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# Acid and enzymatic extraction of collagen from Atlantic cod (*Gadus Morhua*) swim bladders envisaging health-related applications

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## ABSTRACT

Atlantic cod is processed industrially for food purposes, with several by-products being directed to animal feed and other ends. Looking particularly into swim bladders, the extraction of collagen can be a valuable strategy for by-product valorization, explored in the present work for the first time. Collagen was extracted using acetic acid (ASCsb) and pepsin (PSCsb) with yields of 5.72% (w/w) and 11.14% (w/w), respectively. SDS-PAGE profile showed that the extracts were compatible with type I collagen. FTIR, CD and XRD results suggest that the PSCsb structure underwent partial denaturation, with microDSC showing a band at 54 °C probably corresponding to a melting process, while ASCsb structure remained intact, with preserved triple helix and a denaturation temperature of 29.6 °C. Amino acid composition indicates that the total content of proline-like amino acids was 148/1000 residues for ASCsb and 141/1000 residues for PSCsb, with a hydroxylation degree of about 37%. The extracts exhibited a typical shear thinning behavior, interesting property regarding their further processing toward the development of biomaterials. In this regard, assessment of metabolic activity of human fibroblast cells cultured in the presence of collagen extracts with concentrations up to 3 mg/mL revealed the absence of cytotoxic behavior. Collagen extracts obtained from Atlantic cod swim bladders shown attractive properties regarding their use in cosmetic or biomedical applications.

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## KEYWORDS

Fish Collagen; extraction; swim bladders; Atlantic cod; marine biomaterials

## 1. Introduction

Atlantic cod (*Gadus morhua*) is one of the most important commercial fish species in Portugal [1], having a unique status in national culture, from its production to the

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Portuguese gastronomy. In the case of the cod processing industry, about 40% (w/w) of raw-material resulted in by-products such as swim bladders, viscera, skin, bones and fins [2], which may represent a sustainable resource of interest to the economy of food supplements, feed and value-added compounds (biopolymers, enzymes, bioactive compounds) and from this to other with higher added-value. In fact, different ingredients, such as proteins, lipids, taurine, collagen, calcium, and phosphorous can be extracted from cod by-products [3], opening several biotechnological application possibilities.

The main part of the fish by-products resulting from the fisheries and food processing industries is still directed for fish silage, fishmeal and oil production, mainly used for animal feed [3,4]. Nevertheless, cod by-products have already been used, for example, for the extraction of collagen [5,6] and fish leather production from skin, the extraction of calcium phosphates such as hydroxyapatite from bones [7], the isolation of enzymes from liver, as well as other valuable compounds from viscera or muscle [4]. In this project, swim bladders were removed from salt-cured cod (*Gadus morhua*) and used to extract fish protein - collagen. The swim bladder or air bladder is an internal gas-filled organ that contributes to the ability of many bony fish (but not cartilaginous fish) to control their buoyancy, avoiding the waste of energy in swimming. Additionally, the swim bladder functions as a resonating chamber, to produce or receive sound and it is evolutionarily homologous to the lungs [8,9]. The swim bladder consists of three main layers: tunica externa, submucosa, and mucosa. The tunica externa is composed of a dense layer of connective tissue [9], being a potential source for the production of collagen.

Collagen is the main protein of connective tissue and the most abundant in vertebrate and invertebrate organisms [6,10–12]. It represents about 30% of total proteins, having a fundamentally structural role in connective tissue as skins, tendons, ligaments and bones, being the most prevalent component of the extracellular matrix (ECM) [13]. There are more than 20 collagens reported so far, differently distributed in animal tissues, being type I collagen the most studied in marine species [6]. Collagens of marine origin have gained more attention and importance because of their high extraction yield and availability, no risk of pathogen infection compared to land-based animals (cows, pigs, poultry, etc.) collagen, minor regulatory and ethical issues and no religious barriers [12,14,15]. Marine collagen from fish, sponges and jellyfish is increasingly used in various biomedical, cosmetics and pharmaceuticals applications [6,16,17]. As examples, collagen was studied to form hydrogels and porous scaffolds for tissue engineering and regeneration purpose [18–21], as a food supplement but also as an ingredient in cosmetics [5], with potential application by incorporation in formulations for creams and lotions. These strategies foster the establishment of highly profitable processes, which corresponds to a shift to a molecular level of utilization of by-products, where the biomolecules recovered would be applied outside the primary sector, with clear economic, environmental and biotechnological benefits [10].

The objective of this work was to address the extraction of collagen from Atlantic cod swim bladders, obtained as marine by-products from the processing of cod for food purposes using a treatment based in salting and drying. The extracted collagens were extensively characterized regarding their physical and chemical properties, as well as examining the rheology behavior and eventual cytotoxicity considering the future application in cosmetics and biomedical context.

## 2. Materials and methods

### 2.1. Materials

Swim bladders from Atlantic cod (*Gadus morhua*) were removed after a salting processing step and kindly offered by a local industry, Soguima (Guimarães, Portugal). The swim bladders were transported to the laboratory and stored at -20 °C until further use. All reagents used were of analytical grade and used as received.

### 2.2. Extraction of collagen

The isolation of collagen from cod swim bladders was performed by an acid and pepsin extraction methodology, similar to the one commonly followed for fish skins, adapted from Kaewdang et al. [22] and Lu et al. [23]. The swim bladders were cleaned several times with distilled water, for removal of salt and muscle debris, and cut into small pieces to enhance the extraction. To remove non-collagenous proteins, the swim bladders were rinsed with 0.15 M NaOH (1:10 w/v) for 3 hours under stirring, changing the solution every 1 hour. The swim bladders were then washed several times with cold distilled water until neutrality and further defatted by immersion in 10% (v/v) 2-propanol with a 1:10 (w/v) ratio for 24 hours under stirring. The resulting materials were further washed with abundant and cold distilled water and freeze-dried for subsequent collagen extraction. Pre-treated swim bladders samples were placed in 0.5 M acetic acid solution (1:10 w/v) for 3 days under stirring, followed by centrifugation at 20,000 g during 30 min at 4 °C. The supernatant, rich in acid soluble collagen (ASCsb) was collected and the remaining biomass were re-extracted with 10% pepsin added to 0.5 M acetic acid to obtain pepsin soluble collagen (PSCsb), also followed by centrifugation at 20,000 g during 30 min at 4 °C. To precipitate the collagens, both supernatants were salted out by adding NaCl to a final concentration of 2.6 M in 0.05 M Tris-HCl (pH 7.5) and left overnight. The precipitate was removed by centrifugation at 20,000 g during 30 min at 4 °C and re-suspended in 0.5 M of acetic acid. The solution was dialyzed first against 0.1 M acetic acid for 2 days, then against 0.02 M acetic acid for 2 days, and finally against distilled water until pH 7. All the previous procedures were performed at 4 °C to avoid collagen denaturation. The solution was freeze-dried and stored at room temperature until further use.

### 2.3. Characterization of the extracted collagen

#### 2.3.1. Extraction yield

The extraction yield was determined as the ratio between the final product (weight of freeze-dried collagen extract) and the initial product (wet weight of by-products biomass) (Equation (1)).

$$\text{Yield of collagen (wet)}(\%) = \frac{\text{Weight of collagen (g)}}{\text{Weight of wet swim bladders (g)}} \times 100$$

### **2.3.2. Fourier-Transform infrared (FTIR) spectroscopy**

The samples ASCs, PSCs (acid soluble collagen and pepsin soluble collagen from cod skin) ASCsb and PSCsb were respectively mixed and analysed with potassium bromide (KBr) pellets. The infrared spectra of collagen structures were obtained with a Shimadzu- IR Prestige 21 spectrometer in the spectral region of  $4000\text{--}800\text{ cm}^{-1}$  for collagen with resolution of  $2\text{ cm}^{-1}$ , as an average of 32 scans.

### **2.3.3. Amino acid analysis**

The amino acid content of ASCs, PSCs, ASCsb and PSCsb were determined by quantitative analysis using a Biochrome 30 apparatus (Biochrome Ltd., Cambridge, U.K.). Briefly, the samples were completely hydrolysed and separated by an ion Exchange column. After post-column derivatization by ninhydrin, the samples were analysed at two wavelengths: 440 and 570 nm. An internal standard of norleucine was used to determine the concentration of amino acids in the sample.

### **2.3.4. Micro differential scanning calorimetry**

Micro-DSC measurements were carried out with a high-sensitivity SETARAM Micro DSC III microcalorimeter, using  $700\text{ }\mu\text{L}$  samples in stainless steel cells to determine the denaturation temperature. Nitrogen was used as purge gas to avoid humidity condensation on the circulating coils of the microcalorimeter. All collagen samples were dissolved at  $5\text{ mg/mL}$  in  $0.5\text{ M}$  acetic acid, under stirring, at  $4\text{ }^\circ\text{C}$ . DSC scans of  $5\text{ mg/mL}$  of collagen (Coll) from calf skin Bornstein and Traub type I (Sigma) was used as reference material. The mass of sample was weighed directly in the measure cell and solution of  $0.5\text{ M}$  acetic acid was also placed in the reference cell during DSC scans. Measurements were made at  $1\text{ K min}^{-1}$  scan rate in the temperature range of  $5\text{--}80\text{ }^\circ\text{C}$ .

### **2.3.5. Circular dichroism analysis**

Circular dichroism (CD) measurements of the ASCsb and PSCsb were performed using a Jasco Model J1500 spectropolarimeter (Jasco, U.K.) using a quartz cylindrical cuvette (Hellma, Germany) with a path length of  $2\text{ mm}$ , at  $4\text{ }^\circ\text{C}$ . The cuvette was filled with  $600\text{ }\mu\text{L}$  of sample ( $0.1\text{ mg/mL}$ ) for each measurement. CD spectra were obtained by continuous wavelength scans (average of three scans) from  $180$  to  $240\text{ nm}$  at a scan-rate of  $50\text{ nm/min}$ .

### **2.3.6. X-ray diffraction (XRD)**

XRD measurements were obtained using a conventional Bragg–Brentano diffractometer (Bruker D8 Advance DaVinci, Germany) equipped with  $\text{CuK}\alpha$  radiation, produced at  $40\text{ kV}$  and  $40\text{ mA}$ . Data sets were collected in the  $2\theta$  range of  $5\text{--}30^\circ$  with a step size of  $0.02^\circ$  and  $1\text{ s}$  for each step. The average crystallite size was estimated with the Bragg equation (Equation (2)):

$$d\text{ (}\mathring{\text{A}}\text{)} = \lambda / 2\sin\theta \text{ (}\lambda_{\text{CuK}\alpha 1} = 1.5406\text{)}$$

### 2.3.7. SDS-PAGE analysis

Dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared using reagents from Sigma SDS-PAGE reagents and casted on a Biorad Mini Protean II System. Freeze-dried collagen was dissolved in 0.5 M acetic acid (10 mg/mL) under stirring. The solutions were then mixed 1:1 (v/v) with loading buffer and heated 10 min at 95 °C to completely denature the proteins. The type I collagen from bovine skin (used as reference) and the samples ASCs, PSCs, ASCsb and PSCsb (20 µL) were loaded onto SDS-PAGE gel, as well as 4 µL of protein marker. Both separating and stacking gels were run at 90 V. After running, the gels were stained in a Coomassie (0.500 g Coomassie Brilliant Blue G-250 (Biorad), 500 mL methanol, 100 mL acetic acid and 400 mL deionized water) staining solution for 30 min. Excess stain was removed with Destain I (80 mL methanol, 14 mL acetic acid and 156 mL deionized water) for 30 min and Destain II (12.5 mL methanol, 17.5 mL acetic acid and 220 mL deionized water) overnight, under stirring.

### 2.3.8. Gel permeation chromatography – size exclusion chromatography

The studied collagen samples (ASCsb and PSCsb) were analysed by gel permeation chromatography with a refractometer, right angle laser-light scattering and a viscosity detectors (TDA 305 – Viskotec, Malvern, UK). Novema Precolumn (10 µm, 8 × 50 mm), Novema 30 Å (10 µm, 8 × 300 mm), Novema 1000 Å (10 µm, 8 × 300 mm) and Novema 1000 Å (10 µm, 8 × 300 mm) were used for the polymers separation. The system was kept at 30 °C and 0.15 M NH<sub>4</sub>OAc/0.2 M AcOH buffer (pH 4.5) was used as eluent, at rate of 1 mL/min. Pullulan 47 kDa and polydispersity index (PDI) 1.07 (PSS standard services) was used to obtain a multidetector calibration (used to obtain the absolute Mw). The dn/dc was established as 0.186 after a literature search [24,25].

### 2.3.9. Rheology

Rheological analyses were performed using a Kinexus pro + rheometer (Malvern Instruments, UK), using the acquisition software rSpace. The measuring system used in these experiments is composed by a stainless steel cone (40 mm of diameter and 4°) and plate geometries. The surface geometry was covered with dodecane to prevent water loss. Rotational experiments were performed in order to obtain shear viscosity as a function of the shear rate, from 0.1<sup>-1</sup> to 1000 s<sup>-1</sup>, at 25 °C. All plots are obtained by the average of at least 3 experiments. These experiments were conducted with two collagen samples (ASCsb and PSCsb) at 3% (w/v) in 0.02 M acetic acid.

### 2.3.10. Cytotoxicity

Cell Expansion - Human fibroblast cell line (MRC-5) was cultured in D-MEM low glucose medium (Sigma-Aldrich) supplemented with 10% FBS (Alfagene) and Pen/Strep (100 U/100 g mL – 1; Life Technologies). Fibroblasts were used at passages 17-22. Cells were incubated at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Media was exchanged every 2–3 days until cells reached a 90% confluence.

**Cell Culture** - Cells were harvested and 15,000 cells were cultured in 24 well-plates. The cells were left to adhere for 4 h and, after that, collagen extracts were added to adherent cells. Extracts were dissolved in the culture medium at different concentrations: 0.25, 0.5, 1, 2, 3, 4, and 5 mg mL<sup>-1</sup>. For all the assays a positive control was performed (no biological extract in the culture medium). Each experimental condition was tested in triplicate and two independent assays were performed.

**Cell Viability** - The metabolic activity of cells, cultured with different extracts' concentrations and time points, was determined by the MTS assay (CellTiter 96 AQueous One Solution, Promega). Basically, the quantity of formazan product is directly proportional to the number of living cells in culture. At days 1, 2, and 3, the culture medium was removed and the testing conditions were rinsed with sterile Phosphate-Buffered Saline (PBS). A mixture of culture medium (without FBS and phenol red) and MTS reagent (5:1 ratio) was added to each well and left to incubate for 3 h, at 37 °C, in a humidified 5% CO<sub>2</sub> atmosphere. Thereafter, the absorbance of the MTS reaction medium from each sample was read in triplicate at 490 nm in a microplate reader (Synergy HT, Bio-TEK). All experiments were performed in triplicate.

## **2.4. Statistical analysis**

All tests were performed in triplicate and the data are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism Software. Differences between the several conditions of the cellular assays were analysed using nonparametric test (Kruskal-Wallis test), as the respective samples did not fit with the assumptions of normality (Shapiro-Wilk);  $p < 0.05$  was considered significant.

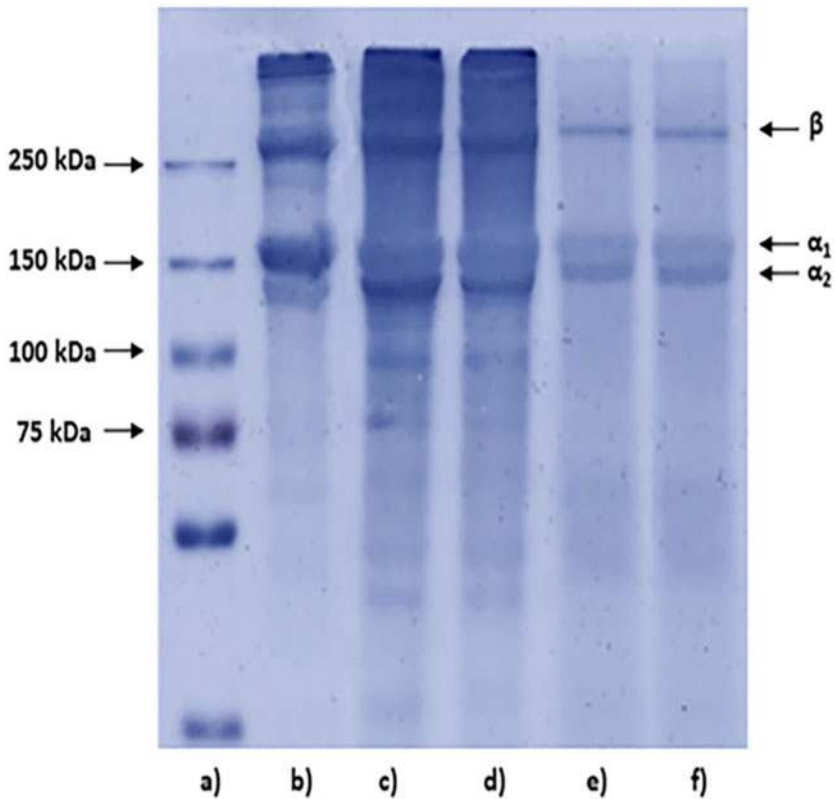
## **3. Results and discussion**

### **3.1. Extraction of collagen**

Acid-soluble collagen (ASCsb) and pepsin-soluble collagen (PSCsb) were extracted from cod swim bladders with a yield of 5.72% and 11.14%, respectively, in respect to the wet weight of raw-material. The results indicated an increase in collagen extraction yield in pepsin digestion because the telopeptide region was cleaved by this enzyme, facilitating the removal of the proteins from the remaining matrix. This is in agreement with previous studies for other fish species and by-products (like skin). Kaewdang et al. (2014) reported that ASC and PSC (acid soluble collagen and pepsin soluble collagen) from yellowfin tuna swim bladders were extracted with a yield of 1.07% and 12.10%, respectively. The differences in yields can be attributed to the different collagen structures in marine fish and the varying crosslinking of collagen fibrils in different by-products [12].

### **3.2. SDS-PAGE analysis**

In order to access structural information, purity and breakdowns of collagen protein upon extraction, sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-



**Figure 1.** Electrophoresis analysis of cod skin and swim bladder collagen. a) protein marker; b) type I collagen from bovine skin; c) ASCs; d) ASCsb; e) PSCs and f) PSCsb.

PAGE) was used to separate proteins fragments according to their size. The collagens extracted from cod swim bladders were submitted to this analysis, together with collagen from cod skin and type I collagen from bovine skin as reference. [Figure 1](#) displays the illustrative results, with the profiles of the several collagen extracts exhibiting the characteristic bands of type I collagen which is considered the gold standard in biomedical industries and tissue engineering approaches. The SDS-PAGE bands profiles of the swim bladder collagens (ASCsb and PSCsb) were quite similar to the equivalent collagens obtained from the skin of the same fish, as shown in [Figure 1](#), demonstrating the feasibility of using swim bladders for the isolation of collagen, as reported for other fish species [12,22,23]. Moreover, the similarity of those profiles with the one exhibited by type I collagen obtained from bovine skin, namely the presence of two distinct  $\alpha$ -chains ( $\alpha_1$  and  $\alpha_2$ ), with molecular weight of about 150 kDa, with  $\alpha_1$  much more intense than  $\alpha_2$  (theoretically, the double), as well as dimers –  $\beta$  chain – at about 250 kDa, is compatible with a classification of swim bladder collagens as type I [6,13]. Nevertheless, based on the electrophoretic patterns of collagens extracted from cod swim bladders, there are differences between ASCsb and PSCsb, suggesting that the crosslinks of collagens were more abundant in ASCsb than in PSCsb. It may be due that some  $\beta$  and  $\gamma$  (trimer) components were cleaved after digestion with pepsin [12].



### 3.3. Chemical characterization of ASCsb and PSCsb

The collagen extracted from cod swim bladders were characterized by FTIR, with the representative spectra being shown in Figure 2. The spectra shown very similar profiles, suggesting that the extracts present similar structures and chemical compositions, with the characteristic peaks of Amide A, Amide B, Amide I, Amide II and Amide III being clearly visible. These findings are similar with other found in literature for other marine species [12,22,26–28], as well as with collagens extracted from cod skin, also displayed in Figure 2 as reference. The N-H stretching vibration typical of intermolecular hydrogen bonding, commonly observed in a range between 3000–3500  $\text{cm}^{-1}$ , is relative to the Amide A band and was here observed at 3400  $\text{cm}^{-1}$ . The absorption characteristic of amide B, relative to the asymmetrical and symmetrical stretch of CH<sub>2</sub> is found at 2935  $\text{cm}^{-1}$  and 2926  $\text{cm}^{-1}$  for ASCsb and PSCsb,

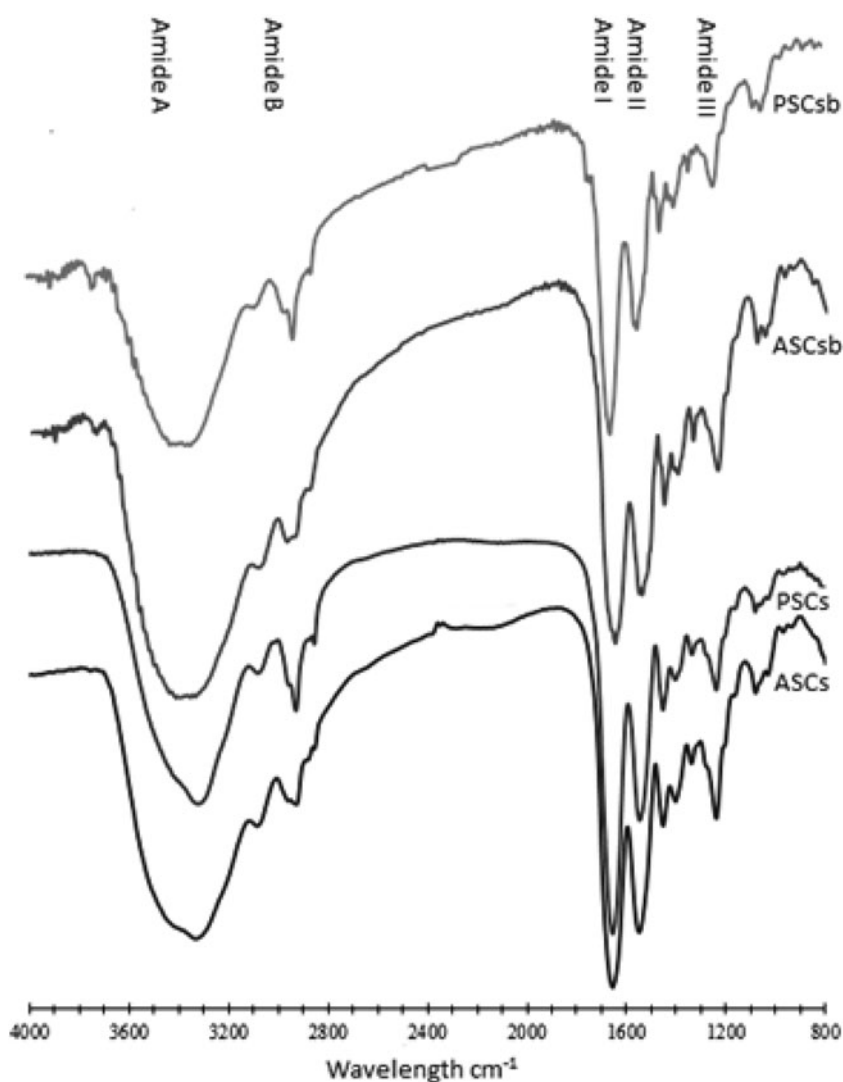


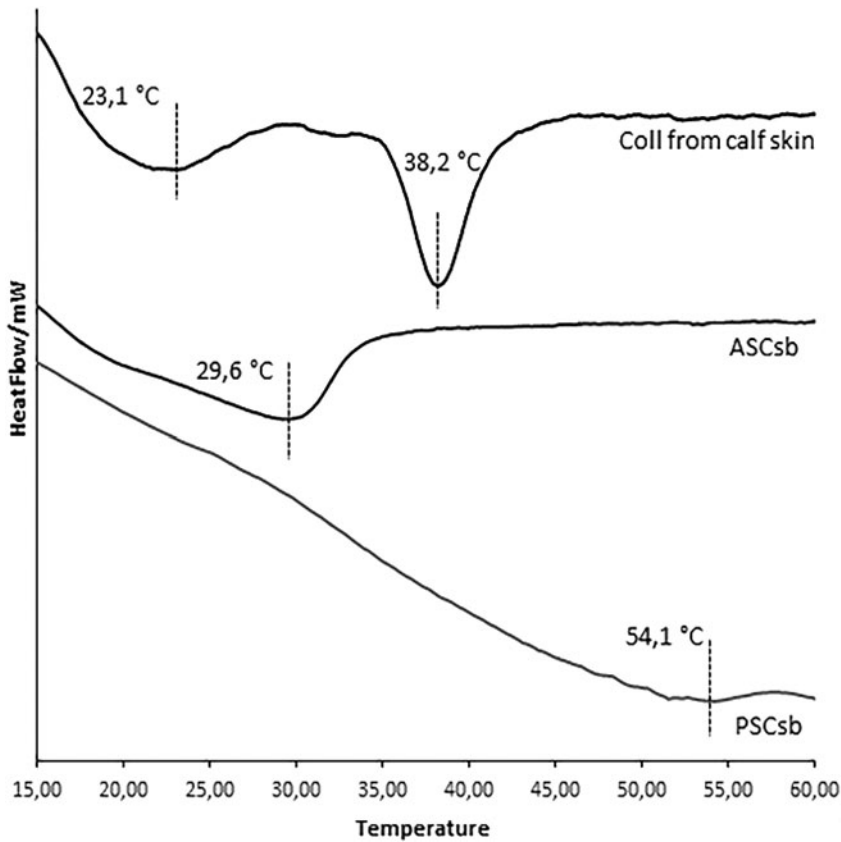
Figure 2. FTIR spectra of ASCs, PSCs, ASCsb and PSCsb from cod.

**Table 1.** Amino acid composition of ASCs, PSCs, ASCsb and PSCsb from cod.

Amino acid	ASCs	PSCs	ASCsb	PSCsb
	Residues per 1000 total amino acid residues			
Asp	49	51	50	51
Thr	22	23	21	22
Ser	68	69	66	72
Glu	74	70	73	71
Gly	348	347	337	338
Ala	121	127	122	126
Cys	3	3	3	3
Val	15	15	13	14
Met	22	18	19	20
Ile	8	8	8	8
Leu	21	18	21	21
Nleu	13	16	14	16
Tyr	3	3	4	4
Phe	16	15	18	17
OhLys	1	1	6	6
His	7	5	6	6
Lys	27	26	27	25
Arg	45	38	46	41
OhPro	50	53	54	53
Pro	89	95	94	88
Total	1000	1000	1000	1000

respectively, while amide I peak that is characteristic of the stretching vibrations of C=O groups of proteins occurred at  $1651\text{ cm}^{-1}$  and  $1649\text{ cm}^{-1}$ , respectively. The absorption band characteristic of amide II was very similar for ASCsb and PSCsb, arising at  $1543\text{ cm}^{-1}$  correspondent to NH bending vibration coupled with CN stretching;  $1454\text{ cm}^{-1}$ , attributed to CH<sub>2</sub> bending; and  $1398\text{ cm}^{-1}/1396\text{ cm}^{-1}$ , deriving from COO-symmetrical stretching, respectively. The band of amide III occurred at  $1337\text{ cm}^{-1}/1336\text{ cm}^{-1}$ , correspondent to NH bending associated with CN stretching; and  $1236\text{ cm}^{-1}/1238\text{ cm}^{-1}$ , relative to C-O stretching, respectively.

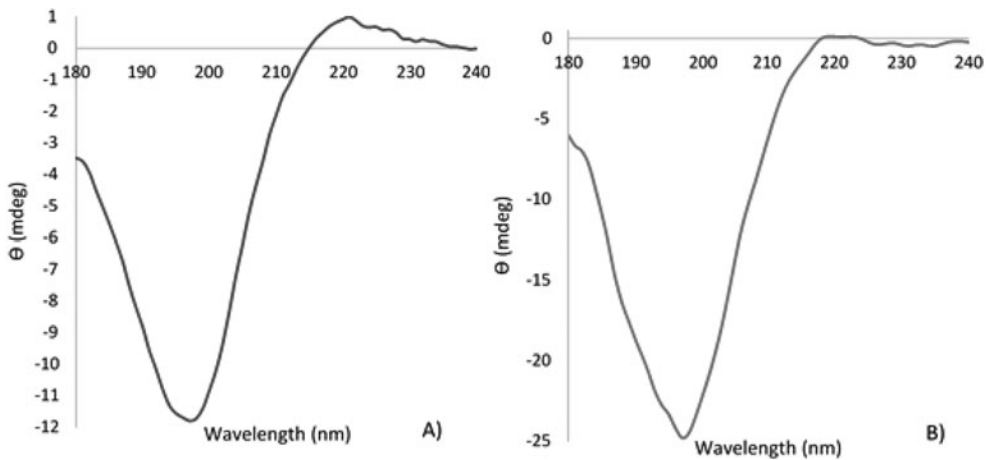
The amino acid composition was analysed in order to understand the quantitative composition of the extracted collagens. The results of this analysis (in n/1000 residues) for ASCsb and PSCsb are presented in Table 1 and compared with ASCs and PSCs, as reference. The amino acid content of collagen extract from cod swim bladders are similar to collagen extract from cod skin and the reported for other species [11,12,22,29]. The extracts are rich in glycine (Gly), alanine (Ala), proline (Pro) and hydroxyproline (OhPro), as characteristic of collagen. Glycine is the major amino acid in all extracts, being about 1/3 of amino acid residues, namely 337 for ASCsb and 338 for PSCsb per 1000 residues, followed by alanine with 122 and 126 residues, respectively. This is consistent with the fact that glycine occurs regularly at every third residue throughout most of the collagen molecules, with some exceptions like the first 14 amino acids from N-terminus and the first 10 from C-terminus [30], the telopeptides. The total proline contents (Pro and OhPro) of ASCsb and PSCsb are 148 and 141 residues/1000 residues, respectively. Proline and hydroxyproline are important for the structural integrity of collagen, being correlated to species (lower in fish than mammalian) and their living temperature [30]. In addition to the total proline contents, the hydroxylation degree is also relevant, namely for the structural integrity and thermal stability of collagens [11,12,22], being determined as 36,49%



**Figure 3.** Micro-DSC spectra of ASCsb, PSCsb and Coll from calf skin (used as reference).

and 37.59% for ASCsb and PSCsb, respectively, similar to the observed for ocellate puffer fish [31].

In order to verify the process of heat-induced denaturation, micro-DSC measurements were carried out. Figure 3 shows the DSC curves describing the heat flow to the collagen samples, ASCsb and PSCsb, as well as Coll from calf skin used as reference, when submitted to heating under inert atmosphere. The ASCsb and PSCsb DSC curves displayed only one endothermic peak, being different from the two obtained for coll from calf skin, used as reference as this is one of the principal sources of medical grade collagen [32]: one at 23.1 °C originated from the breaking of hydrogen bonds among collagen molecules [33] and another at 38.2 °C referring to denaturation process, which is directly related to: the number of amino acids like hydroxyproline [6,33], fish habitat [11], physiological temperature of the fish [34], hydration level of collagen [35] and type of extraction. For the acid extracted collagen, an evident peak was observed at 29.6 °C, corresponding to the denaturation temperature ( $T_d$ ), thus thermally less stable than the one observed for calf skin collagen, but within the higher values described by others for fish collagens, commonly associated with fresh water fish. Surprisingly, the thermogram for PSCsb does not exhibit a well-defined peak attributable to denaturation process, but only a wide band with a maximum at about 54.1 °C, which may be due to a melt-like process, not visible for

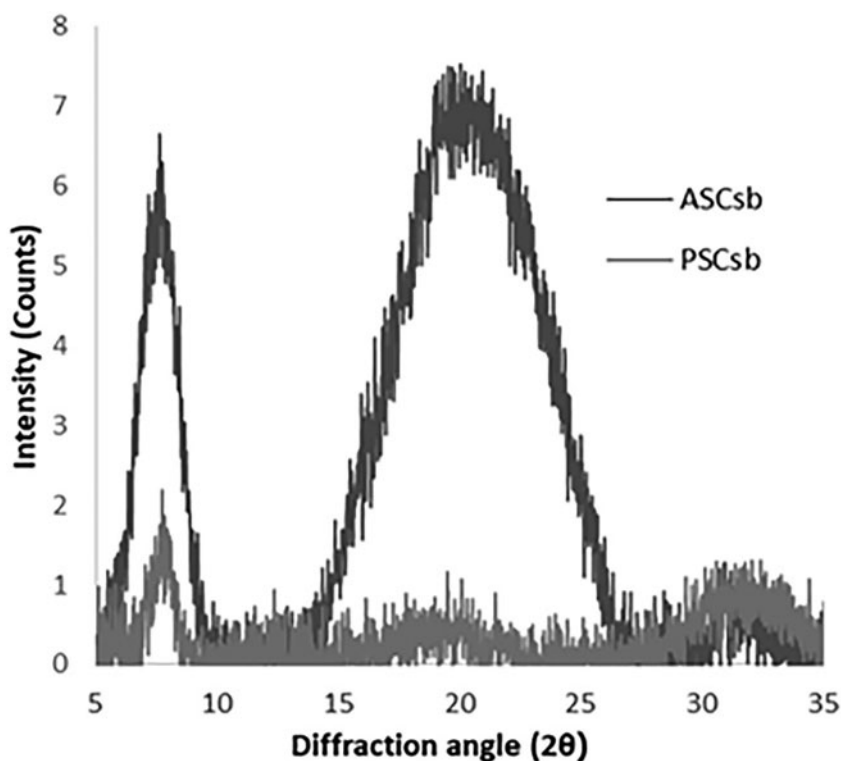


**Figure 4.** CD spectra of A) ASCsb and B) PSCsb from cod, measured at 4 °C.

the other analysed samples. This may be due to the fact that the PSCsb already has some degree of denaturation and less amount of preserved peptide alpha chains, which may be leading to a different structural organization and/or hydration level.

The secondary structure of ASCsb and PSCsb was determined by Circular dichroism (CD) and X-ray diffraction spectroscopy (XRD). CD was performed to confirm that the extracts were in the native state, being an effective technique to identify whether the triple helical structure was intact. ASCsb CD spectrum shown in Figure 4 depicts a positive peak at 221 nm (maximum positive cotton effect), characteristic of the presence of triple helix, and a negative peak at 198 nm (maximum negative cotton effect), with a crossover point (zero rotation) at approximately 215 nm, compatible with the observed by other authors [36,37]. However, the spectrum of PSCsb shows only a very small positive peak at 219 nm and a well-defined negative peak at 197 nm, which is indicative of a partially denaturation of the protein and consecutive loss of triple helix, which supports the discussion made above for the thermal characterization of the samples.

In a complementary analysis, the XRD spectra of both collagen samples were also obtained, as this technique is able to detect patterns as the ones that would be defined by the presence of preserved protein secondary structure. For both ASCsb and PSCsb spectra (Figure 5) one can identify two peaks, characteristics of collagen [36,38], with corresponding diffraction angles ( $2\theta$ ) of 7.64° and 20.07° for ASCsb, and 7.74° and 20.11° for PSCsb. The first sharp peak is associated with the triple helical structure of collagen [36] and from the Bragg equation one can determine the corresponding  $d$  value: 11.56 Å and 11.41 Å for ASCsb and PSCsb, respectively, indicating the distance between the molecular chains within the triple helix. From the value of the second broad peak angle it is possible to calculate the distance between skeletons, determined as 4.41 Å and 4.42 Å for ASCsb and PSCsb, respectively. These results are closed to that observed for grass carp and Nile tilapia [36,39], corresponding to the diameter of a collagen molecule with a triple helix structure and a single left-handed propeller chain. The two peaks are much more evident in the ASCsb spectrum, reflecting that ASCsb is in its native and non-denatured conformations, while PSCsb should have



**Figure 5.** XRD spectra of ASCsb and PSCsb from cod.

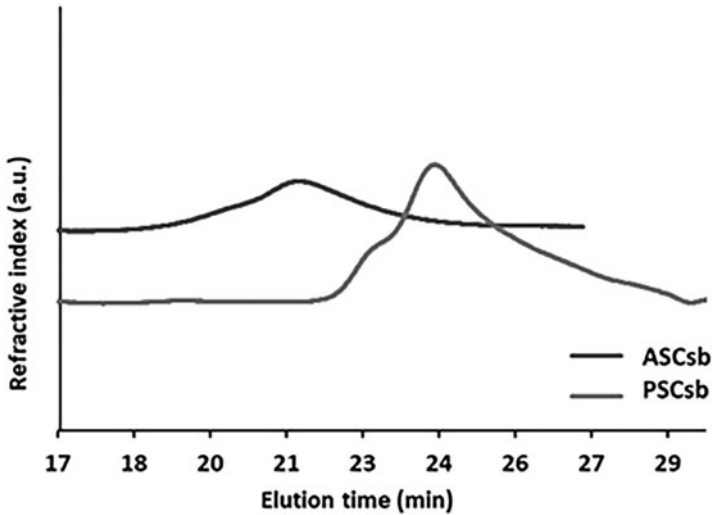
experienced some degree of denaturation, in agreement with the observed previously on the CD and thermal analyses.

### **3.4. Molecular weight determination by GPC-SEC**

A polymeric solution contains at least hundreds of polymer chains. These chains are not homogenous in nature. They probably differ in several physicochemical properties, the molecular weight being one of them [40]. The GPC-RALS method allows the determination of the Number Average Molecular Weight ( $M_n$ ) and Weight Average Molecular Weight ( $M_w$ ), as well as the polydispersity ( $M_w/M_n$ ) [41]. The  $M_n$  can be defined as Total mass of material divided by the total number of molecules, meanwhile the  $M_w$  can be described as the total mass of material multiplied by the molecules mass. The molecular weight of a substance, particularly a polymer, is a key chemical characteristic that can dramatically influence the material mechanical performance, particularly the viscosity and rheological behavior [42]. The results show that the higher molecular masses correspond to ASCsb (Table 2). This is confirmed by the elution times showed in the chromatograms, where high molecular weight molecules will elute first. These chromatograms are wide, indicating broad molar mass distribution. This is in concordance with the PDI values obtained. Although not manifested in its PDI, PSCsb has probably a subtle tendency to form aggregates. This particularity can be deduced from the form of its RI curve, which presents a lump in

**Table 2.** Molecular weights and polydispersities of the studied collagens. The average values of the obtained Number Average Molecular Weights (Mn), Weight Average Molecular Weights (Mw) and polydispersities (Mw/Mn) are displayed, together with the corresponding standard deviations.

Collagen	Mn (kDa)	Mw (kDa)	PDI
ACSsb	128.8 ± 6.4	182.9 ± 7.9	1.420 ± 0.010
PSCsb	23.6 ± 2.7	28.7 ± 1.7	1.227 ± 0.150



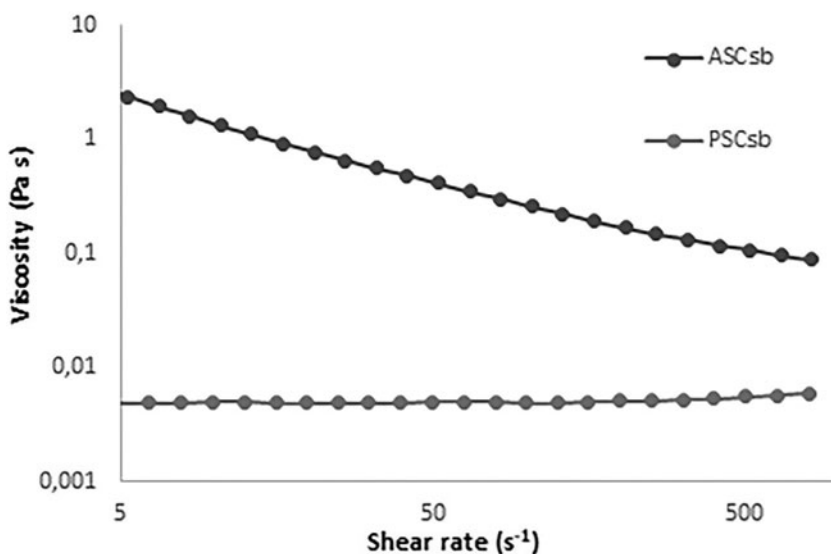
**Figure 6.** Gel permeation chromatograms of the studied collagens. The obtained refractive indexes (RIs) are expressed at the corresponding elution times.

its left hillside (Figure 6). The ACSsb result is in agreement with the SDS-PAGE profile but the same is not observed in the result of PSCsb, possibly due to the conformation of the molecule.

### 3.5. Rheological behavior of collagen extracts

The viscosity behavior of a 3 wt. % aqueous solution of the extract obtained as a function of shear rate was evaluated, not only regarding the characterization of the materials but also toward application development, with the obtained results described in Figure 7.

Flow behavior was described by the fitting of the experimental data (shear stress-shear rate) with the power law model (Equation (3)):  $\tau = k\dot{\gamma}^n\eta$ , where  $\tau$  is the shear stress (Pa),  $\dot{\gamma}$  is the shear rate ( $\text{s}^{-1}$ ),  $k$  is the consistency coefficient (Pa.sn) and  $\eta$  is the flow behavior index (Ma et al., 2014). ACSsb and PSCsb presented a flow behavior index  $\eta = 0.33 \pm 0.01$  and  $\eta = 0.61 \pm 0.16$ , respectively, characteristic of non-Newtonian fluids (for Newtonian liquid,  $n = 1$ , pseudo plastic fluid  $n < 1$  and swelling plastic fluid  $n > 1$ ). Moreover, ACSsb and PSCsb samples exhibits a typical shear thinning behavior for different shear rates, characterized by a decrease in the viscosity with increasing shear rate and a linear relationship between the shear rate and shear stress [26]. This shear-thinning (pseudo plastic) behavior, also present in hyaluronic



**Figure 7.** Viscosity curve of ASCsb and PSCsb (3 wt %) extracted from cod.

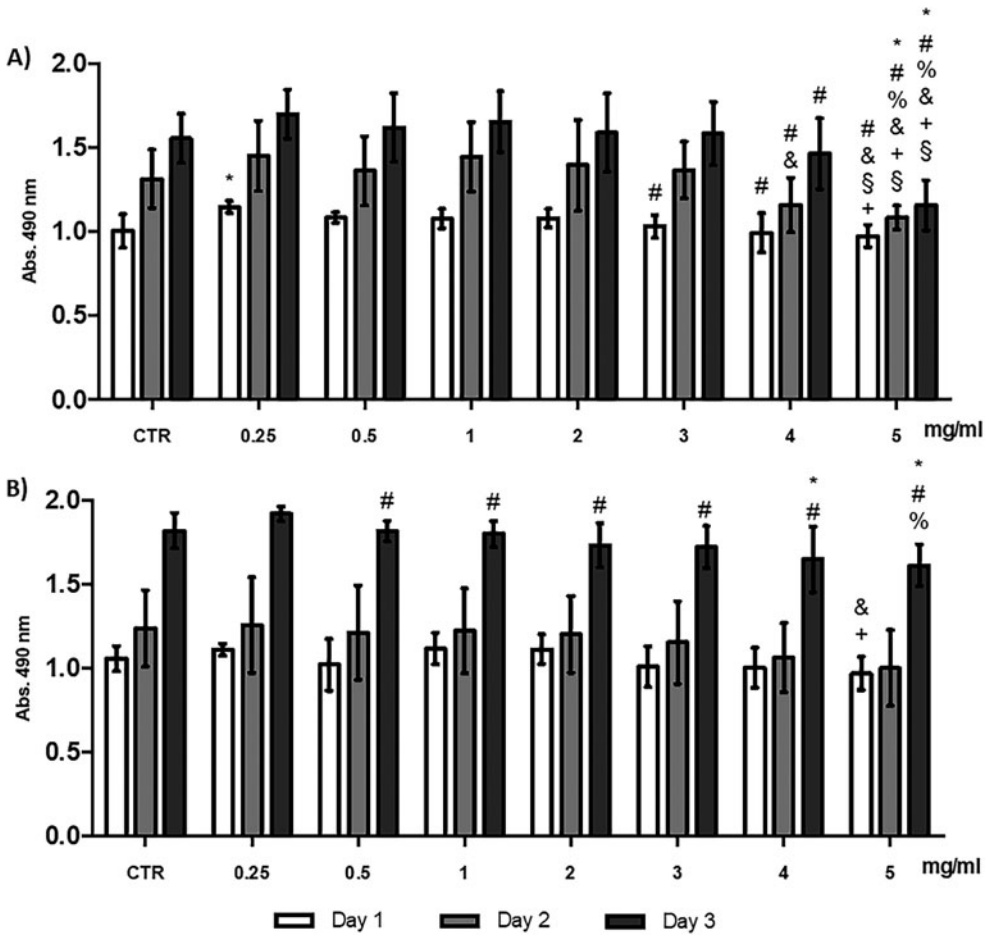
acid aqueous solutions [43,44], reveals to be an interesting rheological property regarding its further processing, namely on the production of different types of biomaterials, including injectable systems, envisaging biomedical application, namely on tissue engineering and regeneration, but also as component in cosmetic formulations.

### 3.6. Cytotoxicity

In the present study, the eventual cytotoxic effect of collagen extracts over MRC-5 human fibroblasts was evaluated by assessing their metabolic activity after culturing the cells in the presence of varying concentrations of extracts (0.25 – 5 mg/mL) for 1, 2, and 3 days (Figure 8A and B), by using the MTS assay. No significant variation in the metabolic activity of MCR-5 cells was observed after 1 day of incubation, which is in accordance with previously published results demonstrating that fish collagen is not cytotoxic after 1 day of incubation at concentrations below 1% (10 mg/mL) [32]. Nevertheless, a slight cytotoxicity was observed with the higher concentrations of ASCsb extract (5 mg/mL, day 2 and 3) and PSCsb extract (4 and 5 mg/mL, day 3). Despite the differences observed for the higher concentrations in respect to the control, maybe caused by experimental artifact, cell viability is still quite high. Therefore, these results are quite promising when envisaging the use of these collagens isolated from Atlantic cod swim bladder in biomedical context, namely on the development of biomaterials (hydrogel, membrane or other).

## 4. Conclusions

A feasible methodology for the extraction of ASCsb and PSCsb from Atlantic cod swim bladders was established, with the respective products being compatible with



**Figure 8.** Metabolic activity of human fibroblast cell line (MRC-5) cultured in the presence of A) ASCsb and B) PSCsb, at different concentrations, during 1, 2 and 3 days. Data were considered statistically different if  $p < 0.05$ . \* indicates significant differences when compared to CTR; #, when compared to 0.25 mg/mL; %, when compared to 0.5 mg/mL; &, when compared to 1 mg/mL; +, when compared to 2 mg/mL; \$, when compared to 3 mg/mL.

type I collagens, according with the SDS-PAGE profile. The results of the FTIR, XRD and CD analyses indicated that the acid extraction rendered collagen with preserved triple helix structure, while extraction with pepsin – although capable to increase the collagen production yield – had an adverse effect over the triple-helical structure, resulting in partial denaturation of collagen. The collagen extracts solutions obtained presented a physical viscous appearance, with interesting rheological and cytotoxicity behavior envisaging their biomedical application, namely to be processed further as biomaterials for tissue engineering, given the shear thinning behavior and neglected effect over human fibroblasts viability. Swim bladders can thus be considered as a good alternative source for the production of collagens, in a biotechnological strategy enhancing the value of fish by-products.



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