); 48 h IASC—0.5 M acetic acid + ultrasonication (15 min) + vigorous mixing (1 h)	ASC: ~6% (dwb) PSC: ~20% (dwb) IASC: 37.08–40.20% (dwb)	SDS-PAGE Amino acid	[94]
Spiny dogfish ( <i>Squalus acanthias</i> )	Skin	Gelatin	Alcalase, 2.1 h + water at 50 °C, 4.6 h	—	Amino acid FTIR TGA	[97]
Carp ( <i>Probarbus jullieni</i> )	Skin	Gelatin	0.02 M sulphuric acid + 0.05 M acetic acid; ultrasonication (30 min); water at 55 °C, 6 h	62.12%	Amino acid SDS-PAGE FTIR	[93]

ASC acid-soluble collagen, PSC pepsin-soluble collagen, dwb dry weight basis, wwb wet weight basis

and gelatin, through the absorption in the amide I region (1600–1700  $\text{cm}^{-1}$ ) [98, 99]. Although the infrared spectra of these macromolecules are quite similar there are some differences that can be pointed out. Gelatin derived from fish skin presented a FT-IR spectra slightly different from ASC and PSC. The lower intensity of amides I, II, III bands suggested a higher degree of disorder in gelatin and the loss of most of the triple helix structure originally exhibited by collagen [100]. The triple helical structure of collagen is also evaluated through FT-IR absorption ratio of amide III to 1450  $\text{cm}^{-1}$  band, which should be close to 1 for native collagen [101, 102] and for collagen extracts [93]. According to the authors, these results confirmed that ultrasound or pepsin treatment had no impact on the native structure of collagen.

X-ray diffraction (XRD) and circular dichroism (CD) are two powerful techniques used to assess the helical content of a collagen sample. Díaz-Calderón et al. [103] have studied the influence of extraction variables (pH and time) on the structure of salmon gelatin. X-ray diffraction patterns of the extracts showed the presence of two characteristic peaks. The first peak is related to the triple helix structure, indicating the distance between the molecular chains while the second peak indicates the distance between skeletons. The authors were able to establish a correlation between the intensity of the first peak and the extraction conditions. Therefore, a higher intensity was found for mild conditions (pH 5/2 h), which results in gelatin with higher triple helix content (9.0%). On the other hand, the most aggressive conditions (pH 3/5 h) have registered a less intense peak and a



lower triple helix content (3.1%). In another study [104], CD was applied to evaluate the secondary structure of ASC and PSC from the skin of Nile Tilapia. CD measures the differences in absorption of left-rotated and right-rotated circularly polarized light. The typical CD spectrum of collagen shows a positive and negative peak whose ratio (Rpn) ranges from 0.12 to 0.15 [105]. Both, tilapia skin ASC and PSC showed XRD diagrams characteristic of collagen, while CD spectra showed Rpn values of 0.12 and 0.13, respectively. As so, it was concluded that ASC and PSC maintained the integrity of the triple helical structure.

Collagen and gelatin extracts are commonly studied for their amino acid composition. The amino acid analysis is achieved by a protein hydrolysis, comprising steps such as amino acid separation, identification and quantification by HPLC. As expected, glycine (225–331 residues/1000 residues) is reported as the most abundant amino acid, followed by alanine [79, 91, 106].

The thermal stability of collagen is usually described by the denaturation temperature ( $T_d$ ) and is frequently determined by differential scanning calorimetry (DSC). Sinthusamran et al. [90], compared the thermal stability of ASC extracted from skin and swim bladder of seabass. The results showed that swim bladder gave collagen with higher thermal stability ( $T_{max} = 35.02$  °C), than the one obtained from skin ( $T_{max} = 33.33$  °C). The differences in  $T_{max}$  between ASCs were correlated with the imino acid content, which was slightly higher for the swim bladder extract. A similar conclusion was drawn after  $T_{max}$  analysis of ASC and PSC from the skin of golden carp. A higher  $T_{max}$  value was found for PSC and UPSC due to the higher content of imino acids which might contribute to a better stabilization of the triple helix structure [93].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a variant of polyacrylamide gel electrophoresis, is often used for the characterization of collagen and gelatin extracts (Table 4). The SDS-PAGE patterns of these macromolecules are usually composed by two different  $\alpha$ -chains ( $\alpha 1$ -chain and  $\alpha 2$ -chain) as a main constituent and a major  $\beta$ -chain. In addition, small amounts of the  $\gamma$  component could also be observed in these samples [84, 106].

Scanning electron microscope (SEM) is also frequently described in studies regarding the extraction of collagen/gelatin to investigate the collagen morphology, namely the presence and organization of fibrils [87, 107]. This technique could be used to evaluate the extent of the denaturation in collagen, the microstructure and surface area [100, 108]. The investigation of collagen and gelatin morphology provides a better understanding about possible applications of these products. For example, Zhang and colleagues [100] reported that ASC extracted from Nile tilapia and channel catfish skin possesses a complex fibril structure with high potential for cosmetic applications. Its ability to hold water

is recommended for its application as hydrating agent. By its turn, PSC yielded a almost uniform multilayer morphology, which indicates higher compatibility with biomedical applications.

Apart from the above-mentioned methods of characterization, collagen and gelatin have also been studied for their functional properties including emulsifying, foaming, water and oil holding capacity [95, 96, 108] as well as its mechanical and biological properties [91, 93, 106]. These important functional properties are very relevant in the food chemistry field. For example, the WHC and OHC are related to the texture by the interaction between gelatin and other components, such as water and oils.

## Hydroxyapatite

Hydroxyapatite [ $C_{10}(PO_4)_6(OH)_2$ , HAp], as well as other calcium phosphate-based compounds, are employed as biomaterials, namely in the fabrication of bone implants and bone cements, due of their high biocompatibility [4, 109]. From a chemical and structural point of view, HAp with a Ca/P molar ratio = 1.67, is the most similar material to the inorganic part of bones and teeth [110].

Several methods for HAp synthesis, such as solid-state synthesis, chemical precipitation, hydrothermal treatment, radio frequency thermal plasma and polymer-assisted methods were described in literature [4, 111, 112]. However, these synthesis processes, are biologically hazardous, expensive and complex [110]. Extraction of HAp from by-products of food industry (fish bones and scales) arises as a cheaper and uncomplicated alternative procedure [113, 114]. Also, the HAp obtained from natural sources, is more compatible than the synthetic counterpart.

## Extraction from Bones and Scales

Before extraction of hydroxyapatite itself, soft tissues (such as cartilage and fragments of meat and skin) are removed manually from the bones using hot water [10, 110, 111, 115, 116]. The washed bones can be mixed with an alkaline solution and/or acetone to remove protein, lipids, oils and other organic impurities [117].

Isolation of HAp can be achieved by different methods, such as calcination, alkaline hydrolysis, enzymatic hydrolysis and ionic liquid pretreatment.

Calcination process is the most common way to produce HAp due to its low cost and simplicity. Different calcination temperature and duration of heat treatment result in HAp with different properties, namely in terms of the degree of crystallinity, crystallite size, and surface structure composition [110, 111, 118]. Lower calcination temperatures are recommended to produce medically preferable carbonated



**Table 5** Classification, advantages, and restrictions of the most common techniques used to characterize hydroxyapatite (adapted from [125])

Technique	Advantages	Disadvantages
FTIR	Measurement over a narrow range of wavelengths at a time; no external calibration is required; provides accurate and fast results; identification of contaminants (even at small concentrations)	Inorganic materials are not easily analyzed by FTIR spectroscopy
XRD	Powerful and rapid technique; provides an unambiguous mineral determination; data interpretation is relatively straightforward	Homogeneous and single-phase materials are best for identification of an unknown; peak overlay may occur, and it is worse for high angle reflections
EDS	Provides unique peaks characteristic of the atomic structure of the atoms; quick and versatile technique	Comparatively lower precision than SEM
SEM	Direct visualization, high resolution	Powder aggregation during the sample preparation; sometimes needs TEM to confirm results
TEM	Direct visualization, high resolution	Powder aggregation during the sample preparation, electron beam damage, preference for electron-dense atomic species

HAp, while higher temperatures (800–1000 °C) lead to elimination by heat of carbonated groups of HAp which affect its biological properties [110, 111, 117].

Alkaline hydrothermal hydrolysis is becoming popular nowadays. When applied, nanostructured carbonated HAp particles are produced, which are highly desirable for biomedical applications [117, 119].

All the above methods have associated environmental issues due to the use of acids and alkalis to separate the HAp from fish waste [120]. Huang et al. [112] successfully extracted HAp from tilapia scales by enzymatic hydrolysis (using protease N and flavourzyme). The extracted material showed low crystallinity and nanoparticle sizes with a Ca/P molar ratio of 1.78. However the associated costs, as well as the time required and the need for controlled environment maintenance, make this process more complex.

Ionic liquid was recently presented as a novel approach for extraction of HAp from fish scales [121]. The extracted HAp showed a Ca/P ratio of 1.60, which is comparable with that of human bones. Ionic liquid interacts with collagen by hydrogen bond, dissolving it, while HAp remains undissolved and can be collected as a precipitate by centrifugation. Another advantage of this method is that the ionic liquid can be recycled.

### Characterization of Hydroxyapatite

Various characterization techniques are used to characterize the HAp extracted from fish residue. The details about the advantages and limitations of the most common techniques are presented in Table 5.

Thermal gravimetric analysis (TGA) is often applied to study the thermal stability of the samples [111, 117, 121–123]. Results of TGA analysis, performed on HAp from fish scales, showed one broad endothermic transitions peak in the differential thermal analysis DTA curve,

at temperatures lower than 200 °C, corresponding to the desorption of water. A small peak was detected at about 350 °C, corresponding to the combustion of carbon compounds, indicating that the pretreatment was efficient in removing organic matter from the scales [122].

Functional groups at the surface of extracted HAp are characterized by FT-IR [110, 120, 121, 123, 124]. HAp shows various characteristic peaks in the FT-IR spectrum at 3572 cm<sup>-1</sup> (OH stretching), 1087, 1046, 1032 cm<sup>-1</sup> (P–O stretching) and 631, 602, and 574 cm<sup>-1</sup> (O–P–O bending) (Fig. 3a).

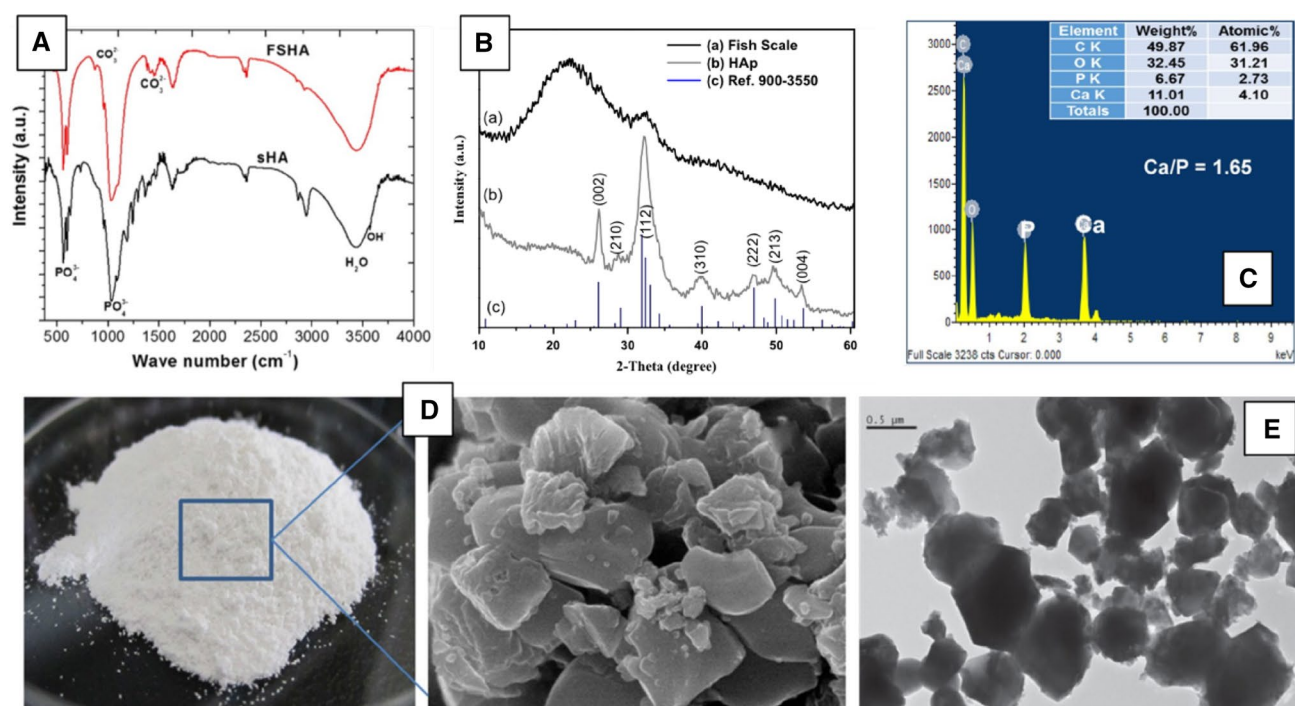
XRD provides extended information about the crystalline structure and is used to confirm the phase composition of HAp [112, 114, 116, 122, 124]. HAp shows a well resolved characteristic peak at 2-theta value of 32.2° (211 plane of geometry) (Fig. 3b).

It is important to determine the amount of possible toxic elements since the HAp is obtained from biological sources [110]. For this, the chemical analysis of the extracted HAp is often performed by inductively coupled plasma atomic emission spectrometry [10, 110, 111, 122] or by flame atomic absorption spectroscopy [114].

Electron dispersive scanning analysis (EDS), used in combination with SEM, is a simpler approach to determine the elemental composition of HAp (in particular the Ca/P molar ratio) (Fig. 3c) [112, 120]. However, it only allows a semi-quantitative analysis of the chemical elements.

Direct visualization using SEM and transmission electron microscopy (TEM) provides detailed information about the particle size, shape, morphology, dispersion and microstructure of HAp (Fig. 3d–e) [111, 114, 116, 123, 125].

Because HAp plays an important role in the biomedical field, cytotoxicity and proliferation studies with different cell-lines are performed to confirm its biocompatibility [110, 112, 113, 117, 120, 123].



**Fig. 3** Common techniques used to characterize Hap isolated from fish scales. HAp shows various characteristic peaks in the FT-IR spectrum (a) at 3438 cm<sup>-1</sup> (OH stretching), 957, 1030 cm<sup>-1</sup> (P–O stretching) and 563 and 602 cm<sup>-1</sup> (O–P–O bending). The XRD profile of Hap (b) shows characteristic diffraction peaks at 26.1, 28.45, 32.90, 40.19, 46.82, 50.05, 53.56°, which correspond to the 002, 210,

112, 310, 222, 213 and 004 planes of the hexagonal HAp unit cell. In EDS technique (c), the unique peaks of Ca, P, and O of HAp appear at their characteristic positions. SEM and TEM (d–e) provides direct information about the morphology, shape, size, and dispersion of Hap. Figures reproduced with permission from [112] (a), [120] (b–c), and [117] (d–e)

## Fish Oils

Lipids from marine origin have a higher unsaturation degree compared with those of terrestrial organisms. That composition is usually determined by an acute identification and quantification of the fatty acids (FAs) content [69, 126]. Long chain polyunsaturated fatty acids (PUFAs) in the *cis* isomeric configuration such as eicosapentanoic acid (C20:5n3, EPA), arachidonic acid (C20:4n6, AA) and docosahexanoic acid (C22:6n3, DHA) are important precursors of prostaglandins, thromboxanes and leukotrienes [4, 127]. These essential FAs, being mostly obtained from the diet, are part of two related groups—omega 3 and omega 6 [128]. With growing awareness of the clinical benefits for this class of compounds, there is an increasing need to locate sources for human consumption. Accumulation of fatty acids in many lean fish species is predominantly in perivisceral organs (e.g. liver and intestine), with uptakes exceeding 50% of the wet weight [128, 129].

## Lipid Extraction

The research and characterization of FAs in fish oil requires prior extraction from raw material followed by

purification/separation [4, 130]. Fishmeal process is the most common source of fish oil, since oil is generated as a by-product in the fishmeal industry. The extraction can be categorized into three categories: physical, chemical and biological. Physical extraction includes homogenizing, heating, pressing and filtering, also regarded as rendering. The bulk of fishmeal and oil is manufactured by the wet rendering method where direct steam is injected into the rendering tank, along with the material being rendered, as opposed to dry rendering, where steam is confined in a jacket that surrounds the tank containing the material [131]. Pressing is carried out to squeeze the oil from the slurry, which is followed by filtration or centrifugation to separate FAs from the miscela [126]. This is the most common and simple procedure to obtain fish oil at the industrial scale and allows the highest extraction yields. However, it is not so feasible when the oil content is low [127, 132].

Table 6 resumes the most common techniques used for lipid extraction. The first developed chemical methodology for the extraction of lipids was based in a liquid–liquid phase with a mixture of polar and non-polar solvents in specific ratios [127, 133]. This methodology serves as a benchmark for comparison between other solvent extraction methods.

**Table 6** A summary of industrial applied extraction methods for oil extraction from fish by-products

Extraction processes	Method	Parameters	Advantages	Disadvantages	Ref.
Physical	Wet rendering	Homogenizing Cooking Pressing Filtering	Standard method in oil and fishmeal industry Use of direct steam flux in tissues for better yield and quality than dry rendering	Addition of chemicals in pressing operation can reduce nutrition value or increase residual chemicals in fishmeal Additional steps for human consumption (refining and polishing to remove impurities)	[129, 130, 132]
	Dry rendering	Same steps as wet rendering	Standard method in oil and fishmeal industry	Indirect steam jacketed vessel for cooking many lead to overheating and unpleasant off-flavors Addition of chemicals in pressing operation can reduce nutrition value or increase residual chemicals in fishmeal Additional steps for human consumption (neutralization, refining and polishing to remove impurities)	[129, 130, 132]
Chemical	Folch Bligh and dyer	Extraction with organic solvents (chloroform, methanol)	Simple and standard method Direct determination of total lipids	Large amount of toxic/carcinogenic solvents; Time consuming	[118, 122]
	Soxhlet	Extraction with non-polar organic solvents (hexane; toluene; petroleum ether)	Simple and standard method; Can be operated with non-chlorinated solvents; Few steps of manipulation and extraction cycles	Lower yields than liquid-liquid methods; Special equipment required; Conditions difficult to be controlled (continuous flow of solvents); Time consuming	[123]
	Supercritical fluid extraction (SFE)	Extraction with CO <sub>2</sub> , sometimes with addition of a polar solvent (methanol)	Rapid and less labor consuming; No organic solvents; Non-destructive (lipids can be further used)	Very expensive equipment; Supply of CO <sub>2</sub> needed	[123, 124]
	Autolysis	Use of endogenous enzymes and addition of acidic catalyst	Production of silage rich in small peptides and amino acids Enzymes promote release of oil	Time consuming (reduces hydrolysates quality) Conditions difficult to be controlled	[123]
Biological	Hidrolysis	Use of exogenous enzymes (proteases and lipases) and addition of acidic or alkaline catalyst	Rapid and less labor consuming that autolysis Use of commercial low cost and food grade enzymes	Favorable conditions for hydrolysis of free fatty acids and oil-water emulsion	[123]

The Soxhlet extraction is based on solid–liquid extraction and excludes the need for filtration. However, its extraction efficiency is highly dependent on the properties of the organic solvent [126]. This methodology easily extracts triacylglycerols (TAGs) but the extraction of polar complex lipids like phospholipids is rather difficult, usually requiring a more polar co-solvent [126]. The system temperature required for evaporation and number of cycles must be considered, since it can influence lipid yield. Although solvent extraction is widely used for analytical purposes at the lab scale it has some drawbacks in the fish processing industry due to high energy costs, environmental concerns and restrictions of the use of solvents in the food industry.

The supercritical fluid extraction (SFE) is the most important breakthrough in lipid extraction, being considered as a potential replacement for the traditional organic solvent methodology [126, 134]. At supercritical conditions, fluids acquire special proprieties (gas-like low viscosity and high diffusivity and liquid-like high densities), enhancing the solvation and flow properties and making extraction faster and more effective [126, 127]. Supercritical CO<sub>2</sub> is widely used as solvent, being considered a green solvent due to its low toxicity, cost and flammability [130, 135]. Additionally, using this technique fish oil can be fractionated into different components according to their molecular weight or degree of saturation. The major drawback for SFE-CO<sub>2</sub> is related to the very expensive cost of the apparatus [126].

Biological methodologies include enzymatic oil extractions and silage production through the use of enzymes. These methods have become popular to recover fish oils since it can be simpler and cheaper regarding investment cost and energy expenses [127, 130].

Silage is the fish digest recovered using internal enzymes from viscera and acid for stability, in a process referred to autolysis. The process forms fish silage from muscle and aqueous solution rich in small peptides and aminoacids, and the enzymes promote the release of oil. If exogenous enzymes are used, the process is called hydrolysis. Addition of exogenous enzymes (lipases and proteases) sourced from either animal, vegetable or microbial, requires no organic solvents and makes hydrolysis highly controllable with reduced reaction time [136, 137]. Enzymatic reaction provides milder conditions in terms of temperature, which protects FAs from oxidation, isomerization, and other undesirable reactions, yielding better results than thermal procedures [4, 138]. Another biological process for production of fish ensilage combines minced fish material with a carbohydrate source (sugar or molasses) or organic acids (lactic acid) and microorganism. The success of this fermentation is dependent of production of lactic acid by microorganism that must be sufficient to establish a pH below 4,5 [129].

Each technique has its own advantages and drawbacks and leads to different profiles of FAs concentrates. For

example, SFE is suitable for the recovery of lipids as fatty acids esters, while acylglycerols can be obtained by enzymatic methods [4].

## Fatty Acids Characterization and Quantification

To determine the FAs composition a three-step analysis is carried out, namely extraction, derivatization to their methyl ester forms and analysis.

One-dimensional thin Layer Chromatography (TLC) with silica gel has been used for lipid class separation for many years, mainly for inexpensive and flexible requirements [139, 140]. Commonly, the solvent elution system consists in a mixture of polar/non-polar solvents. One of the drawbacks in TLC characterization is co-elution of some classes with similar polarity, requiring a previous small-scale procedure of isolation based on a column of silica gel with polar/non-polar solvents.

Some methods of analysis of fatty acids as their methyl esters rely on a previous saponification of lipids before converting into FAMES. This method consists on heating the extracts under reflux with an excess of dilute aqueous ethanolic alkaline solution. The saponifiable layer contains the FAs and the non-polar hydrocarbons, long chain alcohols, sterol and glycerol ether are separated in the non-saponifiable layer [140].

The esterification of FAs to FAMES, to improve their volatility, is performed by derivatization using an alkylation reagent, before gas-chromatography (GC) analysis. There are many esterification methods described in literature, each varying the derivatization reagent since no single reagent will suffice, giving a choice for the analyst to best fit the circumstances. The commonest reagent for the preparation of FAMES is HCl in methanol. A solution of concentrated sulfuric acid in methanol used for esterification is recommended [141]. A comparison of different derivatization reagents and mechanisms is well presented in the Ref. [140]. The former methodology is nowadays being neglected for in situ transesterification, that allows a faster processing of samples. In situ transesterification refers to the direct transesterification of lipids in a matrix without prior lipid extraction and saponification. This procedure is gaining recognition as a lipid measurement process [134, 142] and can be carried out using both acid and base derivatization reagent, or a combination of both [143, 144].

GC is the preferred technique in characterization and quantification of FAMES. The flame ionization detector (FID) is universally used in fatty acid quantification, with high sensitivity and stability, low dead volume and fast and linear response time linear over a wide range [140]. Mass spectrometry (MS) coupled with a GC gives detailed spectrometric information of FAMES, offering two important advantages over FID: the ability to confirm the identity of



analytes based on spectral information, and separation of peaks from a noisy background or coeluting peaks if unique ions are available [145].

Nuclear magnetic resonance (NMR) spectroscopy analysis of commercial fish oil has been used to provide discrimination concerning the nature, composition, refinement, and/or adulteration or authentication of products [146]. Furthermore, NMR provides a complementary analysis for characterization and quantification of FAMES by GC-FID. Other advantages of NMR include simple sample preparation and short analysis time. However, the cost of the equipment is the most prohibitive limitation of its use.

## Conclusions

Every year, worldwide, FPI is responsible for the production of high amounts of biowaste. Despite some of these residues are already used, a more sustainable exploitation of these natural resources is an asset. Fish by-products, such as bones, scales, skin and viscera have been studied as potential raw materials for isolation of added-value compounds, with different applications on several fields. Although several processes are already described in the literature, it is possible to isolate different compounds from the same by-product. Both collagens and hydroxyapatites can be obtained from scales/bones, corresponding to an integral valorization of these morphological parts, heretofore regarded as waste. Essential FAs can be isolated from viscera, as well as proteolytic enzymes for several industrial purposes. These enzymes can be also applied in enzymatic hydrolysis processes for processing other morphological parts, operating in a loop (e.g. isolation of hydroxyapatite from the scales using endogenous enzymatic hydrolysis).

Because some of the processes are industrially achievable, research regarding the valorization of the FPI by-products may result in the generation of economically attractive products. More importantly, this will result in a significant reduction of wastes, giving rise to a more beneficial alternative to the disposal methods currently employed.

This review is focused on the techniques for extraction and characterization of added-value materials from FPI waste. Methods and techniques developed for the extraction and characterization of materials from other sources can be easily adjusted to take in consideration the specific features of fish by-products. Industrial processes are feasible, thus simultaneously permitting the reduction of waste and the obtainment of added-value materials.

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## Compliance with Ethical Standards

**Conflicts of interest** The author declares no conflict of interest.

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