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Determination of collagen types and mineral contents in fish skin and collagen-containing skin-derived protein hydrolysates before and after *in vitro* simulated digestion

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An *in vitro* digestion model was established to characterize the types of collagens in skin of cod, white fish, and salmon as well as their collagen-containing skin-derived protein hydrolysates (CSPH) before and after digestion. Moreover, the mineral content and their bioaccessibility were evaluated. Finally, the presence of heavy metals was evaluated to assess the safety of these products. The results showed that white fish protein exhibited a high digestibility, reaching up to 92%. Among the collagen products, salmon collagen had the highest digestibility (~73%). Protein identification revealed that the emPAI of type I collagen in digested skin and CSPH was higher than that of undigested samples. In addition, raw skins had higher contents of P, K, Ca and Mg, and the mineral content of CSPH was lower than that of unprocessed skins. Among the minerals studied, Ca and Cu showed the highest bioaccessibility in raw skin cod, being 32% and 26%, respectively. The bioaccessibility of Cu in raw skin salmon was also higher (~34%). Moreover, in CSPH, Mg, K and Cu can be easily digested and absorbed. Regarding heavy metals, As and Pb were below the respective safe limits in all raw skins and CSPH, while Hg and Cd were not detected in the fish CSPH. Fish-derived collagen has gained significant attention due to its numerous health benefits, high bioavailability, and superior sustainability compared to animal collagen. Moreover, different types of collagens offer distance roles and advantages in the body. However, there are limited reports on how collagen structure and type may change during the digestive process. This study seeks to deepen our understanding of the economic value of fish collagen, as well as the mechanisms of its absorption and digestion. By investigating processes, the research aims to provide a clearer insight into the physiological effects of fish-derived collagen, which can inform the development of tailored collagen supplementation programs based on specific health needs.

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1. Introduction

The growth of the global population has brought about an increase in the demand for food. As an important part of the food industry, fisheries and aquaculture provide a large amount of food to meet human needs.[1] According to a report by the Food and Agriculture Organization of the United Nations (FAO), fish food consumption continued to grow at an average annual rate of 3% from 1961 to 2018.[2] However, the increase in food fish production has been accompanied by the generation of waste, and the discarding of these wastes has also caused economic losses.[3] Extracting desirable compounds from fish biomass (including proteins, bioactive peptides, fish oils and minerals, *etc.*) not only reduces waste during fish processing, but also promotes the recovery of high-added-value compounds for human consumption with positive health impact.[4]

Collagen is the most abundant protein in mammals, accounting for 30% of total animal protein. Collagen can be obtained from animal skin, cartilage, *etc.* For example, pigs and cows are popular sources of collagen.[5,6] However, the development of animal-derived collagen has been limited due to risks for zoonotic diseases or allergic reactions. At the same time, religious beliefs are also issues that need to be considered when developing animal collagen products.[7] In this

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sense, marine organisms are also considered as potential sources of collagen. In recent years, fish-derived collagen has become a market-attractive product to meet people's demand for collagen, and has entered multiple industries such as cosmetics, food and medicine. It not only solves the dependence on mammalian collagen, but also helps alleviate environmental problems.[8]

The structure of collagen has been well characterized and can be divided into 28 types based on its structure, including fibrous (such as I, II, and III, *etc*.) and non-fibrillar (such as IV, VI, and VII, *etc*.).[9] Among them, type I collagen is the most common type of collagen and consists of two identical α1 chains and one α2 chain. The α1 chain usually consists of repeats of the Gly-XY sequence, where X and Y are proline and hydroxyproline respectively. Type I collagen is widely used in biomedicine, cosmetics and pharmaceutical industries due to its good biodegradability, biological activity and low immunogenicity.[10,11] In addition, fibrous type II and type III collagen are also the main collagen types, accounting for 80–90% of the total collagen content in mammals together with type I collagen.[12] It is worth noting that the denaturation temperature of fish-derived collagen is relatively low, mostly between 25–30 °C, which is lower than the denaturation temperature of mammalian collagen (about 40 °C), making fish collagen widely used in the fields of medicine.[1] In addition, it also exhibits a variety of physiological activities. Fish-derived collagen is applied to the cosmetics industry for its anti-aging properties, as well as its anti-bacterial capacity also make it a focus in the fields of health care and food industry.[13,14] According to recent studies, research on fish-derived collagen is mostly focused on extraction and industrial application. However, in the background of increasing demand for collagen, it is necessary to explore more possibilities for its application while seeking economical and reliable sources of collagen.

In our study, three species of fish (cod, white fish and salmon) and their collagen-containing skin-derived protein hydrolysates (CSPH) were selected as research subjects, and a static *in vitro* digestion model was established to explore the changes in collagen types and the bioaccessibility of minerals before and after *in vitro* digestion. Moreover, its safety was determined by evaluating the hazardous contaminants (heavy metals), aiming to develop potential fish collagen sources that are more nutritious to humans.

2. Materials and methods

2.1. Reagents

Ammonium carbonate ($(NH_4)_2CO_3$), sodium hydroxide (NaOH), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$) and calcium chloride dihydrate ($CaCl_2(H_2O)_2$) were purchased from Sigma-Aldrich (St Louis, MO, USA); pepsin (975 units per protein, porcine), pancreatin (8 × USP specifications, porcine) and porcine bile were also acquired from Sigma-Aldrich (St Louis, MO, USA); potassium chloride (KCl), sodium chloride (NaCl), hydrogen nitrate (HNO_3), hydrogen peroxide (H_2O_2), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$) and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Merck (Darmstadt, Germany).

2.2. Sample collection and processing

Sample 1. Unprocessed skins: skin from Atlantic cod (*Gadus morhua*), salmon (*Atlantic salmon*) and white fish (*Platichthys flesus*) were purchased from a local Norwegian company selling the fish fillets as food and frozen immediately at −20 °C in polyethylene bags and carton prior to further processing.

Sample 2. Collagen-containing skin-derived protein hydrolysates (CSPH): 3 CSPH from three fish species: cod, white fish, and salmon, were provided by Seagarden (Avaldsnes, Norway).

Skin collagen was extracted by acid hydrolysis and digested using a mixture of proteases (180 mL Papain from Enzybel and 180 mL Neutrase from AB Enzymes, 360 mL per 4000 kg skin). Prior to extraction, the fish skin was pretreated with water to remove salt and blood impurities, followed by alkali (NaOH 45%, 10 L per 4000 kg skin) treatment to remove the non–collagenous proteins. Following the extraction of collagen with acetic acid, the preparation was filtered using active carbon and then concentrated by evaporation to around 45% moisture level. The extract was then dried using spray drying technology (GEA dryer MS 850, GEA, Dusseldorf, Germany). Nutritional, mineral, and microbiological analyses for the collagenous products (supplements) were performed by ALS Laboratory, Oslo, Norway, SynLabs, Stjørdal Norway, and Slab, Stord, Norway.

2.3. Sample preparation for proteomic analyses, protein quantification and collagen type identification

Prior to protein quantification and collagen types identification, pretreatment was carried out for both unprocessed skin and CSPH, as detailed below:

(a) Unprocessed skins: An appropriate amount of the tissues was homogenized with 300 μL of RIPA buffer. Protein extraction was performed with Sample Gringing kit, Cytiva (ref. 80-6483-37) according to the manufacturer's instructions.

(b) Collagen-containing skin-derived protein hydrolysates (CSPH): Protein extraction of powder collagen (20 mg) was performed using 200 μL RIPA buffer.

2.3.1. Protein content quantification. Protein content was quantified using the Qubit protein BR assay kit (ref. A50669). Specifically, 20 μL of samples/standard were added to an Eppendorf tube and a blank group was set as a control. Then, 150 μL of BR buffer and 30 μL of BR reagent were added, vortexed for 5 seconds to mix thoroughly, and incubated at room temperature for 10 minutes. The results were read on the Qubit quantitation platform and the protein content in the sample was calculated based on the standard curve.

2.3.2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) identification of collagen types. To perform solution protein digestion prior to LC-MS/MS analysis, the procedure for raw fish and CSPH was as follows: Cysteine residues were reduced with 2 mM DTT (DL-dithiothreitol) in 50 mM

ABC at 60 °C for 20 min. Sulfhydryl groups were alkylated with 5 mM IAM (iodoacetamide) in 50 mM ABC for 30 min at room temperature in the dark. Then, excess IAM was neutralized with 10 mM DTT in 50 mM ABC, during 30 min at room temperature. Subsequently, 20 μg sample aliquotes were subjected to trypsin digestion with 400 ng (100 ng μl^{-1}) sequencing grade modified trypsin (Promega) in 50 mM ABC at 37 °C for 4 hours. The reaction was stopped with trifluoroacetic acid (TFA) at a final concentration of 0.1%.

Finally, for all samples tested, 1 μg of the peptide mixtures was diluted to 20 μL with 0.1% formic acid (FA) and loaded onto an Evotip pure tip (EvoSep) according to the manufacturer's instructions.

LC-MS/MS determination: Tandem mass spectrometry analysis (LC-MS/MS) was performed in a Tims TOF fleX mass spectrometer (Bruker). The samples loaded in the Evotip pure were eluted to an analytical column (pepsep C18 8 cm × 100 μm, 3 μm; Bruker) by the Evosep One system and resolved with the 60 SPD chromatographic method defined by the manufacturer. Then, the eluted peptides were ionized in a captive Spray with 1700 V at 200 °C and analyzed in a ddaPASEF mode with the following settings:

Tims TOF fleX: mode: custom; 1/*K*0: 0.6–1.4 V s cm^{-2}; ramp time: 100 ms; duty cycle: 100%; ramp rate: 9.42 Hz; Ms averaging: 1; auto calibration: off.

MS: scan: 100–1700 *m*/*z*; ion polarity: Positive; scan mode: PASEF MS/MS: number of PASEF ramps: 4; total cycle time: 0.53 s; charge minimun: 0; charge maximum: 5; scheduling: target intensity: 12 500, intensity threshold: 1000. Active exclusion: ON.

The system sensitivity was controlled with 20 ng of HELA digested proteins. 1950 proteins identified with the 60 SPD gradient.

Protein identification: The PASER system (Bruker) was used to search the MS and MS/MS data with the Sequest algorithm (ProLuCID) with the following parameters: SwissProt 23.03.10 database; Trypsin specificity; IAM cys-alkylation and taxonomy not restricted. All the proteins with FDR ≤1% were recorded. The Modified Protein Abundance Index (emPAI) was also calculated.

EmPAI index constitutes an established method of estimating protein abundances from peptide counts in a single LC-MS/MS experiment. EmPAI is defined as 10PAI minus one, where PAI (Protein Abundance Index) denotes the ratio of observed to observable peptides.

2.4. Mineral profile and heavy metal determination

The mineral and heavy metal profiles and contents in the samples were determined according to a previously method.[15] Specifically, the samples were placed in a microwave oven (MARS, CEM, Vertex, Spain) for mineralization. 300 mg of samples were weighed into a Telfon digester, HNO_3 (14 M, 4 mL) and H_2O_2 (30% v/v, 1 mL) were added, and the samples were digested at 800 W and 180 °C for 15 min. After digestion, the samples were taken out and cooled to room temperature, after removing nitrogen, samples were filtered, and the volume was made up with distilled water.

The inductively coupled plasma mass spectrometry (ICP-MS) was used to determine heavy metal and mineral content. The conditions were set as follows: carrier gas flow (1.07 L min^{-1}), helium (He) as reactant gas, high-frequency emission power (1550 W), Ar gas flow (15.0 L min^{-1}), nebulizer pump speed (0.10 rps), and radio frequency matching (1.80 V). For the analysis of heavy metals, ^{72}Ge, ^{103}Rh and ^{193}Ir were used as internal standard solutions to correct the fluctuation and drift of the instrument signal. A standard curve of 0–1000 μg L^{-1} was used for the quantitative analysis of As, Cd and Pb, and a standard curve of 0–100 μg L^{-1} was used for the quantitative analysis of Hg. The mineral content was analyzed using ^{45}Sc and ^{72}Ge as internal standard solution, and a 0–1000 μg L^{-1} standard curve was used for quantitative analysis. The value of the correlation coefficient was $R \geq 0.9999$ and each calibration point had an RSD value of ≤5%. At the end of the sample sequence analysis, a calibration pattern was analyzed obtaining an average between the reference and the obtained value around RSD ≤ 5%.

2.5. *In vitro* digestion protocol and bioaccessibility evaluation

A static *in vitro* digestion model was employed to simulate the adult human digestive process. This model is based on the standardized methodology proposed by INFOGEST.[16] The *in vitro* digestion model includes three stages: oral, gastric, and intestinal. Different environments of the digestive tract are simulated by preparing digestion simulation solutions with different pH values: saliva (SSF, pH = 7), gastric juice (SGF, pH = 3) and intestinal juice (SIF, pH = 7).

Oral phase: 2.5 g of sample was mixed with 2 mL SSF by shaking for 1 min. Then, 12.5 μL of $CaCl_2$ was added and the volume was made up to 5 mL with distilled water and shaken for 2 min in a 37 °C water bath.

Gastric phase: 4.5 mL SGF was added to the above mixture and vortexed for 1 minute. Then, 8 mg of pepsin (from porcine gastric mucosa, 2000 U mL^{-1}) and 2.5 mL of $CaCl_2$ were added and vortexed for 1 minute. The pH of the mixture was adjusted to 3 with NaOH (1 M) and HCl (6 M), further diluted to a final volume of 10 mL with distilled water and shaken in a water bath at 37 °C for 2 hours.

Intestinal phase: 5.5 mL SIF were added to the above mixture and shaken for 1 minute. Subsequently, 2.5 mL trypsin (from porcine pancreas, 800 U mL^{-1}), 1.25 mL porcine bile extract and 20 μL $CaCl_2$ were added and vortexed for 1 minute. Then, NaOH (1 M) and HCl (6 M) were used to adjust the pH of the mixture to 7. Finally, the volume was made up to 20 mL and shaken in a water bath at 37 °C for 2 hours. The digested samples were centrifuged at 4000 rpm for 40 minutes at 4 °C, and the supernatant was collected as the bioaccessible fraction (BF) for subsequent analysis, including protein content and digestibility, collagen type determination, mineral content, *etc*. At the same time, a blank group was prepared using deionized water instead of fish samples.

The digestibility of protein is considered to be the protein content in the bioaccessible fraction divided by the total protein content, as shown in formula (1). To calculate the bioaccessibility of the target compounds (minerals), a digestion blank is required to eliminate the interferences coming from the digestion reagents. The bioaccessibility of the target compounds (minerals) can be calculated according to the following formula (2):

$$\text{Protein digestibility}(\%) = \frac{(\text{protein content in BF})}{(\text{total protein content})} \times 100 \quad (1)$$

$$\text{Bioaccessibility}(\%) = \frac{(\text{content in BF})}{(\text{total content})} \times 100 \quad (2)$$

2.6. Statistical analysis

All experiments and measurements of characteristics were repeated at least three times. One-way ANOVA with Dunnett's multiple comparisons test was performed using Statgraphics® Centurion XV (Statpoint Technologies, Inc., USA). GraphPad Prism (GraphPad Software Company, La Jolla, CA, USA) was used for graph plotting. For each analysis, a significant level of 5% was assumed. The error bars presented on the figures correspond to the standard deviations, and letters were used to label the significance of the difference.

3. Results and discussion

3.1. Protein and digestibility

Fig. 1 shows the protein content and digestibility in raw fish and fish CSPH. As can be seen from Fig. 1(A), the protein content of the three CSPH is higher than that of raw fish. Among them, the protein content of white fish CSPH showed the greatest difference compared with raw white fish, which is about 3.2 times that of raw white fish. There were significant differences in the protein content of the three types of raw fish ($p < 0.05$), but there was no significant difference in the protein content of different collagens ($p > 0.05$). Fig. 1(B) shows the protein digestibility of raw fish and fish CSPH, that is the proportion of protein content in the bioaccessible fraction to total protein after *in vitro* simulated digestion. Among the three types of raw fish, white fish protein has the highest digestibility, about 92%, which is significantly different from the protein digestibility of cod and salmon ($p < 0.05$). Moreover, among CSPH, salmon CSPH had the highest protein digestibility, about 73%, while the other two CSPH (from cod and white fish) showed similar protein digestibility, about 45%.

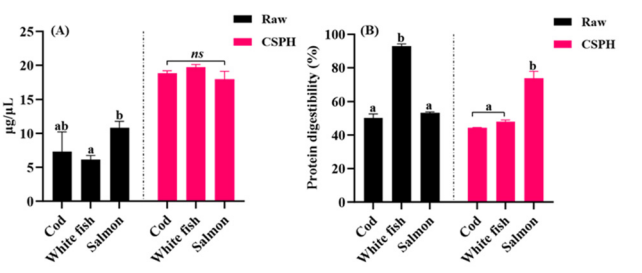

Fig. 1 Protein content and protein digestibility in raw fish and collagen-containing skin-derived protein hydrolysates (CSPH) fish collagen. (A) Protein content, (B) protein digestibility. One-way ANOVA was performed using Duncan's multiple comparison *post-hoc* test to assess statistical significance between samples. Values as mean ± standard deviation. Statistically significant differences ($p < 0.05$) between samples are indicated by lowercase letters, ns means no significant difference.

The digestibility of protein in the gastrointestinal tract determines the nutritional value of the protein. The digestion of protein is a complex process. Protein digestion begins in the oral and mainly occurs in the stomach and small intestine. Under the action of digestive enzymes, protein are hydrolyzed into peptides and amino acids, which are further absorbed by the intestinal lumen.[17] Proteins from different sources have different structures, so different digestive enzymes are needed. Proteins with complex structures are decomposed by specialized enzymes.[18] The differences in protein digestibility between different types of raw fish and fish CSPH can be attributed to the differences in protein structure.

Studies have shown that salmon protein hydrolysates consist of relatively large peptides, with an average molecular weight of 3395 Da.[19] In a related study, Dong *et al.* evaluated the *in vitro* digestion of Atlantic od nutrients subjected to ultrasound treatment, finding that protein digestibility ranged from 35–80.85%.[20] In the study by Hernández-Olivas *et al.*, the protein digestibility of various fish was evaluated using a gastrointestinal digestion model designed for the elderly.[21] The degree of hydrolysis of fish protein varies depending on the type of fish, ranging from 50% to 70%. Hake protein showed slightly lower digestibility compared to other fish proteins. This difference may be attributed to the role of lipids, which form stable lipid–protein aggregates through covalent bonds, thereby reducing protein digestibility. Additionally, lipid hydrolysis and oxidation may influence muscle membrane permeability, altering the activity of enzymes involved in protein hydrolysis.

It is known that increased protein digestibility means that more peptides and amino acids are released during digestion in the stomach and long intestine, whereas lower protein digestibility will result in higher amounts of undigested protein entering the colon and participate in the fermentation of gut microbiota, increasing the risk of intestinal disease.[22,23] Therefore, it is necessary to consider both protein content and digestibility when evaluating the nutritional value of food ingredients. Additionally, proteins from different sources may produce products with different physical properties after degradation in the digestive system. Wen *et al.*[24] compared the *in vitro* digestion products from beef, pork, chicken and fish protein and found that pork protein showed the greatest digestibility, while fish and chicken protein had more similar products after digestion. Jiang *et al.*[25] evaluated the impact of protein oxidation caused by different cooking methods on protein structure and *in vitro* digestion. The study showed that

cooking (boiling, steaming and roasting) increased the digestibility of protein and reduced the particle size of protein after intestinal digestion. Among them, the protein in the steamed ingredients had the highest digestibility, about 67%, thus steaming can be considered as an effective method to improve protein digestibility of fish. Exploring appropriate methods to improve the digestibility of fish protein could help reduce negative impacts on human health and maximize their nutritional quality.

3.2. Determination of collagen type

Tables 1 and 2 show the collagen types in raw fish and fish CSPH samples before and after *in vitro* digestion. The exponentially modified protein abundance index (emPAI), that is the number of observed peptides divided by the number of observable peptides per protein (Ishihama *et al.*, 2005), was used as the evaluation metric.[26] The raw cod and raw white fish samples contained three types of collagens, namely I, II, V and I, III, XII, respectively. Raw salmon also contained four types of collagens, namely I, II, V and XII. After *in vitro* digestion, the collagen types identified in raw cod and salmon were reduced to two types, namely type I and type II, whereas the types of collagens in raw white fish after *in vitro* digestion, were type I and III. Type I collagen is one of the most widely used in the fields of biomedicine, food, and cosmetics, *etc*. The estimated emPAI in our samples, including chain α-1 and α-2 of type I collagen in digested raw fish were higher than that in undigested raw fish, respectively: cod (0.325 *vs.* 1.247), white fish (0.280 *vs.* 1.250) and salmon (0.731 *vs.* 1.710).

Table 2 shows collagen types in fish CSPH samples before and after *in vitro* digestion, type I α-2 collagen the most abundant in all studied CSPH before digestion: cod CSPH (emPAI 0.822), white fish CSPH (emPAI 0.066) and salmon CSPH (emPAI 0.374). Furthermore, similar to raw fish, the emPAI of type I collagen in digested fish CSPH was higher than that of undigested fish CSPH. Meanwhile, emPAI is considered to be proportional to protein abundance. This study shows that digestion can increase the abundance of type I collagen, and that high which may have the potential to impart biological properties such as antioxidant capacity and low antigenicity to the sample.[27] Currently, more research is focused on the changes in the biological activity of collagen before and after digestion. For instance, Guo *et al.* investigated the multifunctional biological activities of Alaska pollock skin collagen using an *in vitro* digestion model.[28] Their findings revealed that the collagen hydrolysate produced during the gastric stage exhibited strong angiotensin-converting enzyme inhibitory activity (IC_{50}: 2.92 ± 0.22 mg mL^{-1}), which was higher than that of the hydrolysate obtained after intestinal digestion (IC_{50}: 0.49 ± 0.02 mg mL^{-1}). Similarly, Liang *et al.* explored the antioxidant capacity of tilapia skin collagen through a simulated gastrointestinal digestion process.[29] The results showed that, following *in vitro* digestion, the collagen was broken down into oligopeptides or amino acids with reduced surface hydrophobicity and exhibited enhanced antioxidant activity against DPPH free radicals and linoleic acid peroxidation. However, there are few reports on the effects of *in vitro* digestion on different types of collagens.

Table 1 Determination of collagen types in raw fish before and after *in vitro* digestion

Sample	Type of collagen	emPAI	Type I collagen (emPAI)
Raw cod	Collagen α-1(I)	0.143	0.325
	Collagen α-1(II)	0.035	
	Collagen α-1(V)	0.023	
	Collagen α-2(I)	0.182	
Digest raw cod	Collagen α-1(I)	0.398	1.247
	Collagen α-1(II)	0.030	
	Collagen α-2(I)	0.849	
Raw white fish	Collagen α-1(I)	0.219	0.280
	Collagen α-1(III)	0.039	
	Collagen α-1(XII)	0.014	
	Collagen α-2(I)	0.061	
Digest white fish	Collagen α-1(I)	0.472	1.250
	Collagen α-1(II)	0.049	
	Collagen α-1(III)	0.099	
	Collagen α-2(I)	0.778	
Raw salmon	Collagen α-1(I)	0.218	0.731
	Collagen α-1(II)	0.030	
	Collagen α-1(V)	0.049	
	Collagen α-1(XII)	0.006	
	Collagen α-2(I)	0.513	
Digest raw salmon	Collagen α-1(I)	0.549	1.710
	Collagen α-1(II)	0.049	
	Collagen α-2(I)	1.170	

Table 2 Determination of collagen types in fish collagen-containing skin-derived protein hydrolysates (CSPH) before and after *in vitro* digestion

Sample	Type of collagen	emPAI	Type I collagen (emPAI)
Cod CSPH	Collagen α-1(XV)	0.056	0.822
	Collagen α-2(I)	0.822	
Digest cod CSPH	Collagen α-1(I)	0.515	1.293
	Collagen α-2(I)	0.778	
	Collagen α-2(XI)	0.021	
White fish CSPH	Collagen α-1(III)	0.059	0.066
	Collagen α-1(XV)	0.035	
	Collagen α-2(I)	0.066	
Digest white fish CSPH	Collagen α-1(I)	0.445	1.112
	Collagen α-1(II)	0.049	
	Collagen α-2(I)	0.667	
Salmon CSPH	Collagen α-1(I)	0.081	0.455
	Collagen α-1(XV)	0.035	
	Collagen α-2(I)	0.374	
Digest salmon CSPH	Collagen α-1(I)	0.428	0.514
	Collagen α-1(XIII)	0.066	
	Collagen α-2(I)	0.086	

3.3. Heavy metal

Heavy metals are one of the most important causes of contamination of fish products by hazardous chemicals. Fig. 2 shows the content of our raw fish and fish CSPH in the heavy metals As, Pb, Cd, and Hg. Hg and Cd were not detected in the fish CSPH. As and Pb were detected in all raw fish and fish CSPH. For the same fish, As content in raw fish was significantly higher than that in fish CSPH, while the difference in

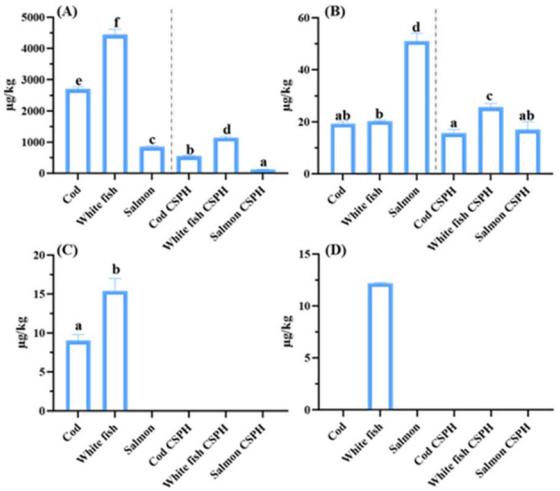

Fig. 2 Heavy metal content in raw fish and collagen-containing skin-derived protein hydrolysates (CSPH). (A) As, (B) Pb, (C) Hg, (D) Cd. None of the fish CSPH surpass the EU maximum allowed levels of heavy metals in products for human consumption: Cd (0.050 mg kg^{-1}); Hg (0.5 mg kg^{-1}); Pb (0.3 mg kg^{-1}) (Regulation (CE) 1881/2006). One-way ANOVA was performed using Duncan's multiple comparison *post-hoc* test to assess statistical significance between samples. Values as mean ± standard deviation. Statistically significant differences ($p < 0.05$) between samples are indicated by lowercase letters.

white fish was most significant, respectively 4447 μg kg^{-1} *vs.* 1149 μg kg^{-1} (raw white fish *vs.* white fish CSPH). The Pb content in raw salmon was higher than other samples, about 51 μg kg^{-1}, while in the other samples were between 17–25 μg kg^{-1}. The Pb content of all samples was below the maximum allowable limit (0.3 mg kg^{-1}). As can be seen in Fig. 2(C) and (D), Hg was only detected in raw cod and raw white fish, and Cd was detected in raw white fish, while all concentrations were well below the maximum allowable limit (Hg: 0.5 mg kg^{-1}; Cd: 0.050 mg kg^{-1}).[30] It is important to highlight that As and Pb contents in fish CSPH were lower compared to raw fish, except for Pb in white fish CSPH. None of the fish CSPH surpass the EU regulation maximum levels of the heavy metals for human consumption: Cd (0.050 mg kg^{-1}); Hg (0.5 mg kg^{-1}); Pb (0.3 mg kg^{-1}).[30] Both the raw fish and fish CSPH in this study can be considered as uncontaminated foods ingredients within the EU permitted limits. In a similar study, Karl *et al.*[31] measured the levels of Cd and Pb in cod from Norwegian, Barents and Greenland Sea. Likewise, the levels of Cd and Pb in all samples did not exceed permissible levels set by EU regulations. For white fish, there are studies in literature that focus on the accumulation of As.[32,33] The liver and gills of white fish are the main sites where As accumulates. Although there is accumulation in bones and skin, the content is lower. Heavy metals are non-degradable and can persist in the environment, remaining in fish tissues for long periods of time. Considering that fish is an important source of protein and polyunsaturated fatty acids for humans, the issue of heavy metal contamination in fish warrants attention.[34]

3.4. Mineral content

Macro and trace minerals play important roles related to human health. The lack of minerals can lead to immune dysfunction, developmental delay, and osteoporosis. Sometimes dietary minerals may not be enough to meet the body's needs, so a supplement may be necessary. Fig. 3 shows the mineral content in raw fish and fish CSPH. It can be seen that in raw fish, the contents of P, K, Ca and Mg were higher, while the content of Zn was the lowest. There were higher contents of Mg, P, K, Zn and Cu in white fish compared to the cod and salmon. The contents of Fe and Ca were higher in salmon (Fig. 3(C) and (F)), which were 12.90 mg kg^{-1} and 3948.00 mg kg^{-1}, respectively. There was none found any Zn in the analyzed CSPH. The content of cod CSPH in Fe (5.07 mg kg^{-1}) was higher than that in white fish and salmon CSPH (Fig. 3(C)). Moreover, the content of salmon CSPH (1770.00 μg kg^{-1}) in Cu was higher than that of cod and white fish CSPH (Fig. 3(G)). Overall, the mineral content of fish CSPH was lower than that of raw fish. Relatively high levels of minerals Mg, K and Ca were analyzed in all the fish CSPH studied. Previous research has shown that fish is a great source of Ca and P, which are necessary nutrients for maintaining bone development.[35] Reasonable supplementation of Ca and P can prevent rickets, osteoporosis, *etc*. In addition, Mg is also involved in the body's bone metabolism and development. Fe has an important impact on the body's hematopoietic function. Lack of Zn can cause growth retardation, cognitive impairment and immune system diseases.[36–38] However, the mineral content in fish is closely related to its species, growth environment, feed, *etc*. Fish with high mineral content can not only be used as dietary mineral supplements, but also promote the development of the fish processing industry and reduce fish waste.

3.5. Bioaccessibility of minerals

The bioaccessibility of minerals in raw fish and fish CSPH was assessed by the INFOGEST *in vitro* static digestion model. Fig. 4 shows the bioaccessibility of minerals in raw fish and fish CSPH. It can be seen that the bioaccessibility of Ca and Cu was higher in cod, 31.89% and 26.26%, respectively, followed by K, 15.22%. The absorption rates of Fe and Zn were approximately 0, indicating that they cannot be absorbed and utilized by the upper gastrointestinal tract (Fig. 4(A)). Similar to cod, the bioaccessibility of Fe and Zn in white fish was 0, while Ca and Cu showed higher bioaccessibility than other minerals, 14.08% and 17.93%, respectively (Fig. 4(B)). For salmon, Cu presented the highest bioaccessibility, which was 33.63%. The bioaccessibility of Mg, P and K did not show significant differences ($p > 0.05$), while the bioaccessibilities of Ca, Fe and Zn were all around 0% (Fig. 4(C)). Fig. 4(D) and (E) shows the bioaccessibility of minerals in the fish CSPH. It can be found that in cod and salmon CSPH, just Mg, K and Cu can be digested and absorbed, while in white fish CSPH, just Mg and K can be digested. However, their bioaccessibility levels were similar to those observed in raw fish samples. Currently, there are few studies about the bioaccessibility of minerals in

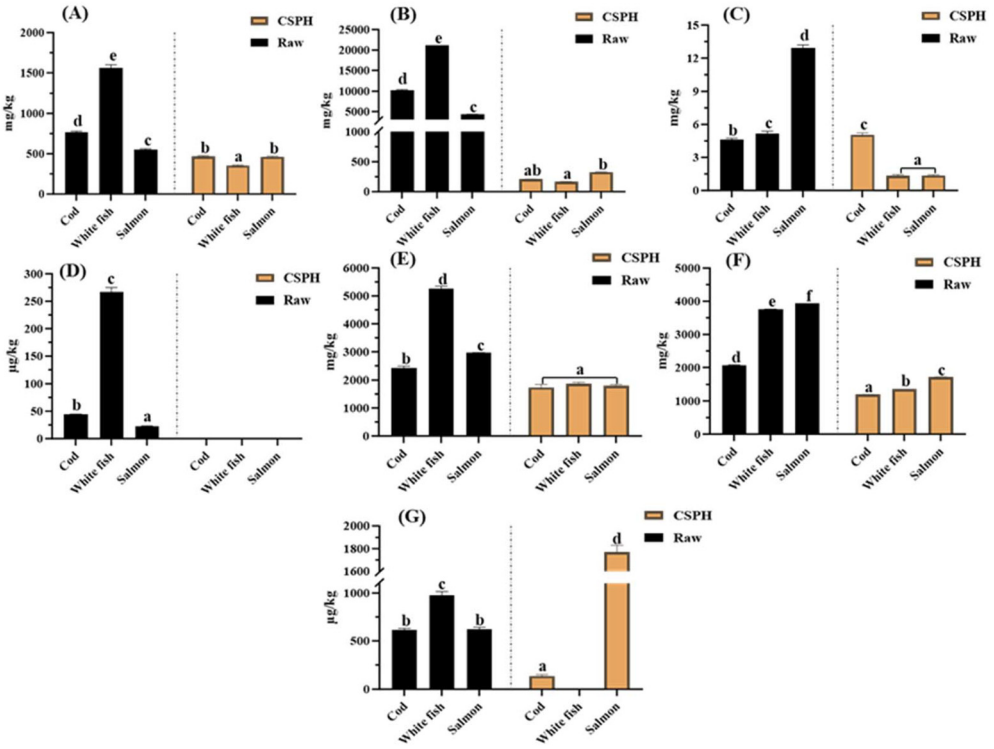

Fig. 3 Mineral content in raw fish and collagen-containing skin-derived protein hydrolysates (CSPH). (A) Mg, (B) P, (C) Fe, (D) Zn, (E) K, (F) Ca, (G) Cu. One-way ANOVA was performed using Duncan's multiple comparison *post-hoc* test to assess statistical significance between samples. Values as mean ± standard deviation. Statistically significant differences ($p < 0.05$) between samples are indicated by lowercase letters.

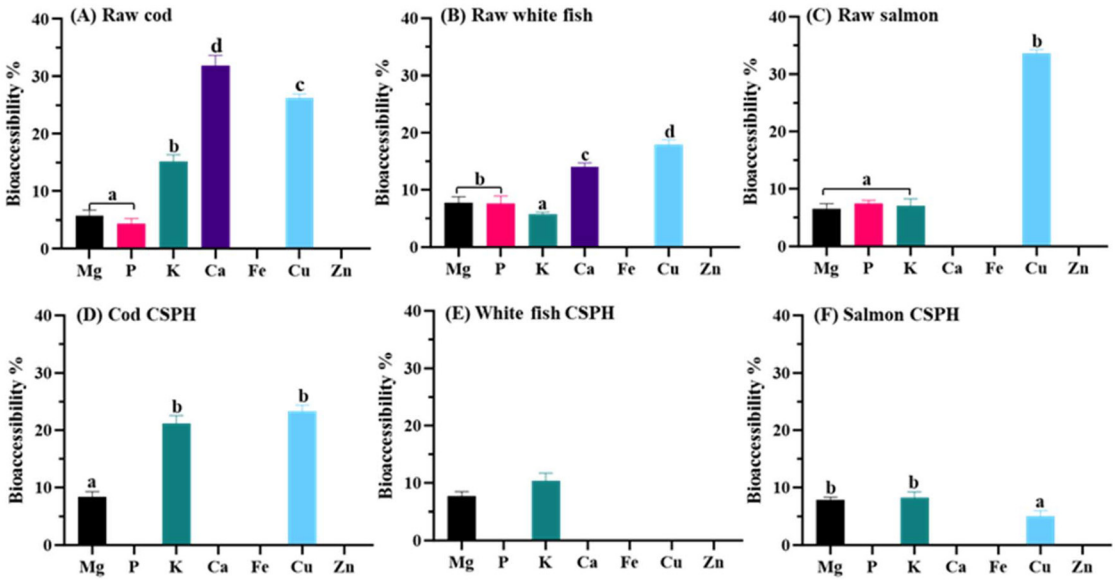

Fig. 4 Bioaccessibility evaluation of minerals in raw fish and collagen-containing skin-derived protein hydrolysates (CSPH). (A) Raw cod, (B) Raw white fish, (C) Raw salmon, (D) Cod CSPH, (E) White fish CSPH, (F) Salmon CSPH. One-way ANOVA was performed using Duncan's multiple comparison *post-hoc* test to assess statistical significance between samples. Values as mean ± standard deviation. Statistically significant differences ($p < 0.05$) between samples are indicated by lowercase letters.

fish-derived products. Ever *et al.*[21] evaluated the bioaccessibility of calcium in various fish, ranging from 20–94%. Among them, the protein content may have a negative impact on the bioaccessibility of calcium, and the salting-out effect produced by free amino acids will reduce the solubility of calcium, thereby affecting the bioaccessibility. De la Fuente *et al.*[39]

explored the bioaccessibility of minerals in fish (salmon and mackerel backbones and heads) protein hydrolysates. The highest bioaccessibility of minerals was found in salmon and mackerel head hydrolysates for Fe, about 100%, followed by Se in salmon backbone hydrolysates, close to 95%. Jensen *et al.*[40] explored the bioaccessibility of minerals in cod and salmon bones, the results showed that the bioaccessibility of Mg in bone meal was up to 74%, the bioaccessibility of P was up to 11%, and the bioaccessibility of Ca was up to 14%. Thus, bone meals have potential as a food supplement and feed. The bioaccessibility of minerals is influenced by various factors such as species, processing methods, and living environment. When considering fish as a mineral supplement, it is important to evaluate not only the mineral content but also their bioaccessibility. A holistic approach that considers the absorption efficiency of various nutrients, including minerals, and applying specific methods to enhance nutrient absorption can help maximize resource utilization efficiently.

4. Conclusions

From the results of this study, it can be concluded that raw white fish and salmon CSPH showed high protein digestibility and can be considered as protein supplements. Meanwhile, *in vitro* digestion process increases the emPAI of type I collagen in raw fish and fish CSPH, which is one of the most widely used collagens in biomedicine and food industries. In addition, the possibility of fish as a mineral supplement has also been explored, and they have potential in supplementing Mg, P, K, Ca, *etc*. Among these minerals, Ca and Cu showed higher bioaccessibility in raw skin cod and Cu in raw skin salmon. In CSPH, Mg and K can be digested and absorbed. As can be seen, both raw fish and fish CSPH showed potential as human collagen and mineral supplements. Overall, raw fish and fish CSPH have the potential to be considered as protein, collagen and mineral supplements for the body. The heavy metal content below the regulatory maximum level further provides safety assurance for their application in food, pharmaceutical and cosmetic industries. This study can provide new suggestions and directions for the development of marine fish resources.

Author contributions

Conceptualization: Francisco J. Barba, Zouhir el Marsni and Katerina Kousoulaki; methodology: Min Wang, Noelia Pallarés and Zouhir el Marsni; software: Min Wang and Noelia Pallarés; validation: Zouhir el Marsni and Francisco J. Barba; formal analysis: Min Wang; investigation: Francisco J. Barba and Katerina Kousoulaki; resources: Zouhir el Marsni; Francisco J. Barba and Katerina Kousoulaki; writing – original draft preparation: Min Wang and Yixuan Liu; writing – review and editing: Noelia Pallarés, Katerina Kousoulaki and Francisco J. Barba. All authors have read and agreed to the published version of the manuscript.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Conflicts of interest

The authors report no conflict of interest.

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