applied sciences

MDPI

Article

Skin Byproducts of *Reinhardtius hippoglossoides* (Greenland Halibut) as Ecosustainable Source of Marine Collagen

Eva Martins [1,2,*], Rita Fernandes [1,2], Ana L. Alves [1,2], Rita O. Sousa [1,2], Rui L. Reis [1,2] and Tiago H. Silva [1,2,*]

[1] 3B's Research Group, I3Bs—Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Avepark–Parque de Ciência e Tecnologia, Barco, 4805-017 Guimarães, Portugal
[2] ICVS/3B's—PT Government Associate Laboratory, Braga, 4710-057 Guimarães, Portugal
* Correspondence: eva.biotec@gmail.com (E.M.); tiago.silva@i3bs.uminho.pt (T.H.S.)

Abstract: Collagen is a ubiquitous protein present in the extracellular matrix of all major metazoan animals, with approximately 28 different human collagen types described in the literature, each with unique physicochemical properties. Collagens found broad application in the cosmeceutical, pharmaceutical, and biomedical fields and can be isolated from environmentally sustainable sources such as marine byproducts, which are abundant in the fish processing industry and are highly appealing low-cost sources. In this study, marine collagen was isolated from the skins of Greenland halibut (*Reinhardtius hippoglossoides*), an unexplored byproduct from fish processing plants, using three different collagen extraction methods, due to the use of distinct salting-out methods using a solution of 2.6 M NaCl + 0.05 M Tris-HCl pH = 7.5, (method I); a combination of 0.7 M NaCl followed by a solution of 2.3 M NaCl + 0.05 M Tris-HCl pH = 7.5 (method II); and one method using only 0.9 M NaCl (method III), yielding *COLRp_I*, *COLRp_II*, and *COLRp_III* collagens. These extracted type I collagens were produced with a yield of around 2 and 4% and characterized regarding the physicochemical properties, considering possible biotechnological applications. This work evidenced that the typical triple helix structure conformation was preserved in all extraction methods, but influenced the thermal behavior, intrinsic morphology, and moisture capacity of the collagens, with interest for biotechnological application, as the incorporation as an ingredient in cosmetic formulation. Furthermore, the use of collagen isolated from skin byproducts represents a high economic value with decreasing collagen cost for industrial purposes and is also an environmentally sustainable source for industrial uses.

Keywords: marine collagens; fish skins; byproduct valorization; active ingredient; circular economy

Citation: Martins, E.; Fernandes, R.; Alves, A.L.; Sousa, R.O.; Reis, R.L.; Silva, T.H. Skin Byproducts of *Reinhardtius hippoglossoides* (Greenland Halibut) as Ecosustainable Source of Marine Collagen. *Appl. Sci.* **2022**, *12*, 11282. https://doi.org/10.3390/app122111282

Academic Editor: Chiara Cavaliere

Received: 10 October 2022
Accepted: 5 November 2022
Published: 7 November 2022

1. Introduction

Collagen is the most abundant extracellular matrix protein present in mammals, which typically has a characteristic triple-helix structure that easily enables its identification [1,2]. This specific protein structure is stabilized by intra- and inter-chain hydrogen bonding responsible for the strong and rigid nature of most tissues, constituted by continuous repetition of the glycine-X-Y sequence, where X and Y are frequently proline and hydroxyproline amino acids [3,4]. Hitherto, collagen has been described in humans in 28 different types, with a variation in molecular arrangement and functional properties, and accounts for 25–35% of the total protein mass, collagen being the major constituent of the dermal skin layer, connective tissues, cartilage, tendons, and bone [5–7].

Furthermore, each type of collagen has unique physicochemical properties, molecular weight, sequence, and amino acid composition, which influence the quality of the collagen [8–11]. The main conventional sources of collagen for industrial use are bovine

and porcine origin. Nevertheless, some religious constraints have been raised on its use and other sustainable alternatives of collagen have been appearing, including the ones resulting from the better utilization of marine byproducts as a low-cost effective form of collagen [12]. Moreover, marine collagen is associated with low immunogenicity and lack of risk of transmission of diseases or zoonoses, fewer regulatory issues, and a high potential for industrial applications [13–15]. The advances in the know-how of processes and technologies have been enabling the isolation of high-added-value products from marine byproducts such as valuable proteins and biomolecules like collagen, gelatins, and bioactive peptides [16].

Nowadays, collagen from marine origin has been extracted from different raw materials such as the fish skins of starry triggerfish (*Abalistes stellatus*) [17], Atlantic codfish (*Gadus morhua*), and salmon (*Salmo salar*) [18], the swim bladders of giant croaker (*Nibea japonica*) [19], Atlantic cod (*Gadus Morhua*) [20], and yellowfin tuna (*Thunnus albacares*) [21], the arm of octopus (*Callistoctopus arakawai*) [22], shark (*Prionace glauca*) and ray (*Zeachara chilensis* and *Bathyraja brachyurops)* cartilage [23], from the bell and oral arms of jellyfish (*Acromitus hardenbergi Stiasny*) [24], and fish scales of sardines [25], to name a few. The physicochemical characteristics of collagen vary between marine species, habitats, age, and extraction methods [26]. Furthermore, collagens are frequently solubilized using an acetic acid treatment that is followed by polypeptide protonation [27,28].

Indeed, collagen has exceptional properties such as biocompatibility [29,30], biodegradability, low toxicity, and low antigenicity, and is a key element in wound healing [19,31], being of utmost importance to biomedical, pharmaceutical, and cosmetics industries [12,32–37]. In the skincare and cosmetic areas, marine collagen can be used for wound dressing [38–40], for dermal moisturizing applications as a promising ingredient in cosmetic formulations due to a good capacity to retain water [18], for an antioxidant ingredient to provide protection against UV radiation due to stimulating collagen synthesis, minimize the lipid skin degradation and photo-aging effect [41–43], or as skin-hydrating and firming agents principally following several applications [44–46]. Recently, a drink containing fish collagen hydrolysates showed an improvement in skin parameters such as hydration, wrinkles, pores, and collagen content in human skin [47], being a new application of collagen and consequently adding value to marine byproducts.

This work used Greenland halibut *Reinhardtius hippoglossoides* (Walbaum, 1793) skins as a byproduct for the extraction of valuable collagen protein and further characterization. To our knowledge, the extraction of Greenland halibut collagen and, so far, its valorization has never been reported. This protein was isolated using three salting-out methods and the physical and chemical properties of the extracted protein were surveyed. This fish species was selected for being a commercially valuable species widely distributed in vast areas of North Atlantic, Pacific, and Arctic waters. Furthermore, byproducts from this species are extremely easy to obtain from a local fish processing industry, due to their commercial value regarding use in food chain systems but are currently still mostly directed to low-value applications. The main goal of this study is to demonstrate that skin byproducts from Greenland halibut fish could be a sustainable source for the extraction of high-value protein, namely collagen, which can be an alternative source of collagen for industrial applications mainly in the cosmetic field. Interestingly, the properties of collagen biopolymer can be tunable by slightly changing steps in the extraction methodology.

2. Materials and Methods

2.1. Marine Byproducts

Greenland halibut (*Reinhardtius hippoglossoides*) skins, kindly offered by Fundación CETMAR (Vigo, Spain), were obtained frozen from a fish processing company and kept at −20 °C in our laboratory facilities until the collagen extraction procedure.

2.2. Collagen Extraction from Fish Skins

Collagen is a thermosensitive protein, and, for this reason, the experimental extraction was performed at 4 °C to avoid its temperature denaturation (Figure 1). Firstly, skins were washed with abundant distilled water and cut into small pieces (around 1 cm^2) for the removal of any exogenous material. Following that, skins were pre-treated with a 10% ethanol solution (1:10 w/v), changed twice daily for 48 h to remove fat, followed by a 0.1 M NaOH (1:10 w/v) solution changed twice daily for three days, under stirring, to remove non-collagenous proteins [18,48,49]. The remaining biomass was washed with cold distilled water until the pH reached neutral and further extracted with 0.5 M acetic acid (1:20 w/v) for 4 days, followed by centrifugation at 20,000× g for 30 min at 4 °C. The supernatants were then divided into three equal portions, and salting-out overnight with equal volume of 2.6 M NaCl + 0.05 M Tris-HCl pH = 7.5 (method I); or by adding NaCl until obtaining a concentration of 0.7 M, followed by equal volume of a solution of 2.3 M NaCl + 0.05 M Tris-HCl pH = 7.5 (method II); or by adding NaCl until obtaining a concentration of 0.9 M (method III), corresponding to *COLRp_I*, *COLRp_II*, and *COLRp_III*, respectively. Each collagen precipitate was collected as a pellet by centrifugation, at 20,000× g for 30 min at 4 °C and resuspended in 0.5 M AcOH (acetic acid) solution. The excess of salts was removed by dialysis using cellulose membrane with a cut-off of 14 KDa, against 0.1 M AcOH for 2 days, 0.02 M AcOH for an extra 2 days, and then against distilled water until pH = 7. Finally, the solutions were separated, identified according to the salting-out method, stocked at −80 °C, freeze-dried (0.060 mbar vac., −65 °C temp.), and stored at room temperature until further characterization.

2.3. Characterization of Collagen Protein

The collagen extraction yield was calculated using the following Equation (1):

$$\text{Yield of collagen } (\%) = \frac{\text{Weight of collagen after freeze} - \text{drying (g)}}{\text{Weight of initial wet skins (g)}} \times 100 \quad (1)$$

The physicochemical properties of the *COLRp_I*, *COLRp_II*, and *COLRp_III* collagens were investigated by SDS-PAGE electrophoresis to determine the purity of the extracted marine collagens, amino acid analysis to access the content of amino acids, Fourier transform infrared (FTIR) spectroscopy for visualization of amino collagen bonds, circular dichroism (CD) to evaluate the secondary collagen structure, whereas thermal stability of the protein was assessed by rheology and micro-differential scanning calorimetry (micro-DSC). Additionally, the morphology and chemical composition of the collagens were examined by SEM and EDS analyses and moisture regain properties of the collagens were analyzed.

2.3.1. SDS-PAGE gel Electrophoresis

The purity of collagen was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The gel was prepared using the SDS kit (Sigma-Aldrich®, St. Louis, MO, USA) prepared with 3% stacking and 7.5% resolving gel cast on the Mini Protean® 3 Cell System (Bio-Rad, Hercules, CA, USA). Each freeze-dried collagen was dissolved at 1.5 mg/mL in 0.5 M acetic acid. Subsequently, samples were mixed with loading buffer reagent (Bio-Rad) at a 1:1 (v/v) ratio and were denatured at 95 °C for 15 min. For each well, 20 µL of each collagen and 4 µL of PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific™, Vilnius, LT, USA) were loaded into the gel and ran at 90 V for 45 min. After electrophoresis, the gel was stained with Coomassie Blue R 250 (Bio-Rad, Hercules, CA, USA) for 30 min, followed by destaining solution I (32% methanol, 5.6% acetic acid, and 62.4 % deionized water) for 30 min and destaining solution II (5% methanol, 7% acetic acid and 88% deionized water) until protein bands were adequately visualized.

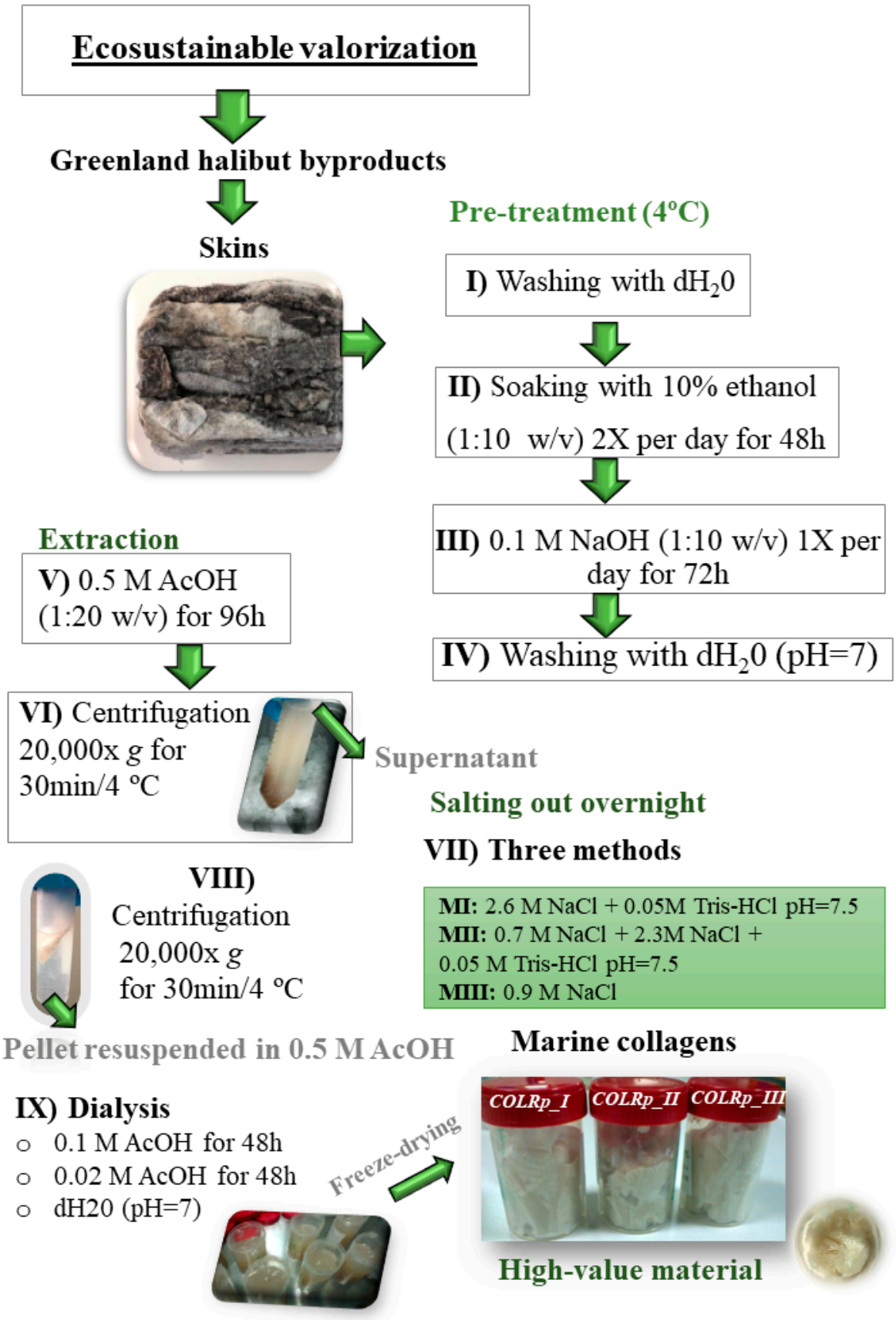


Figure 1. Schematic representation of extraction of marine collagens using the skin byproducts from *R. hippoglossoides* fish as valuable raw material.

2.3.2. Circular Dichroism

The secondary structure of collagens was analyzed using the Jasco J-1500 circular dichroism (CD) spectrometer (JASCO Corp., Tokyo, Japan) at 4 °C. CD spectra were acquired by continuous wavelength scans (in triplicate) at a scanning speed rate of 50 nm/min. Measurements were obtained between 180–240 nm wavelengths under a nitrogen atmosphere, using a cylindrical quartz cuvette and an optical path length of 2 mm. Spectra were performed at a collagen concentration of 0.1 mg/mL in 0.05 M acetic acid solution.

2.3.3. FTIR Measurement

The IRPrestige 21 spectrometer (Shimadzu) was used to obtain FTIR spectra of freeze-dried collagens in potassium bromide (KBr) pellets, in the range from 4000 to 800 cm^{-1}, data acquisition rate of 2 cm^{-1} per point, and an average of 32 scans.

2.3.4. Rheology

Rheological properties of the collagens were assessed on a Kinexus Pro + rheometer (Malvern Instruments Ltd., Worcestershire, UK) using 1% (w/v) collagen in 0.02 M acetic acid solutions, acquired with rSpace software. The measuring system was composed of an upper stainless-steel parallel plate with 20 mm diameter at a 1 mm gap. Collagen solutions were heated from 15 to 45 °C at a rate of 0.5 °C/min. The rheological parameters as storage modulus (G′), loss modulus (G″), complex viscosity (η*), and the loss tangent (tan δ = G″/G′) were measured. Data represented mean values from three replicates.

2.3.5. Amino Acid Analysis

Amino acid analysis was performed at the Center for Biological Research of the Spanish National Research Council (CIB-CSIC, Madrid, Spain). Briefly, 5 mg of each collagen was hydrolyzed in 6N HCl (110 °C, 20 h), using a Biochrom 30 (UK) amino acid analyzer. The hydrolysate was vaporized and the residue was dissolved in a sodium citrate buffer pH 2.2. Subsequently, samples were separated using a cation column in the amino acid analyzer at 570 nm.

The percentage of marine collagen hydroxylation was calculated following Equation (2), whereas pyrrolidine amino acid content was the sum of hydroxyproline (OHPro) and proline (Pro) amino acids:

$$\text{Hydroxylation } (\%) = \frac{\text{OHPro content}}{\text{pyrrolidine amino acid content}} \times 100 \quad (2)$$

2.3.6. Micro-Differential Scanning Calorimetry

Micro-DSC analysis was assessed to determine the denaturation temperature of the collagen proteins. Micro-DSC collagen spectra were performed using 700 µL of each sample in the stainless steel cells of the Micro DSC III microcalorimeter (Setaram), performed at the Faculty of Biotechnology, Universidade Católica Portuguesa (Porto, Portugal). Collagen solutions were dissolved at 5 mg/mL in 0.5 M acetic acid at 4 °C and the solvent was used as a reference cell. Measurements were performed at a 1 K/min scan rate from 5 to 80 °C and nitrogen gas (99.9%) was used as a purge gas to avoid humidity condensation on the circulating coils of the microcalorimeter.

2.3.7. Moisture Regain

Moisture regains of collagens were evaluated by the variation of the weight after incubation of freeze-dried collagens in an atmosphere system with controlled humidity. After 72 h of drying in the oven, collagens were weighted (T0) and transferred to a closed atmosphere system (desiccator) at 57% constant relative humidity, at room temperature.

Collagens were reweighed after 48 h, and humidity regains were calculated and expressed as percentages of dry weight using Equation (3);

$$\text{Moisture regain } (\%) = \frac{\text{Weight of water in the collagen}}{\text{Dry weight of collagen}} \times 100 \quad (3)$$

2.3.8. SEM and EDS Analyses

The morphology of the marine collagens was visualized using a scanning electron microscope (JSM-6010 LV, JEOL, Tokyo, Japan), using beam energy of 10 keV at 250×, 500×, and 1000× magnifications. Samples were sputter-coated with an electrically conducting layer of gold (Au) using the EM Leica ACE600 sputter coater. Energy-dispersive X-ray spectroscopy (EDS) was performed to evaluate the chemical composition of freeze-dried marine collagens using the INCAx-Act PentaFET Precision Oxford Instruments.

2.3.9. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

Collagens were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) to determine the presence of heavy metals of the fifteen elements (Ag, Al, As, Au, Ca, Cd, Fe, K, Mg, Mn, Na, P, Pb, Sr, and Zn). 1 mg/mL of each hydrogel was dissolved in 2% H_2SO_4 and after the complete dissolution was filtered using 0.45 µm. Each sample solution was prepared in 5% HNO_3 (ratio 1:9) for ICP determination. The ICP-OES analysis was performed using an ICP JY 2000-2 spectrometer (HORIBA Jobin Yvon, Stow, MA, USA) with the absorption wavelengths of λ = 328.07; λ = 309.27; λ = 197.19; λ = 242.79; λ = 317.933; λ = 228.80; λ = 259.94; λ = 766.49; λ = 279.55; λ = 257.61; λ = 588.99; λ = 213.62; λ = 220.35; λ = 407.77; λ = 206.20 for Ag, Al, As, Au, Ca, Cd, Fe, K, Mg, Mn, Na, P, Pb, Sr, and Zn, respectively.

The concentrations were calculated from standard calibration curves prepared using standard solutions from 0 to 10 ppm. All ICP standard solutions (reagent grade 1000 mg/L) from (Merck, Darmstadt, Germany) were used for preparation of calibration standards.

2.4. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software. The performed one-way ANOVA (analysis of variance), Student's tests, and the Tukey post hoc test were conducted to determine the significant differences among the conditions. All analyses were conducted using triplicates (n = 3), with results being expressed as mean values and standard deviations. The level of significance was set at * $p < 0.05$.

3. Results and Discussion

R. hippoglossoides fish skin byproducts were used as sources for the production of collagen by using acetic acid extraction and three different salting-out approaches. The three collagen extracts (*COLRp_I*, *COLRp_II*, and *COLRp_III*) were characterized for understanding the physicochemical properties and uniqueness of each method.

3.1. Yield and SDS-PAGE Pattern

Acid-solubilized collagens were produced with a yield of 3.8%, 2.5%, and 1.6%, corresponding to *COLRp_I*, *COLRp_II*, and *COLRp_III* (Figure 2A), showing yield differences related to the methodology used. The highest collagen yield was obtained with method I, which showed a similar yield to the one obtained with other marine species, namely *Diodon holocanthus* balloon fish (4%) [46] and *Paracentrotus lividus* sea urchin (4.93%) [50]. The purity of extracted collagen was evaluated by SDS-PAGE, showing a similar profile between the isolated fish collagen and the bovine collagen standard used as a reference for type I collagen. Figure 2B displayed the patterns of the isolated collagens from the *R. hippoglossoides*, confirming the presence of two alpha (α1 e α2) chain subunits with protein molecular weight nearby 130 kDa, a characteristic feature of type I collagen, and also observed a β dimer band with a protein weight of around 250 kDa. Typically, the

α1 chain had the highest intensity band, compatible with the ratio of 2 α1 chains for 1 α2 chain in each type I collagen molecule. However, *COLRp_II* and *COLRp_III* suggest having a decrease in the prevalence of β band compared to the *COLRp_I* collagen with all fish collagens showing a more intense β band than bovine collagen, associated with the presence of intermolecular crosslinking. In the literature, the fish collagen (codfish) had a similar collagen pattern with investigational collagens (*R. hippoglossoides*) and weight of α (α1 e α2) and β chains [20], a phenomenon also identified in other marine species as barramundi (*Lates calcarifer*), tilapia (*Oreochromis niloticus*) [51,52], striped catfish (*Pangasianodon hypophtalamus*) [53], and small-spotted catshark (*Scyliorhinus canicula*) [48].

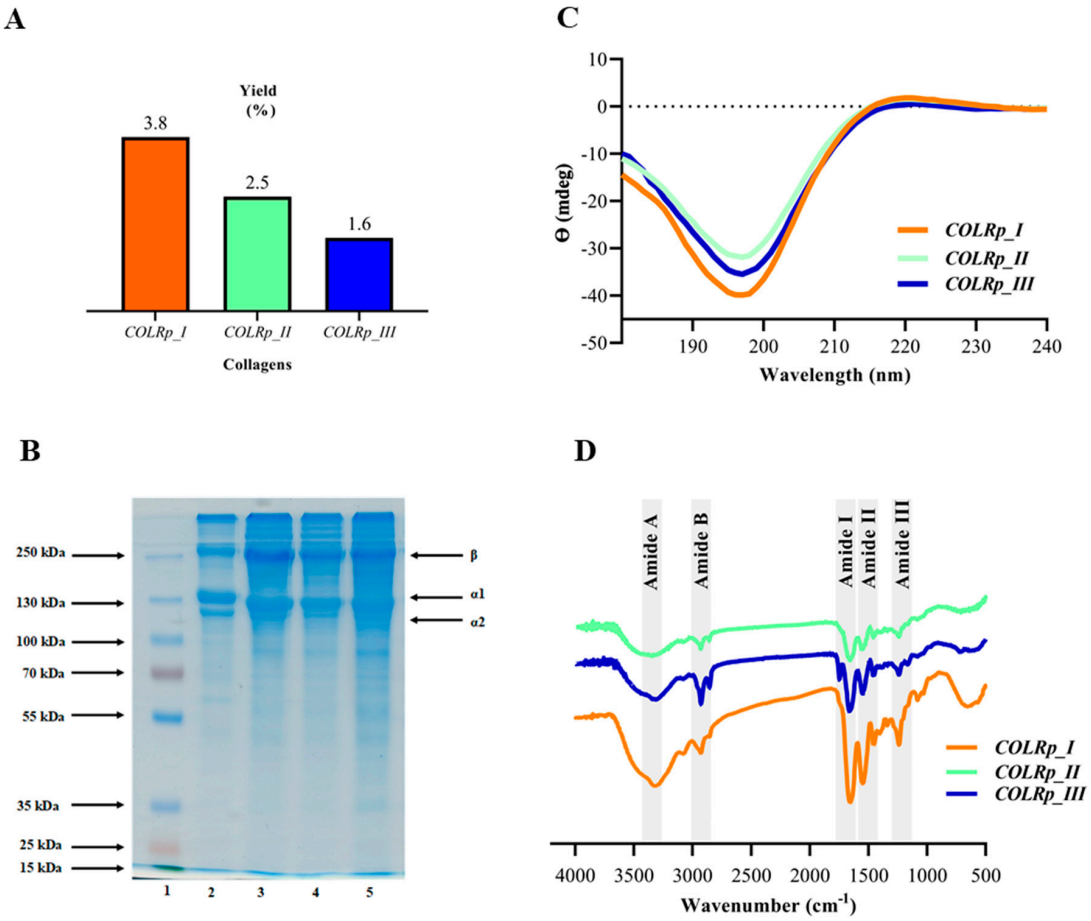


Figure 2. Results from the characterization of *R. hippoglossoides* fish collagens isolated from skin byproducts using three methods. (**A**) Extraction yield. (**B**) SDS-PAGE gel patterns, whereas number 1 represents protein molecular weight marker; 2 denotes standard collagen from bovine skins (*COL_Bovine*); 3, 4, and 5 represent *COLRp_I*, *COLRp_II,* and *COLRp_III* collagen profiles, respectively. (**C**) Circular dichroism. (**D**) FTIR spectra.

3.2. Circular Dichroism

Circular dichroism (CD) is a spectroscopic technique used to survey the stability and folding properties of a protein under certain conditions, namely in situations of protein denaturation (such as temperature, chemical compounds, and changes in pH). CD spectra evaluated the conformation of the secondary structure through the molecular arrangement of the three polypeptide chains in three extracted marine collagens at 4 °C, as visible in Figure 2C. The spectra of *COLRp_I*, *COLRp_II*, and *COLRp_III* showed a similar minimum negative peak at 197 nm, indicating that the protein molecular exhibits a random coils conformation or the disarrangement of collagen polypeptide chains. These results agree with other studies [54,55]. Besides that, collagens have a positive peak characteristic of

triple helix conformation in the range of 216 to 232 nm (*COLRp_I* and *COLRp_II*) and 218 to 225 nm (*COLRp_III*) with a maximum peak at about 220 nm. This CD profile demonstrated that the native structure and biochemical properties of the extracted collagen protein are well-preserved independently of the extraction methods tested.

3.3. FTIR Measurement

The secondary structure and functional groups of the *R. hippoglossoides* collagens showed the five main amide bonds (amides A, B, I, II, and III), visualized in Figure 2D. Regarding amide A, the absorption band is an N-H vibrational coupling group present typically in the intermolecular hydrogen bonds between 3000 and 3500 cm^{-1} [56]. In the FTIR spectra it was observed that the amide A peaked at 3319, 3338, and 3315 cm^{-1} for *COLRp_I*, *COLRp_II,* and *COLRp_III,* respectively. These values were in the range described to other marine species such as sturgeon fish [57] or sea cucumber [58]. Concerning amide B, corresponding to the asymmetric and symmetric stretching of CH_2 groups, it typically presents peaks between 2933 and 2869 cm^{-1} [56], and were here detected at 2927 cm^{-1} for *COLRp_I* and 2926 cm^{-1} in both other collagens (*COLRp_II* and *COLRp_III*). Amide I of three collagens displayed the C=O stretching of the carboxyl groups at 1654 cm^{-1}, 1656 $cm^{-1,}$ and 1660 cm^{-1} for *COLRp_I*, *COLRp_II,* and *COLRp_III,* respectively. According to the literature, the frequencies of amide I bands are in the range of 1600 and 1700 cm^{-1} [59]. Furthermore, amide I may participate in the formation of hydrogen bonds between the carbonyl group (C=O) and the N–H group, which is responsible for the triple helical structure [57,60]. The absorption characteristic of amide II was relative to NH bending vibration coupled with CN stretching between 1500 and 1600 cm^{-1}. The absorption characteristic of amide II was 1548 cm^{-1} for *COLRp_I* and *COLRp_III*, and 1544 cm^{-1} for *COLRp_II,* corresponding to the NH bending of collagen. Regarding the band of amide III, it was observed at 1238 cm^{-1}, for *COLRp_I*, and *COLRp_III*, and 1240 cm^{-1} for *COLRp_II* and 1238 cm^{-1}, being related to C-N stretching vibrations and N-H bending, indicating the existence of a helical structure [58].

The qualitative spectrum of *R. hippoglossoides* collagen structure provided by FTIR analysis showed peaks of amides and their resemblance to type I collagen. Additionally, FTIR analysis could contribute to understanding the conformation of the collagen molecule. The ratio of the intensity of amide III and the observed signal at 1450 cm^{-1} can give information about the presence of triple helix conformation on the structure of collagen molecules when the ratio is near 1 [61]. In this study, *COLRp_I*, *COLRp_II,* and *COLRp_III* collagens showed 1.17, 1.16, and 1.17 ratios, respectively, confirming the preservation of triple helix conformation in all extracted collagens, being in consonance with CD results. Moreover, characteristic peaks of proteins are N-H bending and C=O, N-H, and C-N stretching, which were also identified in the marine collagens.

3.4. Rheology

Rheology studies evaluate how materials deform or flow in response to the forces and or stresses applied. The variations in temperature ranges influence the structure of the collagen molecule and consequently affect its rheological behavior. The effect of temperature on each collagen was determined by measuring the complex viscosity (η*) and loss angle (tan δ) in the range of 15–45 °C. The temperature of the melting (Tm) or gelation point is considered when the decrease in η* reached 50% of the initial value (M1) or another way to measure the temperature corresponding to the maximum peak value of tan δ achieved (M2). All extracted collagens had a Tm of 29.9 °C using M1 or a distinct temperature melting of 22.3 °C, 19.5 °C, and 16.9 °C (Figure 3A) using M2 determination, for *COLRp_I*, *COLRp_II,* and *COLRp_III,* respectively, likely due to a lower content of amino acids as hydroxyproline in *COLRp_II* and *COLRp_III* compared to the *COLRp_I*.

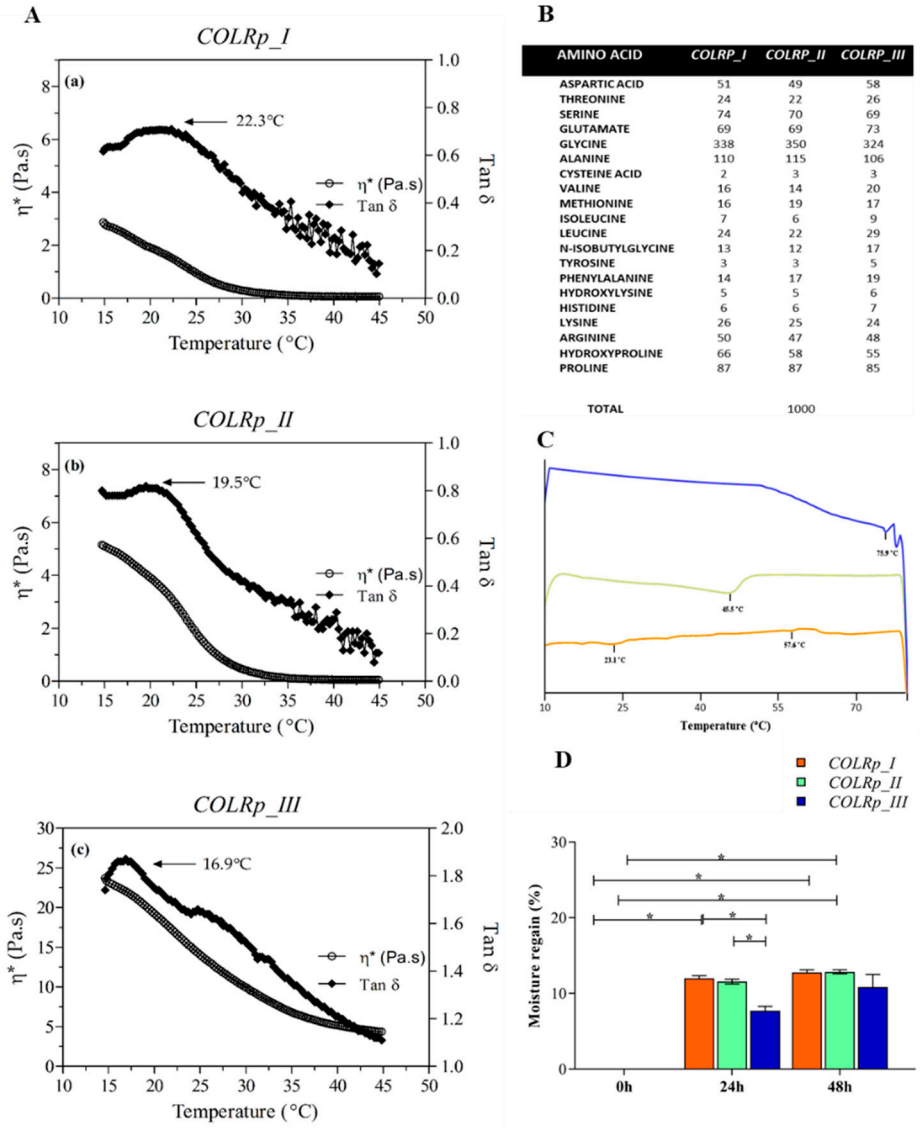


Figure 3. Physicochemical properties of Greenland halibut skin collagens. (**A**) Rheological properties of *COLRp_I*, *COLRp_II,* and *COLRp_III* collagens presented as the temperature dependence of η* (Pa.s) and tan δ. (**B**) The amino acid contents of collagens. (**C**) Micro-DSC thermograms. (**D**) Moisture regain (%) of extracted collagens. Statistically significant differences were determined using one-way ANOVA following a post hoc Tukey´s test. The asterisks (*) denote a statistically significant difference ($p < 0.05$). Values represent the mean ± SD of at least three individual measurements.

3.5. Amino Acid Analysis

In general, collagen has a high content of glycine, alanine, hydroxyproline, and proline amino acid, with the standard amino acid sequence in the triple helix region being Gly-X-Y, where X and Y are often proline and hydroxyproline. Accordingly, the amino acid composition of the extracted collagens is particularly rich in glycine, alanine, and proline (87), with glycine being the most abundant amino acid in *COLRp_I*, *COLRp_II,* and *COLRp_III* collagens, accounting for 33.8%, 35.0%, and 32.4% of the total amino acid composition, respectively. These values are in agreement with the percentage reported by Wu et al. [62] for silver carp collagen. Furthermore, hydroxyproline and proline (pyrrolidine acids) are known to influence the temperature stability of collagen, with marine-origin collagens exhibiting typically a lower denaturation temperature and viscosity than terrestrial mammals collagens [63]. Greenland halibut collagens contained 153 (*COLRp_I*), 145 (*COLRp_II*), and 140 (*COLRp_III*) residues of pyrrolidine acids, similar to the values detected for other

marine collagens such as Atlantic cod (*Gadus morhua*) [64] or carp (*Cyprinus carpio*) [65]. Furthermore, the hydroxylation degree is a relevant parameter to evaluate the collagen thermal stability and helix structure [1]. The hydroxylation degree was 43.1% for *COLRp_I*, 40% for *COLRp_II*, and 39.2% for *COLRp_III*. Indeed, the degree of the hydroxylation of *COLRp_I* collagen was the highest, at least 3% which is directly linked to the higher thermal stability of this collagen molecule [66]. These results point out that the change in NaCl concentration during collagen precipitation has a relevant role in the thermal stability of the isolated protein. It has been reported that collagen thermal stability is dependent on the presence of different ion concentrations [67–69], which also influence the rheological properties of collagen solution. According to Duan et al., for NaCl concentration above 0.1 M, collagen solutions exhibited a salting-out effect, with their pseudo-plastic behavior becoming stronger, without a significant change in thixotropy [70].

The variation of both the pyrrolidine acids contents and hydroxylation degree observed in the extracted collagens is consistent with the variation in melting temperature, supporting the relationship between both features in collagen. Interestingly, the serine amino acid content was 74, 70, and 69 residues for *COLRp_I*, *COLRp_II*, and *COLRp_III*, respectively, being an amino acid reported in the literature as playing a role in enhancing the moisturizing capability of the skin [71], which might be relevant for the development of cosmetic applications using the produced collagens.

3.6. Micro-Differential Scanning Calorimetry

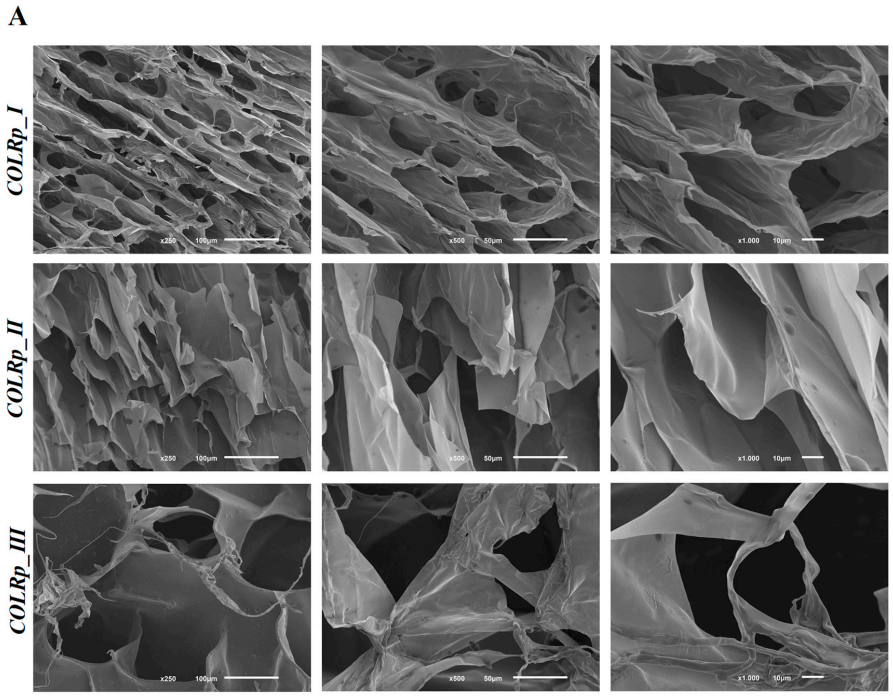
The heat-induced denaturation of collagens was surveyed by micro-DSC measurements (Figure 3C). The *COLRp_I* micro-DSC curve displayed two endothermal peaks, one at 23.1 °C probably relative to the collagen transitional conformation, similar to the observed with the Pacific whiting fish (21.7 °C) [72], and another peak relative to denaturation of the collagen at 57.6 °C. *COLRp_II* and *COLRp_III* collagens showed only one peak at 45.5 °C and 75.9 °C, respectively. According to other studies, endothermal peaks of marine collagen in barramundi (*Lates calcarifer*), tilapia (*Oreochromis niloticus*), and sturgeon fish (*Huso huso*) have shown high thermal behaviors with peak temperatures of 109.6 °C, 113.7 °C, and 92.03 °C, respectively, being associated with the cleavage of peptide chains [51,57,73]. These high thermal behaviors and positive effects on structural collagen stability are beneficial for the development of marine biomaterials as substitutes for mammalian materials. Conversely, marine collagens are commonly reported to show lower thermal stability compared to mammalian origin [26]. Studies have been performed to improve the thermal stability of marine collagen [74–76], showing that the properties of collagen are dependent on pH, temperature, and ionic strength that can enable collagen self-assembly into fibrils and consequently enhance the denaturation temperature to a value higher than 40 °C [77–79]. Nevertheless, the interpretation of the thermal behavior of collagens is not yet fully understood, with the used experimental conditions playing an important role, namely humidity, besides the ones abovementioned, from which the comparison of transition temperatures obtained in different studies should be made carefully.

3.7. Moisture Regain

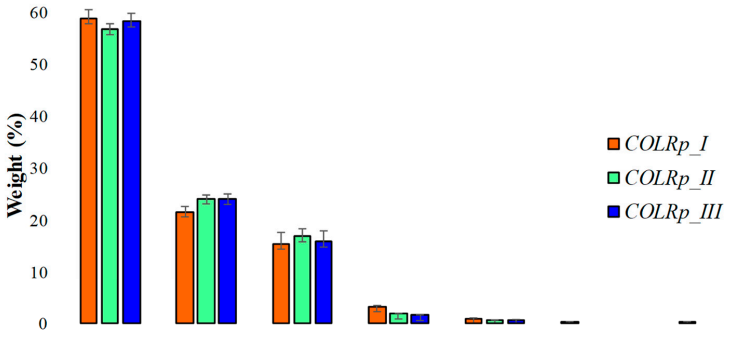
Moisture retaining capacity is an inherent feature of collagen that is widely practical and functional in cosmetic preparation formulation. Collagen is an intrinsic water-binding protein with humectant properties [80]. The produced collagens were shown to absorb about 10% of their weight in water after up to 48 h exposure to a 57% humidity atmosphere. *COLRp_I* demonstrated statistically significant differences between 0–24 h and 0–48 h moisture regains, whereas *COLRp_II* and *COLRp_III* demonstrated significant differences between 0–48 h (Figure 3D). After 24 h in constant relative humidity, *COLRp_III* revealed statistical differences between *COLRp_I* and *COLRp_II*, decreasing the sample's weight by 4.3% and 3.9%, respectively. Distinctively, *COLRp_III* collagen was precipitated with a low concentration of NaCl (0.9 M), which suggested decreasing the percentage of water regain compared to the other collagens.

3.8. SEM and EDS Analyses

Scanning electron microscopy (SEM) was used to examine the surface of collagen and energy-dispersive X-ray spectroscopy (EDS) was used to examine the most abundant chemical elements in the samples. The results suggested a similar morphology surface between the collagens, with the "cellular" structure typically obtained upon freeze-drying of polymeric solutions. In all cases, the cell walls are just a few microns thick, with slight differences in porosity and mean pore size, apparently increasing in the sequence *COLRp_I* < *COLRp_II* < *COLRp_III* (Figure 4A). In addition, the three highest elements detected (EDS does not detect hydrogen) in the collagen samples were carbon, oxygen, and nitrogen, with weights around 57–59%, 22–24%, and 15–17%, respectively, which are in agreement with the contents of these elements in proteins. Interestingly, other elements such as chlorine, sulfur, aluminum, and sodium were also identified, most probably due to the presence of salts in the samples (Figure 4B).



B



Collagens	C	O	N	Cl	S	Al	Na
				Wt (%) ± σ			
COLRp_I	58.7 ± 1.7	21.6 ± 1.0	15.4 ± 2.2	3.2 ± 0.2	0.9 ± 0.1	0.2 ± 0.1	0
COLRp_II	56.6 ± 1.1	24.1 ± 0.7	16.8 ± 1.5	1.8 ± 0.1	0.6	0	0.2 ± 0.1
COLRp_III	58.1 ± 1.6	24.0 ± 1.0	15.8 ± 2.1	1.5 ± 0.1	0.6 ± 0.1	0	0

Elemental chemical composition

Figure 4. SEM and EDS analyses of Greenland halibut skin collagens, with observation of morphological features and quantification of the elemental composition.

3.9. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

Fifteen elements were measured in the marine collagen by ICP (Table 1) to ensure that the extracted collagens were free of heavy metals or toxic elements. Elements such as calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), strontium (Sr), and zinc (Zn) were absent in all collagen samples. Apart from that, silver (Ag), arsenic (As), and phosphorus (P) were detected in low amounts in *COLRp_I* compared to the other evaluated collagens. Otherwise, cadmium (Cd) was only identified in *COLRp_I*. Interestingly, aluminum (Al), gold (Au), and lead (Pb) were absent in *COLRp_II*. However, the Pb element was detected with the highest value in *COLRp_III* compared to *COLRp_I*. In summary, the presence of some target elements was identified in the produced collagens, but in all cases in small amounts and well below the limits for elemental impurities recommended by the European Medicines Agency (ICH guideline Q3D (R1) on elemental impurities). These elements can be naturally present in marine byproducts due to accumulation in fish tissues, but the determined quantities show that the respective products do not raise any concerns regarding security for further use.

Table 1. Quantification of chemical elements (ppm) including metals, semi-metals, and non-metals in the extracted collagens and reference values according to ICH guideline Q3D (R1) on elemental impurities.

Chemical Elements (ppm)	*COLRp_I*	*COLRp_II*	*COLRp_III*	Reference Value
Ag	0.01	0.1	0.12	15
Al	0.04	0	0.01	500
As	0.60	1.2	0.72	1.5
Au	0.07	0	0.08	10
Ca	0	0	0	-
Cd	0.02	0	0	0.5
Fe	0	0	0	150
K	0	0	0	-
Mg	0	0	0	under deliberation
Mn	0	0	0	70
Na	0	0	0	-
P	0.29	0.37	0.49	-
Pb	0.08	0	0.32	0.5
Sr	0	0	0	300
Zn	0	0	0	150

4. Conclusions

In the present study, collagen was isolated from the skin of Greenland halibut *R. hippoglossoides* fish, thus proposing an approach for the valorization of this byproduct of fish processing, given the biotechnological value of that protein. Extraction was performed with an acetic acid treatment and three different methods were used for the precipitation (salting-out) of collagens by varying NaCl concentration and pH. The characterization of the produced extracts indicated slight differences in the amino acid composition, thermal stability, and morphology, showing that these are tunable and envisaging a specific application, such as cosmetics, due to the reported differences in moisture capacity of the isolated collagens. In general, the increase in NaCl concentration, conjugated with barely neutral pH, resulted in collagen with lower relative viscosity, higher pyrrolidine acids, and subsequently, superior melting temperature, whereas apparently affecting positively the humectant potential, relevant if envisaging the use of collagen in a cosmetics context, namely as an ingredient in a skincare formulation. With the help of this work, we are able to show that marine byproducts can be a reliable source for collagen extraction, giving value to the byproducts and enabling the valorization of raw materials that are quite abundant in the fish manufacturing sector.

Author Contributions: Conceptualization and formal analysis E.M. and T.H.S.; investigation, E.M., R.F., A.L.A. and R.O.S.; writing—original draft preparation, E.M.; writing—review and editing, T.H.S.; funding acquisition, T.H.S. and R.L.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Union Transborder Cooperation Programme Interreg España-Portugal 2014-2020 (POCTEP), within the European Regional Development Fund (ERDF), under the project 0302_CVMAR_I_1_P.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors acknowledge Julio Maroto (CETMAR—Centro Tecnológico del Mar, Fundación CETMAR, Vigo, Spain), who kindly provided the skin byproducts.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sipilä, K.H.; Drushinin, K.; Rappu, P.; Jokinen, J.; Salminen, T.A.; Salo, A.M.; Käpylä, J.; Myllyharju, J.; Heino, J. Proline hydroxylation in collagen supports integrin binding by two distinct mechanisms. *J. Biol. Chem.* **2018**, *293*, 7645–7658. [CrossRef] [PubMed]
2. Rahman, M.A. Collagen of extracellular matrix from marine invertebrates and its medical applications. *Mar. Drugs* **2019**, *17*, 118. [CrossRef] [PubMed]
3. Fratzl, P. Collagen: Structure and mechanics, an introduction. In *Collagen*; Springer: Berlin/Heidelberg, Germany, 2008; pp. 1–13.
4. Sorushanova, A.; Skoufos, I.; Tzora, A.; Mullen, A.M.; Zeugolis, D.I. The influence of animal species, gender and tissue on the structural, biophysical, biochemical and biological properties of collagen sponges. *J. Mater. Sci. Mater. Med.* **2021**, *32*, 1–12. [CrossRef] [PubMed]
5. Heino, J. The collagen family members as cell adhesion proteins. *Bioessays* **2007**, *29*, 1001–1010. [CrossRef]
6. Haug, I.J.; Draget, K.I.; Smidsrød, O. Physical and rheological properties of fish gelatin compared to mammalian gelatin. *Food Hydrocoll.* **2004**, *18*, 203–213. [CrossRef]
7. Liu, D.; Liang, L.; Regenstein, J.M.; Zhou, P. Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). *Food Chem.* **2012**, *133*, 1441–1448. [CrossRef]
8. Ehrlich, H.; Wysokowski, M.; Żółtowska-Aksamitowska, S.; Petrenko, I.; Jesionowski, T.J. Collagens of poriferan origin. *Mar. Drugs* **2018**, *16*, 79. [CrossRef]
9. Uzel, S.G.; Buehler, M.J. Nanomechanical sequencing of collagen: Tropocollagen features heterogeneous elastic properties at the nanoscale. *Integr. Biol.* **2009**, *1*, 452–459. [CrossRef]
10. Langasco, R.; Cadeddu, B.; Formato, M.; Lepedda, A.J.; Cossu, M.; Giunchedi, P.; Pronzato, R.; Rassu, G.; Manconi, R.; Gavini, E.J.; et al. Natural collagenic skeleton of marine sponges in pharmaceutics: Innovative biomaterial for topical drug delivery. *Mater. Sci. Eng. C Mater. Biol. Appl.* **2017**, *70*, 710–720. [CrossRef]
11. Le, T.M.T.; Nguyen, V.M.; Tran, T.T.; Takahashi, K.; Osako, K.J. Comparison of acid-soluble collagen characteristic from three important freshwater fish skins in Mekong Delta Region, Vietnam. *J. Food Biochem.* **2020**, *44*, e13397. [CrossRef]
12. Felician, F.F.; Xia, C.; Qi, W.; Xu, H.J.C. Biodiversity. Collagen from marine biological sources and medical applications. *Chem. Biodivers.* **2018**, *15*, e1700557. [CrossRef] [PubMed]
13. Pozzolini, M.; Scarfi, S.; Gallus, L.; Castellano, M.; Vicini, S.; Cortese, K.; Gagliani, M.C.; Bertolino, M.; Costa, G.; Giovine, M.J. Production, characterization and biocompatibility evaluation of collagen membranes derived from marine sponge Chondrosia reniformis Nardo, 1847. *Mar. Drugs* **2018**, *16*, 111. [CrossRef] [PubMed]
14. Lin, K.; Zhang, D.; Macedo, M.H.; Cui, W.; Sarmento, B.; Shen, G. Advanced Collagen-Based Biomaterials for Regenerative Biomedicine. *Adv. Funct. Mater.* **2019**, *29*, 1804943. [CrossRef]
15. Coppola, D.; Oliviero, M.; Vitale, G.A.; Lauritano, C.; D'Ambra, I.; Iannace, S.; de Pascale, D. Marine collagen from alternative and sustainable sources: Extraction, processing and applications. *Mar. Drugs* **2020**, *18*, 214. [CrossRef] [PubMed]
16. Shahidi, F.; Varatharajan, V.; Peng, H.; Senadheera, R.J. Utilization of marine by-products for the recovery of value-added products. *J. Food Bioact.* **2019**, *6*, 5183. [CrossRef]
17. Ahmad, M.; Nirmal, N.P.; Chuprom, J. Molecular characteristics of collagen extracted from the starry triggerfish skin and its potential in the development of biodegradable packaging film. *Rsc. Adv.* **2016**, *6*, 33868–33879. [CrossRef]
18. Alves, A.L.; Marques, A.L.; Martins, E.; Silva, T.H.; Reis, R.L. Cosmetic potential of marine fish skin collagen. *Cosmetics* **2017**, *4*, 39. [CrossRef]
19. Chen, Y.; Jin, H.; Yang, F.; Jin, S.; Liu, C.; Zhang, L.; Huang, J.; Wang, S.; Yan, Z.; Cai, X. Physicochemical, antioxidant properties of giant croaker (Nibea japonica) swim bladders collagen and wound healing evaluation. *Int. J. Biol. Macromol.* **2019**, *138*, 483–491. [CrossRef]

20. Sousa, R.O.; Alves, A.L.; Carvalho, D.N.; Martins, E.; Oliveira, C.; Silva, T.H.; Reis, R.L. Polymer Edition. Acid and enzymatic extraction of collagen from Atlantic cod (Gadus Morhua) swim bladders envisaging health-related applications. *J. Biomater. Sci. Polym. Ed.* **2020**, *31*, 20–37. [CrossRef]
21. Kaewdang, O.; Benjakul, S.; Kaewmanee, T.; Kishimura, H.J. Characteristics of collagens from the swim bladders of yellowfin tuna (*Thunnus albacares*). *Food Chem.* **2014**, *155*, 264–270. [CrossRef]
22. Nagai, T.; Nagamori, K.; Yamashita, E.; Suzuki, N. Collagen of octopus Callistoctopus arakawai arm. *Int. J. Food Sci. Technol.* **2002**, *37*, 285–289. [CrossRef]
23. Seixas, M.J.; Martins, E.; Reis, R.L.; Silva, T.H. Extraction and Characterization of Collagen from Elasmobranch Byproducts for Potential Biomaterial Use. *Mar. Drugs* **2020**, *18*, 617. [CrossRef] [PubMed]
24. Khong, N.M.; Yusoff, F.M.; Jamilah, B.; Basri, M.; Maznah, I.; Chan, K.W.; Armania, N.; Nishikawa, J. Improved collagen extraction from jellyfish (*Acromitus hardenbergi*) with increased physical-induced solubilization processes. *Food Chem.* **2018**, *251*, 41–50. [CrossRef] [PubMed]
25. Belouafa, S.; Bourja, L.; Villain, S.; Tayane, S.; Bennamara, A.; Abourriche, A.J.; Cities, D.A. Biocomposite Based on Collagen/Calcium Salts Extraction from Sardine Scales. In Proceedings of the 2nd International Conference on Smart Applications and Data Analysis for Smart Cities, Casablanca, Morocco, 27–28 February 2018.
26. Wang, L.; Liang, Q.; Chen, T.; Wang, Z.; Xu, J.; Ma, H. Characterization of collagen from the skin of Amur sturgeon (*Acipenser schrenckii*). *Food Hydrocoll.* **2014**, *38*, 104–109. [CrossRef]
27. Bae, I.; Osatomi, K.; Yoshida, A.; Osako, K.; Yamaguchi, A.; Hara, K.J. Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes. *J. Food Biochem.* **2008**, *108*, 49–54. [CrossRef]
28. Ben Slimane, E.; Sadok, S. Collagen from cartilaginous fish by-products for a potential application in bioactive film composite. *Mar. Drugs* **2018**, *16*, 211. [CrossRef]
29. Song, E.; Kim, S.Y.; Chun, T.; Byun, H.-J.; Lee, Y.M. Collagen scaffolds derived from a marine source and their biocompatibility. *Biomaterials* **2006**, *27*, 2951–2961. [CrossRef]
30. Zhang, J.; Elango, J.; Wang, S.; Hou, C.; Miao, M.; Li, J.; Na, L.; Wu, W. Characterization of Immunogenicity Associated with the Biocompatibility of Type I Collagen from Tilapia Fish Skin. *Polymers* **2022**, *14*, 2300. [CrossRef]
31. Gharehgheshlagh, S.N.; Fatemi, M.J.; Jamili, S.; Nourani, M.R.; Sharifi, A.M.; Saberi, M.; Amini, N.; Ganji, F.J. Therapeutics. A Dermal Gel Made of Rutilus Kutum Skin Collagen-Chitosan for Deep Burn Healing. *Int. J. Pept. Res. Ther.* **2020**, *27*, 317–328. [CrossRef]
32. Avila Rodríguez, M.I.; Rodríguez Barroso, L.G.; Sánchez, M.L. Collagen: A review on its sources and potential cosmetic applications. *J. Cosmet. Dermatol.* **2018**, *17*, 20–26. [CrossRef]
33. Silvipriya, K.; Kumar, K.K.; Bhat, A.; Kumar, B.D.; John, A.; Lakshmanan, P. Collagen: Animal sources and biomedical application. *J. App. Pharm. Sci.* **2015**, *5*, 123–127. [CrossRef]
34. Peng, Y.; Stoichevska, V.; Vashi, A.; Howell, L.; Fehr, F.; Dumsday, G.; Werkmeister, J.; Ramshaw, J. Non–animal collagens as new options for cosmetic formulation. *Int. J. Cosmet. Sci.* **2015**, *37*, 636–641. [CrossRef] [PubMed]
35. Bernhardt, A.; Paul, B.; Gelinsky, M.J. Biphasic scaffolds from marine collagens for regeneration of osteochondral defects. *Mar. Drugs* **2018**, *16*, 91. [CrossRef] [PubMed]
36. Al-Nimry, S.; Dayah, A.A.; Hasan, I.; Daghmash, R. Cosmetic, biomedical and pharmaceutical applications of fish gelatin/hydrolysates. *Mar. Drugs* **2021**, *19*, 145. [CrossRef] [PubMed]
37. Gupta, S.; Sharma, S.; Nadda, A.K.; Husain, M.S.B.; Gupta, A. Biopolymers from waste biomass and its applications in the cosmetic industry: A review. *Mater. Today Proc.* **2022**, *in press*. [CrossRef]
38. Senaratne, L.; Park, P.-J.; Kim, S.-K. Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. *Bioresour. Technol.* **2006**, *97*, 191–197. [CrossRef] [PubMed]
39. Ge, B.; Wang, H.; Li, J.; Liu, H.; Yin, Y.; Zhang, N.; Qin, S. Comprehensive assessment of Nile tilapia skin (Oreochromis niloticus) collagen hydrogels for wound dressings. *Mar. Drugs* **2020**, *18*, 178. [CrossRef] [PubMed]
40. Geahchan, S.; Baharlouei, P.; Rahman, A. Marine Collagen: A Promising Biomaterial for Wound Healing, Skin Anti-Aging, and Bone Regeneration. *Mar. Drugs* **2022**, *20*, 61. [CrossRef]
41. Zhuang, Y.; Hou, H.; Zhao, X.; Zhang, Z.; Li, B.J. Effects of collagen and collagen hydrolysate from jellyfish (Rhopilema esculentum) on mice skin photoaging induced by UV irradiation. *J. Food Sci.* **2009**, *74*, H183–H188. [CrossRef]
42. Shibuya, S.; Ozawa, Y.; Toda, T.; Watanabe, K.; Tometsuka, C.; Ogura, T.; Koyama, Y.; Shimizu, T.J.B. Biotechnology Biochemistry. Collagen peptide and vitamin C additively attenuate age-related skin atrophy in Sod1-deficient mice. *Biosci. Biotechnol. Biochem.* **2014**, *78*, 1212–1220. [CrossRef]
43. Heidari, M.G.; Rezaei, M. Extracted pepsin of trout waste and ultrasound-promoted method for green recovery of fish collagen. *Sustain. Chem. Pharm.* **2022**, *30*, 100854. [CrossRef]
44. Xhauflaire-Uhoda, E.; Fontaine, K.; Pierard, G.E. Kinetics of moisturizing and firming effects of cosmetic formulations. *Int. J. Cosmet. Sci.* **2008**, *30*, 131–138. [CrossRef] [PubMed]
45. Venkatesan, J.; Anil, S.; Kim, S.-K.; Shim, M.S. Marine fish proteins and peptides for cosmeceuticals: A review. *Mar. Drugs* **2017**, *15*, 143. [CrossRef] [PubMed]

46. Salvatore, L.; Gallo, N.; Natali, M.L.; Campa, L.; Lunetti, P.; Madaghiele, M.; Blasi, F.S.; Corallo, A.; Capobianco, L.; Sannino, A. Marine collagen and its derivatives: Versatile and sustainable bio-resources for healthcare. *Mater. Sci. Eng. C* **2020**, *113*, 110963. [CrossRef]
47. Lin, P.; Alexander, R.A.; Liang, C.H.; Liu, C.; Lin, Y.H.; Lin, Y.H.; Chan, L.P.; Kuan, C.M. Collagen formula with Djulis for improvement of skin hydration, brightness, texture, crow's feet, and collagen content: A double-blind, randomized, placebo-controlled trial. *J. Cosmet. Dermatol.* **2020**, *20*, 188–194. [CrossRef]
48. Blanco, M.; Vázquez, J.A.; Pérez-Martín, R.I.; Sotelo, C.G. Collagen extraction optimization from the skin of the small-spotted catshark (S. canicula) by response surface methodology. *Mar. Drugs* **2019**, *17*, 40. [CrossRef]
49. Zaelani, B.; Safithri, M.; Tarman, K.; Setyaningsih, I. Collagen isolation with acid soluble method from the skin of Red Snapper (*lutjanus* sp.). In *IOP Conference Series: Earth and Environmental Science*; IOP Publishing: Bristol, UK, 2019; p. 012033.
50. Ferrario, C.; Rusconi, F.; Pulaj, A.; Macchi, R.; Landini, P.; Paroni, M.; Colombo, G.; Martinello, T.; Melotti, L.; Gomiero, C.; et al. From Food Waste to Innovative Biomaterial: Sea Urchin-Derived Collagen for Applications in Skin Regenerative Medicine. *Mar. Drugs* **2020**, *18*, 414. [CrossRef]
51. Liao, W.; Guanghua, X.; Li, Y.; Shen, X.R.; Li, C. Comparison of characteristics and fibril-forming ability of skin collagen from barramundi (*Lates calcarifer*) and tilapia (*Oreochromis niloticus*). *Int. J. Biol. Macromol.* **2018**, *107*, 549–559. [CrossRef]
52. Li, J.; Wang, M.; Qiao, Y.; Tian, Y.; Liu, J.; Qin, S.; Wu, W. Extraction and characterization of type I collagen from skin of tilapia (*Oreochromis niloticus*) and its potential application in biomedical scaffold material for tissue engineering. *Process Biochem.* **2018**, *74*, 156–163. [CrossRef]
53. Singh, P.; Benjakul, S.; Maqsood, S.; Kishimura, H.J.F. Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). *Food Chem.* **2011**, *124*, 97–105. [CrossRef]
54. Drzewiecki, K.E.; Grisham, D.R.; Parmar, A.S.; Nanda, V.; Shreiber, D.I. Circular dichroism spectroscopy of collagen fibrillogenesis: A new use for an old technique. *Biophys. J.* **2016**, *111*, 2377–2386. [CrossRef] [PubMed]
55. Menezes, M.d.L.L.R.; Ribeiro, H.L.; de Oliveira, M.F.; de Andrade Feitosa, J.P.; Biointerfaces, S.B. Optimization of the collagen extraction from Nile tilapia skin (*Oreochromis niloticus*) and its hydrogel with hyaluronic acid. *Colloids Surf. B Biointerfaces* **2020**, *189*, 110852. [CrossRef]
56. Kozlowska, J.; Sionkowska, A.; Skopinska-Wisniewska, J.; Piechowicz, K. Northern pike (*Esox lucius*) collagen: Extraction, characterization and potential application. *Int. J. Biol. Macromol.* **2015**, *81*, 220–227. [CrossRef]
57. Atef, M.; Ojagh, S.M.; Latifi, A.M.; Esmaeili, M.; Udenigwe, C.C. Biochemical and structural characterization of sturgeon fish skin collagen (*Huso huso*). *J. Food Biochem.* **2020**, *44*, e13256. [CrossRef] [PubMed]
58. Li, J.; Li, Y.; Li, Y.; Yang, Z.; Jin, H. Physicochemical Properties of Collagen from Acaudina Molpadioides and Its Protective Effects against H_2O_2-Induced Injury in RAW264. 7 Cells. *Mar. Drugs* **2020**, *18*, 370. [CrossRef] [PubMed]
59. Veeruraj, A.; Arumugam, M.; Ajithkumar, T.; Balasubramanian, T. Isolation and characterization of collagen from the outer skin of squid (*Doryteuthis singhalensis*). *Food Hydrocoll.* **2015**, *43*, 708–716. [CrossRef]
60. Zanaboni, G.; Rossi, A.; Onana, A.M.T.; Tenni, R. Stability and networks of hydrogen bonds of the collagen triple helical structure: Influence of pH and chaotropic nature of three anions. *Matrix Biol.* **2000**, *19*, 511–520. [CrossRef]
61. Wang, L.; An, X.; Xin, Z.; Zhao, L.; Hu, Q. Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). *J. Food Sci.* **2007**, *72*, E450–E455. [CrossRef]
62. Wu, J.; Kong, L.; Zhang, J.; Chen, W. Extraction and properties of acid-soluble collagen and pepsin-soluble collagen from silver carp (*Hypophthalmichthys molitrix*) scales: Prerequisite information for fishery processing waste reuse. *Int. J. Biol. Macromol.* **2019**, *28*, 2923–2930. [CrossRef]
63. Leuenberger, B.H. Investigation of viscosity and gelation properties of different mammalian and fish gelatins. *Food Hydrocoll.* **1991**, *5*, 353–361. [CrossRef]
64. Sousa, R.O.; Martins, E.; Carvalho, D.N.; Alves, A.L.; Oliveira, C.; Duarte, A.R.C.; Silva, T.H.; Reis, R.L. Collagen from Atlantic cod (*Gadus morhua*) skins extracted using CO_2 acidified water with potential application in healthcare. *J. Polym. Res.* **2020**, *27*, 1–9. [CrossRef]
65. Duan, R.; Zhang, J.; Du, X.; Yao, X.; Konno, K.J. Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). *Food Chem.* **2009**, *112*, 702–706. [CrossRef]
66. Liu, W.; Tian, Z.; Li, C.; Li, G. Thermal denaturation of fish collagen in solution: A calorimetric and kinetic analysis. *Thermochim. Acta* **2014**, *581*, 32–40. [CrossRef]
67. Tan, Z.-J.; Chen, S.-J. Nucleic acid helix stability: Effects of salt concentration, cation valence and size, and chain length. *Biophys. J.* **2006**, *90*, 1175–1190. [CrossRef]
68. Komsa-Penkova, R.; Koynova, R.; Kostov, G.; Tenchov, B.G. Thermal stability of calf skin collagen type I in salt solutions. *Biochim. Et Biophys. Acta (BBA)—Protein Struct. Mol. Enzymol.* **1996**, *1297*, 171–181. [CrossRef]
69. Bianchi, E.; Conio, G.; Ciferri, A.; Puett, D.; Rajagh, L. The role of pH, temperature, salt type, and salt concentration on the stability of the crystalline, helical, and randomly coiled forms of collagen. *J. Biol. Chem.* **1967**, *242*, 1361–1369. [CrossRef]
70. Duan, L.; Li, J.; Li, C.; Li, G. Effects of NaCl on the rheological behavior of collagen solution. *Korea-Aust. Rheol. J.* **2013**, *25*, 137–144. [CrossRef]

71. Kim, H.; Ro, J.; Barua, S.; Hwang, D.S.; Na, S.-J.; Lee, H.S.; Jeong, J.H.; Woo, S.; Kim, H.; Hong, B.J.; et al. Combined skin moisturization of liposomal serine incorporated in hydrogels prepared with carbopol ETD 2020, rhesperse RM 100 and hyaluronic acid. *Korean J. Physiol. Pharmacol.* **2015**, *19*, 543–547. [CrossRef]
72. Kim, J.S.; Park, J.W. Characterization of acid-soluble collagen from pacific whiting surimi processing byproducts. *J. Food Sci.* **2004**, *69*, C637–C642. [CrossRef]
73. Safandowska, M.; Pietrucha, K. Effect of fish collagen modification on its thermal and rheological properties. *Int. J. Biol. Macromol.* **2013**, *53*, 32–37. [CrossRef]
74. Subhan, F.; Ikram, M.; Shehzad, A.; Ghafoor, A.J. Technology. Marine collagen: An emerging player in biomedical applications. *J. Food Sci. Technol.* **2015**, *52*, 4703–4707. [CrossRef] [PubMed]
75. Pallela, R.; Venkatesan, J.; Janapala, V.R.; Kim, S.K. Biophysicochemical evaluation of chitosan-hydroxyapatite-marine sponge collagen composite for bone tissue engineering. *J. Biomed. Mater. Res. A* **2012**, *100*, 486–495. [CrossRef] [PubMed]
76. Liu, S.; Lau, C.-S.; Liang, K.; Wen, F.; Teoh, S.H. Marine collagen scaffolds in tissue engineering. *Curr. Opin. Biotechnol.* **2022**, *74*, 92–103. [CrossRef] [PubMed]
77. Kadler, K.E.; Holmes, D.F.; Trotter, J.A.; Chapman, J.A. Collagen fibril formation. *Biochem. J.* **1996**, *316*, 1–11. [CrossRef]
78. Pal, G.K.; Suresh, P. Comparative assessment of physico-chemical characteristics and fibril formation capacity of thermostable carp scales collagen. *Mater. Sci. Eng. C* **2017**, *70*, 32–40. [CrossRef]
79. Bae, I.; Osatomi, K.; Yoshida, A.; Yamaguchi, A.; Tachibana, K.; Oda, T.; Hara, K. Characteristics of a self-assembled fibrillar gel prepared from red stingray collagen. *Fish Sci.* **2009**, *75*, 765–770. [CrossRef]
80. Peng, Y.; Glattauer, V.; Werkmeister, J.A.; Ramshaw, J.A. Evaluation for collagen products for cosmetic application. *Int. J. Cosmet. Sci.* **2004**, *26*, 313. [CrossRef]