Characterization of codfish gelatin: a comparative study of fresh and salted skins and different extraction methods

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Abstract

The use of alternative sources for gelatin extraction is in demand in today's industries. Fish skins are an economical and sustainable source option. However, there is a lack of information about the preservation state of skins (fresh, frozen, salted, etc.) and how that affects the gelatin yield and properties, and therefore, compromise the final product.

In this study we present a comparative analysis between different reported gelatin extraction processes for fresh and salted codfish (*Gadus morhua*) skins. The extracted products were characterized based on yield of extraction, amino acid composition, molecular weight distribution, rheological properties and gel strength, as well as the cell compatibility of the gelatins envisaging future biomedical applications.

Results showed that extraction method affected the yield and gelatin properties within the same type of fish skin. Thus, it was found that water acidification step, demonstrated higher extraction yield, while other methods produced gelatins rich in OH-proline+proline, promoting enhanced gel strength and rheological properties. There is thus a compromise between yield and gelatin properties that industries need to understand before selecting their gelatin extraction method. Results, also showed that gelatins derived from salted skins demonstrated lower viscoelastic properties and gel strength, when compared with gelatins from fresh skins.

Our research represents a unique comparative compilation of different extraction methods in cod skins differently conserved, as a tool on the quest for the sustainable valorization of fish by-products, included in a circular economy framework.

Keywords

Fish gelatin; extraction; by-products valorization; gel strength; Marine biomaterials

1 Introduction

Gelatin is the product generated by partial hydrolysis of native collagen, the major structural protein of the human body present in several connective tissues (Ward & Courts, 1977). The chemical properties of gelatin are very similar to collagen, composed by repeated units of motif of Gly-X-Y, where X and Y are, predominantly, proline and hydroxyproline (Gomez-Guillen, et al., 2009). Thermal denaturation of collagen leads to the cleavage of hydrogen and covalent bonds destabilizing the triple helix and then, generating a mixture of peptides with heterogeneous molecular weights depending on the source and production processes (Gorgieva & Kokol, 2011). Due to its natural origin, biocompatibility, biodegradability, viscoelastic properties and commercial availability at relatively low cost, gelatin – typically produced from type I collagen – is being widely used for several technological purposes such as in food processing (Ding, et al., 2020; Etxabide, Uranga, Guerrero, & de la Caba, 2017), cosmetic (Chen & Hou, 2016; Sun, Zhang, & Zhuang, 2013) and pharmaceutic (Kang, et al., 2019; Nayak, Babla, Han, & Das, 2016) industries as well in biomedical and tissue engineering applications (Luetchford, Chaudhuri, & Paul, 2020; Negrini, et al., 2019; Tijore, et al., 2018; Yue, et al., 2015). The primary sources of gelatins are from mammal origin namely porcine and bovine skin (46% and 29.4%) and bones (23.1%) (Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011). Due to religious constraints (Halal, Kosher and Hindu) combined with risks associated to zoonosis such as bovine spongiform encephalopathy (BSE), the industry is looking for new reserves of gelatin.

Fishery industries are known for generating tons of by-products every year where more than 30% are skins and bones (Gomez-Guillen, et al., 2002) that can be transformed and used for several trades, from animal feed industry to biotechnological and medical purposes, namely by the extraction of biopolymers with biomedical relevance, such gelatin, for tissue engineering (TE) applications. The tradition of codfish industry is strongly implanted in Galicia (NW of Spain) as well as in Portugal, being one of the top 3 most captured and consumed fish by those countries (Almeida, Karadzic, & Vaz, 2015; Gonzalez-Lopez, 2012; Martín, 2011). Cod products can be available as fresh fish (fillet product) or dried and salted, being the last one the dominant market in the Portuguese context. Our strategic position offers unique and privileged access to its by-products, offering an excellent opportunity to explore them for biotechnological uses, particularly biomedical purposes. The quality of gelatin depends firstly on the raw materials used and on the chosen extraction method and variables such temperature, pH, extraction time and presence of salts have a clear influence on its properties and gelation capacity (Ahmad, et al., 2017).

In this study, the main objective was to evaluate the impact of different extraction methods and codfish skins preservation state in the gelatin retrieval yields and properties. Two types of cod skins, differently preserved, were tested: fresh skins (non-salted) from Galician fishing companies, and salted skins from Portugal cod processing companies. This work represents to the best of our knowledge, the first specific study comparing different reported gelatin extraction processes applied to fish skins distinctly preserved. This study is a valuable tool for entities

looking for a more sustainable and economic source of gelatin, which is made by the valorization of fish industry by-products.

2 Materials and Methods

2.1 Chemical reagents

Acetic and citric acid was purchased from Scharlau (Scharlab s.L., Mas d´En Cisa, Spain), sulfuric acid was bought from Fisher Chemical (Fisher Scientific, Loughborough, UK) and sodium hydroxide was purchased from Analema (Comercial Lab, Vigo, Spain). Sodium chloride, active charcoal, phosphate-buffered saline (PBS), Dulbecco's PBS (DPBS) and Dulbecco's Modified Eagle's medium (DMEM) low glucose were acquired from Sigma-Aldrich (Missouri, EUA). Fetal bovine serum (FBS), antibiotic-antimycotic (100X) were obtained from ALFAGENE® (Carcavelos, Portugal).

2.2 Raw material

Fresh and salted skins from Atlantic codfish (*Gadus morhua*) were provided by fish processing industries, Fandicosta S.A. (Domaio, Moaña, Spain) and Frigoríficos da Ermida, Lda (Gafanha da Nazaré, Portugal), respectively. Cod skins were transported to the laboratory facilities and stored at -20°C until further use. In all cases, the skins were initially cut in portions less than 5 x 5 cm and 500 g of these fragments were processed per batch. After defrosting, skins were cleaned from all impurities, including bones and meat, and washed with distilled water.

2.3 Experimental Design of skin codfish gelatin extraction

Eight different extraction methods were designed and tested firstly on fresh codfish skins, with methods 1 to 6 using gelatin extraction on hot water, and methods 7 and 8 using gelatin extraction on hot acidic solution (**Table 1**). Methods 1 and 2 (M1 and M2) are based on the application of three sequential washes by sodium hydroxide, sulfuric acid and citric acid, followed by thermal extraction on aqueous medium, purification and deodorization of gelatin solution by filtration/active charcoal adsorption/centrifugation and final gelatin drying in oven (S. C. Sousa, Vazquez, Perez-Martin, Carvalho, & Gomes, 2017) (Figure S1, Supplementary Material, and **Table 1**). In M1, chemical treatments were performed at room temperature (RT, 22°C), whereas in M2 they were run at T= 4 ºC. Methods 3 and 4 (M3 and M4) are based on the chemical treatment by citric acid and subsequent water thermal extraction, purification, and deodorization (filtration/charcoal adsorption/centrifugation) of gelatin solutions and oven-drying (Figure S2, Supplementary Material and **Table 1**). In M3, citric processing was performed at RT (22°C), whereas in M4 it was run at T= 4 ºC. Method 5 (M5) was based on the direct thermal extraction of cod skins in water followed by filtration/charcoal adsorption/centrifugation of gelatin solution

and freeze-drying (Figure S3, Supplementary Material and **Table 1**). In method 6 (M6), based on the descriptions of Gómez-Guillén *et al.*, (2001 and 2002) (Gomez-Guillen & Montero, 2001; Gomez-Guillen, et al., 2002), the steps of processing were: sequential treatments using salt (sodium chloride), alkali (3 times) and acetic acid (including an aqueous wash of the skins between them), thermal extraction of soluble gelatin, purification (as described above) and drying in oven (Figure S4, Supplementary Material and **Table 1**). Methods 7 and 8 (M7 and M8) were based on the thermal extraction of gelatin in acidic conditions (phosphoric acid), with a previous alkali treatment, and subsequent purification and deodorization (filtration/charcoal adsorption/centrifugation) of gelatin solutions and oven-drying (Figure S5, Supplementary Material and **Table 1**) (Benjakul, Oungbho, Visessanguan, Thiansilakul, & Roytrakul, 2009). In M7, the alkali treatment was performed at RT (22°C), whereas in M8 it was run at T= 4 ºC. The methods were then applied to salted cod skins, with the exception of methods M3, M4 and M5 that presented a very low yield and viscosity with fresh cod skins (**Table 1**). Each extraction protocol was performed in duplicate.

Table 1. Details on the different methods used for the extraction of gelatin included in this study.

Methods	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**		**M7**	**M8**
Skin preservation type	Fresh and Salted		Fresh		Fresh	Fresh and Salted		Fresh and Salted	
Pre-treatment temperature	22°C	4°C	22°C	4°C	_	22°C	4°C	22°C	4°C
Pre-treatment solutions	0.2% (v/w) NaOH 0.2% (w/v) sulphuric acid 1% (v/w) citric acid		1% (v/w) citric acid		_	0.8 M NaCl 0.2 M NaOH 0.05 M acetic acid		0.4% (v/w) NaOH	
Extraction	water extraction 16 h 45°C							0.2% (v/w) Phosphoric acid 3 h 50°C	
Filtration									
Active charcoal lavage									
Dry by oven 48 h									

2.4 Gelatin Yield

The yield of gelatin extraction was calculated considering the wet weight of skins before extraction and the dry weight of gelatin by using the following equation:

$$Yield\ of\ gelatine\ extraction\ (\%) = \frac{Weight\ of\ dried\ gelatine\ (g)}{Weight\ of\ wet\ skin\ (g)} \times 100$$

2.5 Amino acid profile

The amino acid content of extracted gelatins was determined by quantitative amino acid analysis using a Biochrom 30 series (Biochrom Ltd., Cambridge, U.K.) at Centro de Investigaciones Biologicas of the Spanish National Research Council (CSIC), in Madrid (Spain). First, the samples were hydrolyzed and separated through a column of cation-exchange resin following a procedure developed by Spackman, More and Stein in 1958 (Moore, Spackman, & Stein, 1958). The column eluent was mixed with ninhydrin reagent and eluted at high temperature. This mixture reacted with the amino acids forming colored compounds that were analyzed at two different wavelengths: 440 and 570 nm. An internal standard of norleucine was used for quantitative analysis. The sum of amino acids, in each gelatin sample was used to assess the purity of the extracts regarding the % of protein content. Three independent measurements for each sample were performed for the quantification of the average amino acid contents.

2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis - (SDS-PAGE)

SDS-PAGE was prepared using Sigma SDS-PAGE reagents and casted on a vertical electrophoresis unit from Sigma-Aldrich (Missouri, EUA). Gelatin solutions were prepared by dissolving 5 mg/mL in deionized water at 45 °C under stirring until complete dissolution and then was mixed with 1X Laemmli buffer at a final concentration of 1 mg/ml. The samples were heated in an Eppendorf ThermoMixer C at 60 °C for 30 min and then at 95 °C for 5 min for total protein denaturation and centrifuged at 10.000 g for 1 min to sediment eventual undissolved material. After that, 40 µg of gelatin was loaded to each well and run in a 10% polyacrylamide gel. Also, 4 µL of protein ladder was loaded along with the samples. The electrophoresis was carried out at 25 mA until the frontline reached the lower part of the gel. After the run, the gels were stained with a Coomassie (0.125% Coomassie Blue R 250 (Biorad), 50% Methanol, 10% Acetic acid) staining solution overnight and then soaked in destaining solution (5% Methanol, 7% Acetic acid) overnight.

2.7 Gel permeation chromatography – size exclusion chromatography (GPC-SEC)

The molecular weights of cod gelatins were analyzed by gel permeation chromatography with an Agilent 1260 LC system consisting of quaternary pump (G1311B), injector (G1329B), column oven (G1316A), DAD (G1315C) refractive index (G1362A) and dual angle static light scattering (G7800A) detectors. Proteema precolumn (5 µm, 8x50 mm), Proteema 100 Å (5 µm, 8x300 mm), Proteema 300 Å (5 µm, 8x300 mm) and Proteema 1000 Å (5 µm, 8x300 mm) (PSS, Mainz, Germany) were used for polymer separation. The system was kept at 20°C and 0.15M sodium acetate: 0.2 M acetic acid, pH 4.5 was used as mobile phase, at a rate of 0.5 mL/min. Samples were dissolved at 1.8-2.2 g/L in the GPC mobile phase. All samples seemed fully dissolved, with exception of S7 and S8 from salted skins. To avoid errors due to incomplete dissolution of samples, a refractive index increment (dn/dc) of 0.190 (Blanco, Sanz, Valcarcel, Pérez-Martín, & Sotelo, 2020) was used to estimate the molecular weight.

2.8 Determination of gelatin strength

A standardized protocol (Wainewright, 1977) was used to measure the strength of fresh and salted codfish skins gelatins. As described in literature (Gomez-Guillen, et al., 2001), 6.67% (w/v) gelatin solution was prepared by dissolving 2 g of dried gelatin in 30 mL of deionized water at 45 °C, and after total dissolution, cooled at 4 °C for 16-18 h. Gel strength was measured. using a Stevens-LFRA Texture Analyzer (Hucoa Erlöss S.A., Madrid, Spain) with a 1000 g load cell equipped with a 0.5 inch of diameter Teflon probe. A trigger force of 5 g and a penetration speed of 1 mm/s were used, and gel strength was expressed as maximum force (in g), taken when the plunger had penetrated 3 mm into the gelatin gels, as average of three determinations.

2.9 Rheological behavior

The dynamic rheological properties of the gelatin solutions were measured on a Kinexus Pro+ rheometer (Malvern Instruments, UK) using the acquisition software rSpace. The measuring system was composed by a 4° cone plate geometry (CP4/40 SR1772SS) and a 0.15 mm gap. The experiments were performed following the instructions of Fernández-Díaz *et al.* (2001) (Fernández-Díaz, Montero, & Gómez-Guillén, 2001) with slight modifications. Briefly, a solution of 6.67% (w/v) of each gelatin were dissolved at 45 °C and them cooled at RT. The samples were placed in the plate and the excess removed. The dynamic rheological properties were measured from 2 to 30 °C at a rate of 1 °C/min and then cooled from 30 to 2 °C at a rate of 0.5 °C/min, with an oscillating stress of 3.0 Pa and a constant frequency of 1 Hz. The elastic modulus (G'), viscosity modulus (G'') and the phase angle (tan δ = G''/G') were verified and presented as a function of temperature. The cross-over point of G' and G'' was considered as the melting/gelling point of the gelatins. All plots are represented as the average of at least 3 experiments.

2.10 Biological assessment

2.10.1 Cell culture

In vitro studies were performed using L929 mouse fibroblast cell line (ATCC® CCL-1™). Cells were maintained in DMEM with low glucose supplemented with 10% FBS (Gibco) and 1% antibiotic-antimycotic solution (Gibco), at 37 °C in a humidified atmosphere containing 5% CO_2. Medium was exchanged every 2-3 days and cells were subcultured before they reach confluence. Cells were used between 17 and 22 passage.

2.10.2 Cytotoxicity of codfish gelatins

To assess the cytotoxic effect of gelatins over L929 cells, 15 000 cells were seeded onto 48-well plates and left to adhere for 24 h. Then, the gelatins that were previously dissolved in culture medium, were added to the cells at different concentrations: 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mg/mL. Cells were incubated with gelatins for 24, 48, and 72 h. In each experiment, a negative control (untreated cells), a positive control (cells treated with 5% DMSO), and a background control (medium without cells) were used. Each experiment was performed in triplicate and three independent assays were performed. The cytotoxic effect of gelatins was assessed by evaluation of metabolic activity of cells using MTS assay (CellTiter 96 AQueous One Solution, Promega). In this assay, the quantity of formazan produced is directly proportional to the number of living cells in culture. At the end of the 24, 48 and 72 h incubation periods, the culture medium was removed, and cells were rinsed in sterile 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 atmosphere containing 5% CO_2. After that, 100µL of MTS reaction medium was transferred to a 96-well plate in duplicate and the absorbance was measured at 490 nm in a microplate reader (Synergy HT, Bio-TEK). Results are expressed as percentage relative to the negative control.

2.11 Statistics

Statistical analysis was performed following the specificities of each experiment. For gelatins extraction yields, amino acid analysis, molecular weight and gel strength, a n=2 (replicates of independent batches) were performed and the IC (interval of confidence) with α=0.05 was used. In case of cytotoxicity experiment, Graph Pad Prism 8.01 software (San Diego, CA, USA) was used for multiple variable comparisons by two-way ANOVA followed by Dunnett's test for comparations between samples and the cell control and Tukey's test for multiple comparations between different concentrations. Statistical significance was defined as $p < 0.05$.

3 Results and discussion

3.1 Gelatin extraction and yield

It is known that the type of treatment applied during the extraction process has huge implications on gelatin properties (Gomez-Guillen, et al., 2011; Gomez-Guillen, et al., 2002; Milovanovic & Hayes, 2018). Thus, different methods based on successive rinses in acidic solutions followed by thermal extraction (M1-M4), direct thermal extraction with water (M5), an extraction based on a pre-treatment with salt, alkali and acid solutions followed by thermal extraction with water (M6) and thermal extraction with acid solutions (M7 and M8) (see supplementary material and **Table 1**), were used for the production of gelatin from codfish skins either fresh and salted.

The yield of each type of extraction was evaluated and represented in **Table 2**. It was observed a recovery between 7 and 19% of gelatin, which is consistent with what is reported in literature for the extraction of fish gelatins (Karim & Bhat, 2009). In general, no significant differences were observed in the gelatin yield (%, w of gelatin/w of skin) when using fresh and salted skins with the same extraction method (**Table 1**). However, an accurate comparation can only be made by knowing water and salt content of the samples since this can influence the initial weight of the material. Within each type of skins, M7 and M8 stand out with higher extraction yields ($p<0.05$). It can also be noticed that M7 applied on fresh skin was the best method to recover the largest amount of gelatin ($p<0.05$). This can be related with the use of phosphoric acid (weak acid) in water for the extraction process of gelatin and with the absence of any kind of acidic pre-treatment that normally leads to a loss of collagen through leaching during the series of washing steps (Jamilah & Harvinder, 2002). This strategy allows all collagen present in the skins to remain available for hydrolysis therefore increasing the yield of gelatin extracted by this direct method. Although we have not observed large differences, it seems the protocols where a pre-treatment at 4 °C was applied (M2, M4 and M8) had better yield ratio than the treatments performed at RT, with exception of M8 for fresh skins. This was also observed in the work of Zhou and Regenstein for pollock skin gelatin (Hou & Regenstein, 2004) were a pre-treatment at RT led to a significant loss of gelatin, thus recommending a pre-treatment at low temperatures.

We firstly started to extract the gelatins from fresh cod skins using methods from 1 to 8. After assessing the yield of extraction and observing the viscosity of the resulting gelatin solutions, we discarded M3, M4 and M5 (lower yield and/or low viscosity) and selected the methods M1, M2, M6, M7 and M8 for the extraction in salted skins, since these seemed to be the most promising methods.

Table 2. Results of gelatin yield of extraction from fresh and salted skins of Atlantic cod using the extraction methods described in materials and methods. Values are average ± intervals of confidence for n=2 (replicates of independent batches) and α=0.05.

Yield (%, w of gelatin/w of skin)		
Method	**Fresh Skins**	**Salted Skins**
1	7.49 ± 0.89	6.74 ± 0.67
2	13.92 ± 0.03	12.38 ± 2.27
3	7.11 ± 0.31	-
4	7.36 ± 0.38	-
5	12.70 ± 0.33	-
6	10.81 ± 1.67	9.41 ± 1.03
7	18.52 ± 1.02	14.47 ± 0.80
8	15.19 ± 0.92	15.14 ± 1.27

3.2 Amino acid analysis

The amino acid content is important to evaluate the quality of gelatin, having a predominant role on the properties of this material. Gelatin is derived from thermal hydrolysis of collagen and the respective amino acid sequence can have slightly differences according to the animal species, animal aging, extraction conditions (Haug & Draget, 2011). The most relevant amino acids to consider in the gelatin composition are hydroxyproline (almost exclusive of collagen protein), glycine and proline, as collagens are composed by a sequence of amino acid triplets Gly-X-Y where X is commonly proline and Y is often hydroxyproline. **Table 3** and **Table 4** show the amino acid composition of gelatins extracted from fresh and salted cod skins, respectively. The protein content in samples ranged from 82.3 – 99.8%. In both types of skin, slight but not significant differences on amino acid composition can be observed among the gelatins produced with the different methods used. Considering the amino acid sum of OH-proline + proline and glycine content, methods M1 and M2 revealed slightly higher values, for both fresh and salted skins, than the other methods used. Analyzing the results more closely, for fresh cod skins (**Table 3**), the composition of OH-proline + proline, and glycine was 18.02% and 23.60%, respectively, for M1, and 18.26% and 23.28% for M2 method. For salted cod skins (**Table 4**), the results were similar, with 17.58% and 24.17% for M1 and 18.08% and 23.58% for M2. These results are consistent with those reported by Gusmundsson and Hafsteinsson (Gudmundsson & Hafsteinsson, 1997) and Arnesen & Gildberg (Arnesen & Gildberg, 2007) for codfish gelatin using similar strategy and similar to other cold-water fish skins such as Atlantic salmon (Arnesen, et al., 2007) or Alaska Pollock (Zhou, Mulvaney, & Regenstein, 2006). These specific amino acids are a very important components affecting gelatin properties. It is described that those pyrrolidine amino acids have a critical role in the stabilization of triple helical structure of renatured gelatins (Gomez-Guillen, et al., 2002), specially hydroxyproline due to its ability to form hydrogen bonds by -OH groups. This is an important fact to consider in gelatin strength. Despite the fact that some authors have

indicated that the hydroxyproline content is influenced by the extraction conditions (Nikoo, et al., 2013), this was not observed in the different methods used in our experiments. Also, the different temperature in which the protocols were performed did not demonstrate any significant differences ($p>0.05$), in both skin types, between protocols (M1/M2; M3/M4; M7/M8).

Table 3. Amino acids content of gelatins recovered from fresh cod skins (% or g/100 g total amino acids) using different extraction methods. OHPro: hydroxyproline. Pr: % of protein present, as the sum of amino acids, in the extracted gelatin sample. TEAA/TAA: ratio total essential amino acids for human/total amino acids. Errors are the confidence intervals for n=2 (replicates of independent batches) and α=0.05.

Amino acids	M1	M2	M3	M4	M5	M6	M7	M8
Asp	6.35±0.16	6.14±0.30	6.52±0.01	6.20±0.04	6.48±0.07	6.37±0.24	6.24±0.20	6.37±0.12
Thr	2.56±0.06	2.51±0.09	2.62±0.02	2.50±0.03	2.64±0.01	2.59±0.05	2.51±0.04	2.63±0.10
Ser	6.47±0.05	6.73±0.04	6.52±0.04	6.66±0.46	6.48±0.03	6.61±0.09	6.40±0.22	6.74±0.21
Glu	9.73±0.14	10.09±0.36	10.10±0.13	10.30±0.25	10.36±0.09	10.19±0.17	9.52±0.45	9.68±0.31
Gly	23.60±0.84	23.28±0.02	23.28±0.16	24.10±0.46	23.39±0.29	23.07±0.57	22.98±1.08	22.74±1.38
Ala	8.96±0.14	8.93±0.33	9.15±0.10	9.41±0.15	8.84±0.12	9.05±0.20	8.82±0.33	8.72±0.39
Cys	0.40±0.04	0.35±0.01	0.61±0.05	0.49±0.15	0.57±0.07	0.36±0.03	0.52±0.10	0.58±0.25
Val	2.24±0.04	2.12±0.04	2.19±0.01	1.74±0.06	2.16±0.07	2.07±0.20	2.18±0.08	2.33±0.12
Met	2.33±0.06	2.18±0.01	2.07±0.04	2.20±0.16	2.31±0.21	2.29±0.08	2.19±0.02	2.24±0.20
Ile	1.29±0.04	1.37±0.02	1.49±0.03	1.06±0.03	1.65±0.33	1.49±0.19	1.33±0.05	1.52±0.21
Leu	2.44±0.02	2.49±0.07	2.55±0.01	2.42±0.04	2.78±0.13	2.56±0.11	2.50±0.12	2.61±0.13
Tyr	0.96±0.06	0.83±0.03	0.78±0.03	0.83±0.03	0.81±0.03	0.94±0.09	1.18±0.33	1.14±0.35
Phe	2.19±0.10	1.98±0.09	1.91±0.09	1.93±0.05	2.00±0.10	2.08±0.17	2.30±0.21	2.04±0.25
His	1.14±0.05	1.18±0.12	1.08±0.02	1.00±0.02	1.11±0.00	1.05±0.12	1.30±0.26	1.31±0.28
Lys	3.49±0.42	3.64±0.11	3.53±0.10	3.48±0.20	3.67±0.09	3.64±0.21	3.87±0.39	3.70±0.10
Arg	7.83±0.25	7.91±0.63	8.04±0.14	7.96±0.02	7.60±0.12	7.88±0.34	8.54±0.22	7.99±0.13
OHPro	7.15±0.26	7.41±0.44	7.66±0.22	7.29±0.33	7.33±0.41	7.12±0.34	7.23±0.68	7.35±0.43
Pro	10.87±0.55	10.85±0.58	9.89±0.12	10.43±0.16	9.82±0.24	10.65±0.40	10.40±0.04	10.31±0.12
Pr (%)	97.8±1.2	99.8±4.9	94.8±1.9	98.1±0.7	93.4±3.0	96.8±8.9	85.8±6.1	82.3±5.6
TEAA/TAA (%)	27.8±1.1	27.5±0.6	27.7±0.1	26.0±0.6	28.1±0.5	27.7±1.3	28.9±1.2	28.7±1.4

Table 4. Amino acids content of gelatins recovered from salted cod skins (% or g/100 g total amino acids) using different extraction methods. OHPro: hydroxyproline. Pr: % of protein present, as the sum of amino acids, in the extracted gelatin sample. TEAA/TAA: ratio total essential amino acids for human/total amino acids. Errors are the confidence intervals for n=2 (replicates of independent batches) and α =0.05.

Amino acids	M1	M2	M6	M7	M8
Asp	6.14±0.27	6.38±0.11	6.39±0.03	6.46±0.32	6.19±0.03
Thr	2.43±0.08	2.49±0.07	2.54±0.01	2.58±0.15	2.47±0.05
Ser	6.38±0.17	6.16±0.12	6.62±0.09	6.04±1.03	6.37±0.07
Glu	10.06±0.07	9.98±0.01	10.19±0.04	10.23±0.37	10.08±0.11
Gly	24.17±0.34	23.58±0.28	23.45±0.21	23.10±0.19	24.20±0.23
Ala	9.41±0.02	9.35±0.12	9.37±0.24	9.58±0.02	9.48±0.08
Cys	0.58±0.06	0.59±0.04	0.52±0.04	0.64±0.42	0.61±0.05
Val	1.86±0.12	2.06±0.20	2.09±0.19	1.97±0.21	1.94±0.10
Met	2.35±0.01	2.10±0.16	2.00±0.05	2.24±0.06	2.42±0.16
Ile	1.13±0.07	1.09±0.01	1.22±0.01	1.07±0.13	1.15±0.02
Leu	2.43±0.04	2.39±0.04	2.37±0.01	2.45±0.06	2.48±0.07
Tyr	0.88±0.10	0.90±0.02	0.89±0.07	0.89±0.08	0.95±0.08
Phe	2.05±0.19	2.08±0.24	2.13±0.31	2.23±0.11	2.06±0.09
His	1.01±0.04	1.08±0.02	1.09±0.00	1.13±0.08	1.00±0.02
Lys	3.48±0.03	3.69±0.12	3.69±0.09	3.88±0.50	3.54±0.02
Arg	8.05±0.32	8.02±0.06	7.99±0.18	8.46±0.40	7.98±0.11
OHPro	7.25±0.15	7.54±0.66	7.36±0.97	6.81±0.91	6.99±0.20
Pro	10.33±0.57	10.54±0.26	10.19±0.53	10.26±0.12	10.09±0.07
Pr (%)	90.6±5.7	95.0±1.6	94.7±11.4	83.5±1.4	89.8±7.8
TEAA/TAA (%)	26.7±0.5	27.1±0.5	26.0±0.1	28.0±1.6	27.0±0.4

3.3 Molecular weight distribution

Besides the amino acid content, the molecular weight (Mw) distribution has also a critical role on physical properties of gelatin. The mechanical properties of gelatin (dynamic storage modulus and gel strength) are closely related with the average molecular weight as well as the molecular weight distribution (lower molecular weight fractions give origin to gelatin with low gelling properties by disturbing the formation of a strong network) (Eysturskarð, Haug, Ulset, & Draget, 2009). Therefore, samples from M1, M2, M6, M7, M8 from fresh skins and salted skins were analyzed by SDS-PAGE and by gel permeation chromatography. Throughout the gelatin extraction process, the raw material is submitted to hydrolysis, giving origin to a mix of chains that include α-chains; β-chains and γ-chains. The SDS-PAGE results showed an identical pattern for all samples (**Figure 1**). A type I gelatin pattern was possible to detect by the presence of one γ-chain (a trimer composed of three crosslinked α-chains) at 240-375 kDa; a β-dimmer (composed by two α-chains covalently crosslinked) at 160-250 kDa and two different α-chains (α_1 and α_2) (Gomez-Guillen, et al., 2001) at 80-125 kDa (Boran & Regenstein, 2010). Similar results were presented for codfish skins in previous work (Alves, Marques, Martins, Silva, & Reis, 2017; R. O. Sousa, et al., 2020) as well as in the work of others (Derkach, Kuchina, Baryshnikov, Kolotova, & Voron'ko, 2019; Gomez-Guillen, et al., 2002; Kołodziejska, Skierka, Sadowska, Kołodziejski, & Niecikowska, 2008) and from other types of fish species, such sole, megrim, hake (Gomez-

Guillen, et al., 2002), tilapia (Niu, et al., 2013), unicorn leatherjacket (Kaewruang, Benjakul, & Prodpran, 2013), catfish (Duan, Zhang, Liu, Cui, & Regenstein, 2018) and mackerel (Khiari, Rico, Martin-Diana, & Barry-Ryan, 2017). Considering methods M1 and M2, a lower intensity in γ and β bands can be detected on salted skins compared with fresh ones. This may indicate that the pre-treatment with salt, concomitant with the use of sulphuric acid and citric acid during the washing of skins, contribute to the disruption of covalent bonds in the ancestor collagen protein that gives origin to gelatin, allowing the dissociation of trimeric γ and dimeric β-chains into monomeric α-chains. In M7 from fresh skins, β- and α-chains were not very clear. This could be due to the presence of soluble aggregates limiting the loading of the sample into the gel. Also, strong bands are visible onto the top of the gel, which may indicate the presence of some molecular aggregates that were not completely dissociated.

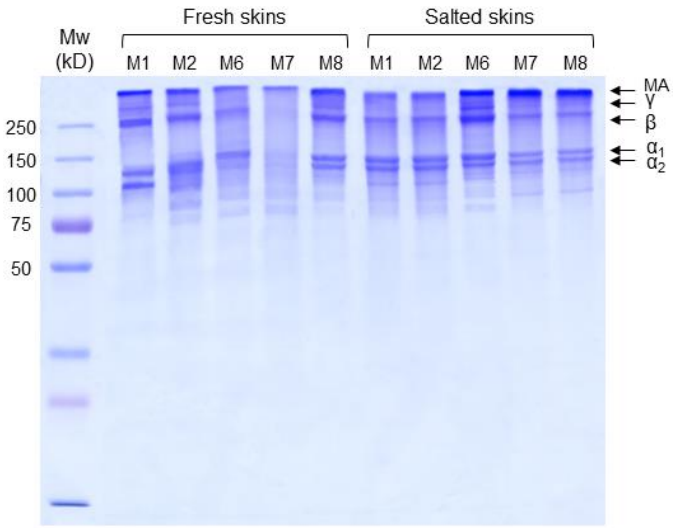


Figure 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of fresh and salted codfish skins for the different extraction methods addressed. Mw: molecular weight. MA: molecular aggregates.

For a precise analysis of molecular weight distributions in gelatin samples, a GPC-SEC analysis was performed, and the data is listed on **Tables 5 and 6** regarding fresh and salted codfish skins, respectively, retrieved from the analysis of the corresponding eluograms displayed in the supplementary material section (Figures S6 and S7). Lower retention times (Rt) corresponded to higher molecular weight species that were eluted first and thus the first peaks can be assigned to the γ (trimmers) and β (dimmers) and the peaks observed at about 48 minutes, corresponding to molecular weight values around 100 kDa, can be associated to the α (monomers) component.

As in SDS-PAGE, the results revealed a heterogenic distribution of the molecular weight of gelatins in all the methods used. It means that the cleavage of inter-chain covalent crosslink and unfavorable breakage of some intra-chain peptide (Zhou, et al., 2006), during the extraction of gelatins, lead to a mixture of fragments with disperse molecular weight that can be from 80 to 250

kDa (Karim, et al., 2009). The polydispersity index (PDI) is a reference of the broadness of molecular weight distributions of a polymer mixture and is calculated by the coefficient of the weight average molecular weight (Mw) to the number average molecular weight (Mn). Often, gelatin present high levels of PDI but those reports usually estimate the molecular weight of the whole distribution instead of each individual peak, such as in Pezron *et al.* (Pezron, Djabourov, & Leblond, 1991) or Farrugia *et al.* (Farrugia, Farrugia, & Groves, 1998). On the other hand, gelatins extracted with PDIs close to 1 should be expected if no intramolecular cleavage has occurred in the chain. Rbii et al. (Rbii, Surel, Brambati, Buchert, & Violleau, 2011) reports a PDI of 1.022 in native gelatin before any treatment. Also, Eysturskarð *et al.* (Eysturskarð, Haug, Elharfaoui, Djabourov, & Draget, 2009) presented a PDI of 1.5 ± 0.2 and suggested that the low PDI obtained from different extraction conditions may suggest some degree of selective hydrolysis by the use of different acids, concentrations, temperatures and extraction times.

In both types of skins, the average distribution of Mw integrates gelatins with Mw < 250 kDa. Considering gelatins from fresh skins (**Table 5**), the ones extracted with methods M6, M7 and M8 present the largest peak corresponding to molecules with an average molecular weight <100 kDa (40.38%), 118.89 kDa (22.41%) and <100 kDa (43.37%), respectively. Another factor is that in M6 and M8 molecular fractions superior to 227.7 kDa and 463.39 kDa, respectively, were not detectable. On the other hand, M1 and M2 present the largest peak at 114.04 kDa (30.15%) and 121.74 kDa (26.00%), which are compatible with the molecular weight of α and β-chains. In the case of gelatins obtained from salted skins (**Table 6**), some differences can be observed, namely in methods M7 and M8 in which, although there was a portion of fragments with low molecular weight (<100 kDa), a large peak area (M7 - 24.38% and M8 - 24.71%) revealed gelatin fragments with high Mw (>500 kDa). This maybe associated to gelatin aggregates. All other methods generate gelatin fragments of lower molecular weight: M1 - 112.83 kDa (29.66%); M2 - 114.32 kDa (28.49%) (corresponding to the molecular weight of α-chains) and M6 - <100 kDa (30.38%). This is in agreement with the SDS-PAGE results that corroborates the presence of gelatin fragments below 100 kDa. Despite the extensive time of incubation at high temperature during the extraction process, it is not unusual the appearance of large molecular weight aggregates that we can see in gelatin from both types of skin. This phenomenon, also observed in SDS-PAGE, can be associated to incomplete dissociation of collagen protein (Meyer & Morgenstern, 2003) that favors the accumulation of γ and β-chain aggregations and less α-chain. In the work of Muyonga *et al.* (Muyonga, Cole, & Duodu, 2004) using gelatin from Nile perch skins, it was shown that extractions at low temperature (50 °C) generates gelatins with higher molecular weight (greater than β dimmers).

Table 5. Molecular weight (kDa) distribution of gelatins from fresh cod skin, according to the peaks shown in Figure S6. Rt: retention time; Mw: weight average molecular weight; Mn: number average molecular weight; PDI: polydispersity index. Values are represented as mean ± confidence intervals (for n=2 and α=0.05).

Method	Peak number	Rt (min)	Mn (kDa)	Mw (kDa)	PDI	Peak area (%)
M1	1-high Mw	34.6-41.7	>500	>500	-	14.82±1.89
	2	42.2±0.0	483.16±7.55	485.72±7.58	1.005±0.000	6.14±0.08
	3	43.3±0.0	350.79±8.62	354.11±8.48	1.010±0.001	10.84±0.50
	4	45.1±0.0	214.62±2.79	219.62±3.40	1.024±0.003	25.08±0.43
	5	48.4±0.0	112.03±1.00	114.04±1.01	1.018±0.000	30.15±3.28
	6-low Mw	50.3-67.6	<100	<100	-	12.98±2.23
M2	1-high Mw	33.9-41.6	>500	>500	-	21.17±2.25
	2	42.1±0.0	502.57±2.57	505.50±2.05	1.006±0.001	6.96±0.25
	3	43.3±0.0	359.98±9.75	363.54±8.68	1.010±0.004	11.47±0.32
	4	45.1±0.1	222.96±14.6	227.05±12.9	1.019±0.009	22.87±0.41
	5	48.4±0.0	120.24±13.3	121.74±12.2	1.013±0.012	26.00±0.43
	6-low Mw	50.6-69.8	<100	<100	-	11.53±3.00
M6	1-high Mw	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	8.21±7.14
	4	46.0±1.3	222.33±18.6	227.71±20.1	1.024±0.004	15.19±7.54
	5	48.6±0.1	114.66±18.6	119.13±16.9	1.040±0.022	36.23±9.31
	6-low Mw	50.3-71.1	<100	<100	-	40.38±5.37
M7	1-high Mw	33.9-41.6	>500	>500	-	19.72±2.51
	2	42.5±0.5	492.28±21.3	494.6±22.5	1.005±0.002	5.55±0.93
	3	43.3±0.0	356.56±2.15	360.32±3.64	1.011±0.005	10.22±1.34
	4	45.1±0.1	215.69±5.78	221.45±5.48	1.027±0.002	21.68±0.20
	5	48.4±0.0	117.11±2.89	118.89±2.79	1.016±0.001	22.41±0.13
	6-low Mw	49.7-69.4	<100	<100	-	20.43±0.55
M8	1-high Mw	-	-	-	-	-
	2	-	-	-	-	-
	3	44.3±0.0	407.85±2.01	463.39±17.7	1.134±0.038	10.08±0.75
	4	45.6±0.2	215.08±1.25	219.03±0.97	1.019±0.001	13.15±1.71
	5	48.7±0.1	113.11±0.98	117.61±0.12	1.040±0.001	33.42±2.28
	6-low Mw	50.3-70.2	<100	<100	-	43.37±4.75

Table 6. Molecular weight (kDa) distribution of gelatins from salted cod skin, according to the peaks shown in Figure S7. Rt: retention time; Mw: weight average molecular weight; Mn: number average molecular weight; PDI: polydispersity index. Values are represented as mean ± confidence intervals (for n=2 and α=0.05).

Method	Peak number	Rt (min)	Mn (kDa)	Mw (kDa)	PDI	Peak area (%)
M1	1-high Mw	35.3-41.7	>500	>500	-	10.48
	2	42.6	496.16	498.68	1.005	4.99
	3	43.8±0.8	381.47±40.6	405.59±80.6	1.061±0.099	8.52±2.16
	4	45.8±1.37	206.64±22.1	213.05±20.3	1.031±0.012	19.00±6.02
	5	48.4±0.0	110.45±12.9	112.83±12.2	1.022±0.009	29.66±1.33
	6-low Mw	50.3-69.5	<100	<100	-	27.35±22.0
M2	1-high Mw	34.6-42.8	>500	>500	-	12.63±2.49
	2	-	-	-	-	-
	3	44.3±0.2	345.82±9.04	349.64±8.21	1.011±0.002	9.16±1.19
	4	45.1±0.0	204.08±3.87	210.55±2.00	1.032±0.010	24.38±2.70
	5	48.4±0.0	112.28±5.38	114.32±5.54	1.018±0.000	28.49±1.14
	6-low Mw	50.3-69.2	<100	<100	-	25.34±0.16
M6	1-high Mw	34.6-41.5	>500	>500	-	15.14
	2	42.1	496.57	499.75	1.006	7.28
	3	43.2	355.70	359.14	1.010	12.12
	4	45.5	216.12	221.76	1.026	16.45
	5	48.4	115.64	117.17	1.013	18.63
	6-low Mw	49.9-70.0	<100	<100	-	30.38±13.3
M7	1-high Mw	34.7-42.7	>500	>500	-	24.38±0.43
	2	-	-	-	-	-
	3	43.4±0.0	372.81±4.37	377.73±2.86	1.013±0.004	10.07±1.80
	4	45.1±0.1	220.65±0.58	226.89±2.09	1.029±0.007	20.33±1.06
	5	48.5±0.0	120.66±1.62	122.66±1.37	1.017±0.003	21.97±3.03
	6-low Mw	49.9-69.6	<100	<100	-	23.26±4.18
M8	1-high Mw	34.1-42.9	>500	>500	-	24.71±2.33
	2	-	-	-	-	-
	3	43.9±0.8	377.40±20.9	381.89±22.1	1.012±0.002	9.09±1.10
	4	45.2±0.1	218.51±10.9	226.26±10.7	1.036±0.003	20.59±0.01
	5	48.4±0.0	119.57±4.92	121.40±5.14	1.016±0.001	19.91±0.25
	6-low Mw	49.9-69.7	<100	<100	-	25.72±3.67

3.4 Gel strength

Gel strength is one of the most important physical characteristic of gelatins, which determines its quality by providing information about the rigidity factor and thus indicating the feasibility for different applications (Kuan, Nafchi, Huda, Ariffin, & Karim, 2016). Gel strengths of the gelatins extracted are presented in **Table 7**, being possible to observe that the different extraction methods and preservation states clearly affected the gel strength of the gelatin.

In general, the gel strength of gelatins from fresh skins was higher than the ones of gelatins from salted skins. Taking in consideration the intervals of confidence, we can claim that in gelatins extracted from fresh skins a higher bloom value was observed for M1 and M2 with 76.50 ± 2.94

g and 82.50 ± 4.90 g, respectively. Considering gelatins extracted from salted skins, it was observed that M1 presented the higher bloom with 43.30 ± 5.88 g ($p<0.05$), followed by M2 and M8, which exhibited intermediate bloom values of 27.17 ± 9.48 g and 23.67 ± 1.96 g, respectively. It is known that gelatins from warm-waters fish present higher bloom, such as grass carp (267 g) (Kasankala, Xue, Weilong, Hong, & He, 2007), Spanish mackerel (291.33 g) (Kusumaningrum, Pranoto, & Hadiwiyoto, 2018) and tilapia (328 g) (Songchotikunpan, Tattiyakul, & Supaphol, 2008). By contrast, gelatins from cold-water fish present inferior gel strength, such as salmon (108 g) (Arnesen, et al., 2007) and Alaska pollock (98 g). This is related not only with the different environments and species used, but also with the different amino acid composition and molecular weight distributions of gelatin of those fishes. As already mentioned in the amino acid analysis, the content of glycine, proline and hydroxyproline greatly influence the final strength of the gel. The pyrrolidine rings of these amino acids play a critical role in the stabilization of the collagen helix and therefore are important for the formation of the gel network. The lower value of gel strengths obtained in this work when compared with gelatins from other fish are related to the lower content of pyrrolidine amino acids (proline and hydroxyproline) (Fernández-Díaz, et al., 2001) from codfish. This correlation is also visible within the present work, since the gelatins produced with the methods M1 and M2, (both with the highest gel strengths) are the ones with higher content of OH-proline + proline. Also, Herrick *et al.* (Herrick, Maziarz, & Liu, 2018) reported the correlation between the molecular weight distribution and the gel strength, affirming that gel strength is mainly dependent on the population at around 100 kDa. This statement is in good agreement with our observations, where the gelatins produced with methods M1 and M2, exhibiting a Mw distribution around 100 kDa, present the higher gel strength, both for gelatins derived from fresh and salted skins. Other important observation is that gelatins extracted from cod skins by Arnesen & Gildberg (Arnesen, et al., 2007) using the same method (M1), and by Fernández-Díaz *et al.* (Fernández-Díaz, et al., 2001), showed levels of gel strength (71 g and ≈ 90 g) very similar to the ones presented in this work for the fresh skins. Again, no significant differences were observed but it seems that protocols M2 and M8 (where a pre-treatment at 4 °C was applied) had higher gel strength than the treatments performed at RT (M1 and M7), with the exception of M2 for salted skins. Gelatins extracted from fresh skins by method M6 presented levels of gel strength lower than the others. This indicate that the presence of sodium chloride may have a negative impact in gelatin quality. Also, gelatins extracted from salted skins were not able to jellify during the maturation time settled for this type of experiment. In this case, the salted nature of skins, associated with the use of sodium chloride in the extraction process may have had an impact in the collagenous structure of the skins. Studies of Choi and Regenstein (Choi & Regenstein, 2000) demonstrated that sodium chloride has a deleterious effect on gel strength through the breaking of hydrophobic and hydrogen bonds responsible for the stabilization of the gel junctions zones, either by directly preventing the bond itself and/or by modifying the structure of the liquid water in the proximity of these sites. Beyond the previously mentioned properties that can interfere with gel strength, the setting time and time of storage are very important factors as mentioned by Arnesen & Gildberg (Arnesen, et al., 2007) work. Indeed, the storage of gelatins

for long periods at low temperatures allow a slow helical regeneration resulting in a gel with higher strength, thus all the measurements in this work were performed using freshly obtained samples and the same setting time (using a standardized protocol) to allow comparison.

Table 7. Gel strength values of gelatins from fresh and salted skins of Atlantic cod produced with methods M1 to M8. Nd: not detected as gelatin did not jellify. Values are average intervals of confidence for n=2 (replicates of independent batches) and α=0.05.

Gel Strength (bloom, g)		
Method	**Fresh Skins**	**Salted Skins**
1	76.50 ± 2.94	43.30 ± 5.88
2	82.50 ± 4.90	27.17 ± 9.48
6	21.75 ± 4.41	ND
7	36.65 ± 0.69	14.49 ± 4.28
8	37.50 ± 0.98	23.67 ± 1.96

3.5 Viscoelastic behavior

The characterization of the dynamic rheological behavior of gelatins is important for the determination of gel forming kinetics and determination of melting and gelling points. The storage/elastic modulus (G'), loss/viscous modulus (G'') and phase angle (δ) are indicators of the elastic energy stored in gel state and the viscous energy dissipated in the solution state (Tau & Gunasekaran, 2016). These parameters are represented in **Figure 2** and **Figure 3** for fresh and salted skins, respectively, during both heating (from 2 to 30 °C) and cooling (from 30 to 2°C) processes. Considering the fresh skins, the heating ramp yielded a decrease in elastic modulus (G') (**Figure 2A**) representing a transition from gel to solution state. For M1 and M2 the decrease was observed between 4 and 10 °C, while in the case of M6 and M7, it was verified between 3 and 7 °C. In the cooling ramp it was observed an increase of G' (**Figure 2D**) at 6 °C for M1, M2 and M7. Methods M6 an M8 showed an increase at lower temperatures (≈3 °C). The increase of G' during the cooling process is related with the transition from solution to gel state caused by triple-helix formation. The differences in G' values at 2 °C between heating and cooling processes could be related with the maturation time during the stabilization of temperature at 2 °C in the beginning of heating ramp program giving the opportunity to gelatins to a quick cold maturation. This phenomenon was also observed in the work of other authors (Gomez-Guillen, et al., 2002; Khiari, et al., 2017). The viscous modulus G'' presented a similar behavior, with a gradual decrease with heating process (**Figure 2B**) and increasing with cooling (**Figure 2E**). The phase angle showed an analogous pattern during heating and cooling of gelatin samples (**Figure 2C and 2F**), with the changes in phase angle indicating a rapid transition from solution to a gel state by formation of junction zones in the three-dimensional network. Taking into account that a low phase angle at low temperatures is an indicator of superior gelling capacity (Gómez-Guillén, Giménez, & Montero, 2005), it is appropriate to consider that M1 and M2 are the ones that

generates gelatins with better gelling ability. For all the methods used, the values of G' were higher than G'' indicating that the elastic behavior of the system was greater than the viscous behavior. Gelatins from methods M1 and M2 presented a G' value almost 5 times higher than the one exhibited by gelatins form M6 and M7. Also, those two materials are the ones with higher melting and gelling temperatures as can be seen in **Table 7**, with 11.68 °C (M1) and 12.01 °C (M2) and 5.35 °C (M1) and 5.97 °C (M2), respectively. As already discussed in the amino acid analysis and gel strength sections, the pyrrolidine amino acid content plays an important role in gelatin stabilization and properties. So, the higher viscoelastic properties of M1 and M2 methods may be due to the presence of a higher content of these amino acids, a good distribution of α and β-chains, as well as its high gel strength (Gomez-Guillen, et al., 2002; Khiari, et al., 2017).

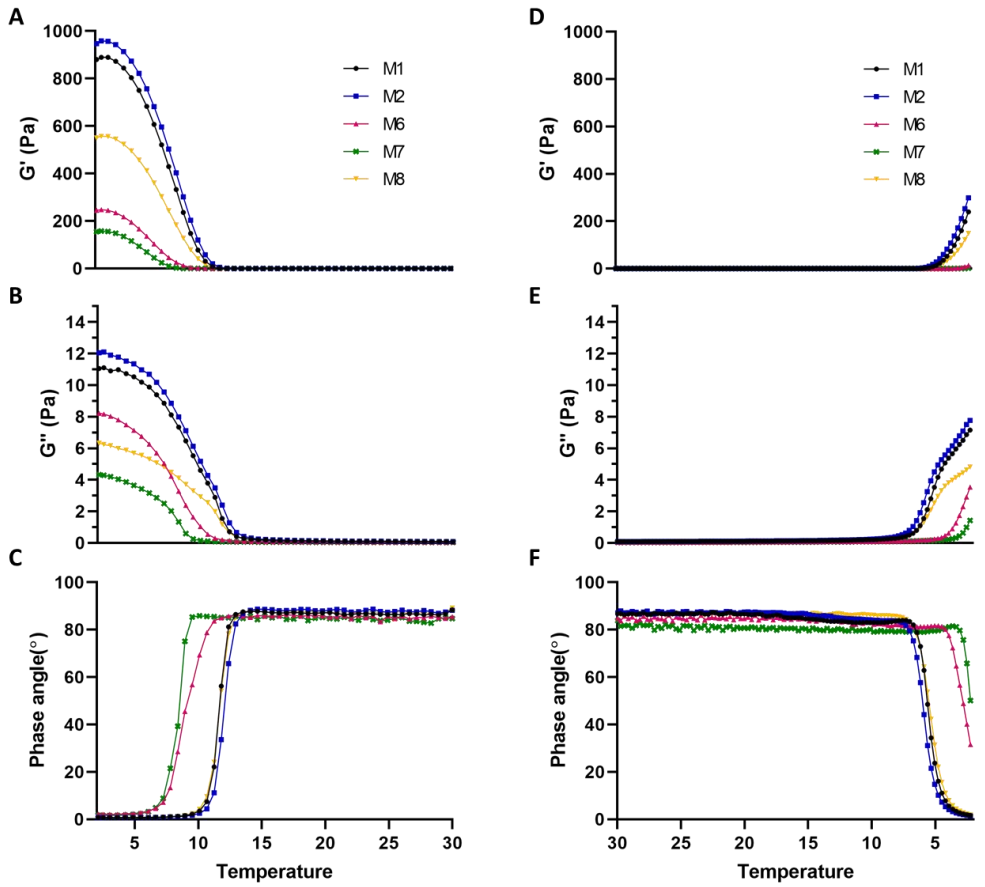
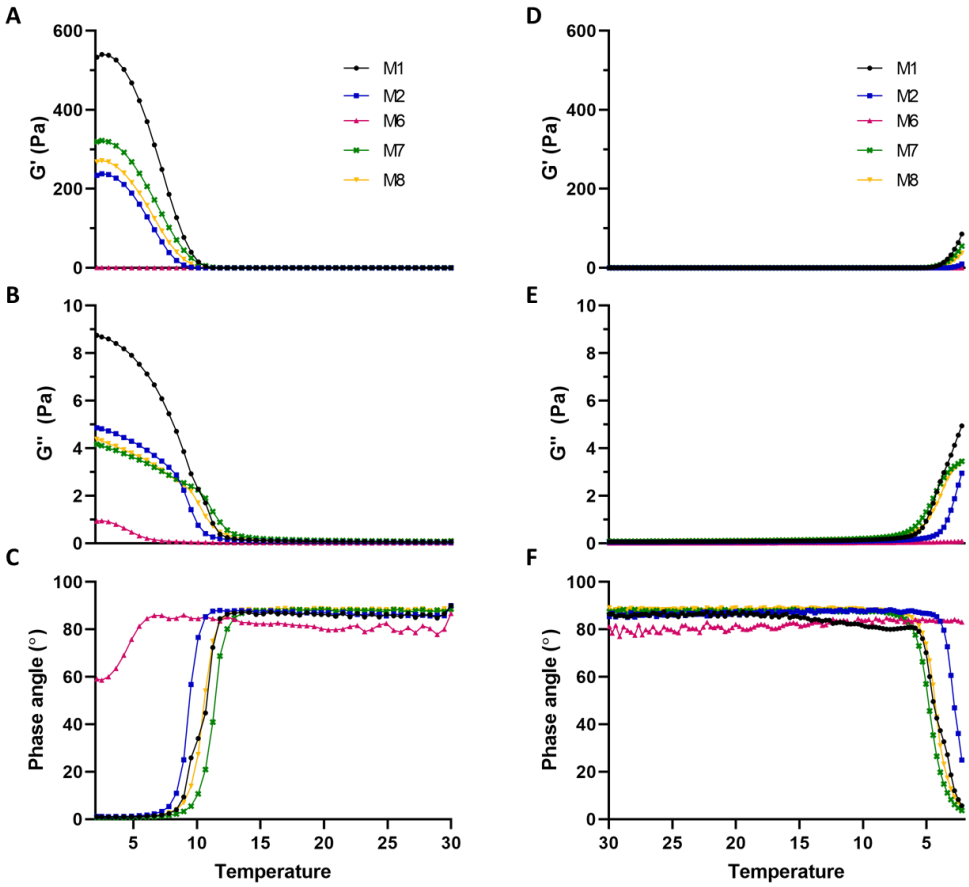


Figure 2. Rheological behavior of gelatins extracted from fresh cod skins. Elastic modulus (G'), viscous modulus (G'') and phase angle (δ) from heating (2 to 30°C, **A**, **B** and **C**) and cooling (30 to 2°C, **D**, **E** and **F**) ramps.

Figure 3 shows the dynamic rheological properties of gelatins derived from salted skins during heating and during cooling. It is evident the difference when compared to the gelatins derived from fresh skins, with lower values of G' and G'' both for heating and cooling ramps being exhibited. Despite the lower values, the same tendency of elastic modulus G' decreasing is detected in the heating ramp (**Figure 3A**) but in this case, it begins earlier, around 3 to 8 °C for

567 methods M2 and M8 and around 4 to 10°C for methods M1 and M7. Method M1 remains with the
568 highest value but with M2 clearly lowest. The viscous modulus G'' (**Figure 3B**) showed a similar
569 behavior with a slightly upper shift on the M2 curve. The cooling ramp indicated an increase of G'
570 (**Figure 3D**) starting from 5 °C for M1, M7 and M8 and from 3 °C for M2. Likewise, G'' also exhibit
571 an equivalent pattern, increasing when decreasing the temperature (**Figure 3E**). Method M6
572 presented a particularly behavior, with flat line present for G' (**Figure 3A**) and a slightly higher
573 value for G'' (**Figure 3B**). This indicates that the viscous behavior is greater than the elastic
574 behavior, associated to a loosen or not cohesive matrix, which is in agreement with the
575 observations during the measurements of gel strength where gelatin obtained from salted skins
576 with method M6 did not jellify. For the remaining strategies, the values of G' were higher than G''
577 indicating that the elastic behavior of the system was greater than the viscous behavior,
578 compatible with a cohesive matrix. Also, an irregular δ pattern (**Figure 3C and 3F**) was detected,
579 suggesting an irregular system with poor capability to form a gel. Also, lower melting and gelling
580 temperatures were detected for these gelatins (**Table 8**).

581



582 **Figure 3.** Rheological behavior of gelatins extracted from salted cod skins. Elastic modulus (G'), viscous
583 modulus (G'') and phase angle (δ) from heating (2 to 30°C, **A**, **B** and **C**) and cooling (30 to 2°C, **D**, **E** and **F**)
584 ramps.

585

M1 and M7 showed to be the ones rendering gelatins with higher melting and gelling temperature, namely 10.37 °C (M1) and 11.30 °C (M7) and 4.45 °C (M1) and 4.69 °C (M2), respectively. These results demonstrated the lower stability of the H-bonded triple helix structure of gelatins extracted from salted skins when compared with the ones extracted from fresh skins. This gives us some indication about the interference of salt in the industrial conservation process of the skins, at least for the rheological properties of the produced gelatins. In this characterization, the effect of temperature during the extraction process was only observed in fresh skins, where M2 and M8 presented better rheological behavior than the RT protocols. In the case of salted skins, this tendency was not verified. Despite the limitations that have been observed, they could be easily overcome and the gel properties could be enhanced by the use of chemical crosslinkers such as transglutaminase (Fernández-Díaz, et al., 2001), pectin (Huang, et al., 2017), or xylitol (Nian, et al., 2018).

According to Gómez-Guillén *et al.* (Gomez-Guillen, et al., 2002), the lower gelling temperatures of gelatins obtained by M6 could be associated with the low molecular weight fragments reported in the GPC-SEC analysis. However, the SDS-PAGE does not corroborate this theory since it was visible stronger bands at higher molecular weights. Either cases, fresh or salted skins derived gelatins, a thermal hysteresis phenomenon is observed. The higher melting temperatures compared with the gelling temperatures are an indication of reluctance to the thermoreversible gel-sol transition that is characteristic of polymeric dispersions (Huang, et al., 2017). Similar results and behavior pattern were obtained for cod gelatin in other works reported in literature (Cai, et al., 2018; Fernández-Díaz, et al., 2001; Gomez-Guillen, et al., 2002; Nian, et al., 2018).

Table 8. Melting and gelling points of gelatins obtained from fresh and salted skins of Atlantic cod under the methods M1 to M8, determined from rheological results. ND: not determined. Values are average intervals of confidence for n=2 (replicates of independent batches) and α=0.05.

	Melting Point (°C)		Gelling Point (°C)	
Method	**Fresh Skins**	**Salted Skins**	**Fresh Skins**	**Salted Skins**
1	11.68 ± 0.19	10.37 ± 1.14	5.35 ± 0.25	4.45 ± 0.97
2	12.01 ± 0.39	9.4 ± 0.49	5.97 ± 0.24	2.76 ± 0.55
6	9.25 ± 1.29	8.37 ± 7.31	3.03 ± 1.78	ND
7	8.52 ± 0.22	11.30 ± 0.11	2.45 ±1.60	4.69 ± 0.38
8	11.63 ± 0.43	10.53 ± 0.36	5.73 ± 0.24	4.23 ± 0.28

3.6 Cell viability of codfish gelatins

Assessing the cytotoxicity of a new material is critical to ensure its safety when biomedical application is foreseen. The cytotoxicity of cod gelatins was assessed using L929 cell line by the MTS assay, which is a colorimetric assay based on the cellular metabolic capacity to reduce a

tetrazolium compound into a formazan product, measured by spectrophotometric techniques (Wang, Henning, & Heber, 2010). This approach is commonly used to evaluate the cytotoxicity of biomaterials and medical devices according to the guidelines established by the competent ISO standard 10993.

L929 cells were exposed to increasing concentrations of gelatin derived from fresh and salted skins during 24, 48 and 72 h, and the results of the MTS assay are presented in Figure 4. In both fresh and salted skins, gelatin concentrations of 4 mg/mL presented the lowest cell viability when compared with control (p-value ≤ 0.05). On the other hand, gelatin concentrations ranging between 0.0625 and 2 mg/mL showed almost no toxicity when compared with control. Regarding fresh skins (**Figure 4A, B, C**), at the concentration of 4 mg/mL, gelatins extracted by method M7 showed higher levels of cytotoxicity to the cells when compared with other methods (p-value ≤ 0.0001). This effect (at 4 mg/mL) was consistent at 24, 48, and 72 h of incubation with the same statistical significance. Regarding salted skins (**Figure 4D, E, F**), the gelatin extracted by method M6 appeared to be the one with higher cytotoxicity to the cells, although the difference between methods was only statistically significant at 24 h of incubation.

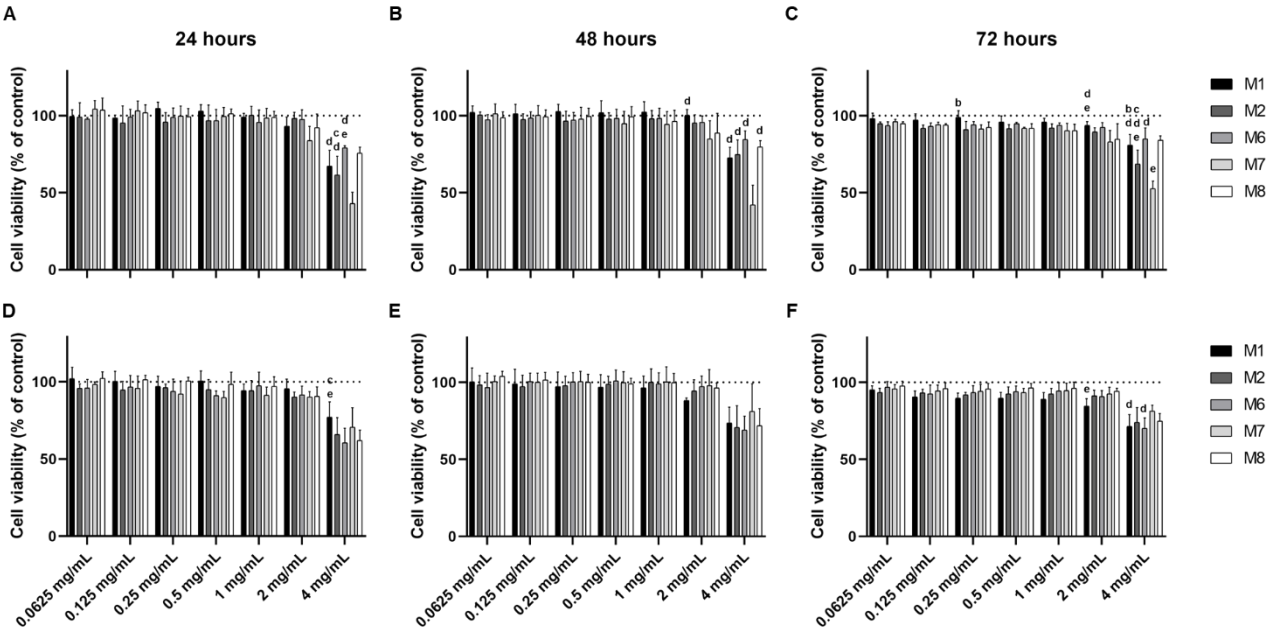


Figure 4. Cytotoxicity of gelatins extracted by different methods over L929 cell line. A-C) Gelatins extracted from fresh cod skins at different concentrations and time-points (24, 48, and 72 h). D-F) Gelatins extracted from salted cod skins at different concentrations and time-points (24, 48, and 72 h), Data were considered statistically different if p-value ≤ 0.05. a indicates significant differences when compared with M1; b when compared with M2; c when compared with M6; d when compared with M7; e when compared with M8.

To verify if some acid residues, resulting from the different extraction processes, could interfere with cell viability, the pH of gelatins dissolved in cell medium was previously assessed. The results of that analysis (data not showed) does not demonstrated any significant differences that could explain the widespread cytotoxicity revealed at 4 mg/mL in both skin types of gelatins. This effect

may be due to other chemical residues derived from the extraction process (e. g salts). Other hypothesis may be related to a gelatin overload for the cells that over time they start to metabolize. Nevertheless, this effect tended to disappear over time. Moreover, it was possible to verify that at concentrations below 2 mg/mL, all gelatins appeared not to affect cell viability.

4 Conclusion

This study is to the best of our knowledge, the first study comparing the physical-chemical properties and yield of codfish gelatin from skins derived from different preservation methods (fresh and salted) using several extraction methods.

From all the methods here studied, M7 and M8 enabled to obtain the highest yields, both for fresh and salted cod skins. It was possible to observed that gelatins extracted at lower temperatures (4ºC) resulted in higher yield percentage of gelatin recovered than the gelatins extracted at RT. Then, in terms of chemical composition, the results were similar for gelatin extracted either from fresh and salted cod skins, although with some highlights in the OH-proline + proline contents for M1 and M2 gelatins. Also, the molecular weight distributions indicated an average Mw<250 kDa for both type of skins throughout the studied methods. Moreover, according to the extracted gelatins physical properties, the methods M1 and M2 lead to gelatins with higher gel strength and viscoelastic properties. However, M1 stands out as better method (concerning gel strength and viscoelastic properties) for salted cod skins, while M2 enabled the production of gelatins with higher quality (better physical and chemical characteristics) with a considerable yield for fresh cod skins. The overall efficiency of extraction can be improved by using other methods, particularly M7 and M8, but with the drawback of producing gelatins with lower gel strength and weak gelling ability, representing lower quality for industrial applications. Thus, the selection of the best method to extract gelatin needs the establishment of a compromise between yield and quality, depending on the foreseen application. Finally, at the biological level, it was possible to verify that using a concentration up to 2 mg/mL of extracted gelatin from either salted and fresh codfish with all the methods, the cell viability is not affected. Thereby, all of the extraction methods and preservation skin fish states used in this study are viable to produce gelatins that can be safely used for wellbeing or medical purposes.

This study shows that the preservation method of the fishing industry by-products has an impact on gelatin extraction, as well as in the choice of extraction methods. Gelatin derived from salted skins presented lower gel strength and therefore is less desirable for industry applications than the one obtained from fresh skins. The salt used during the salting process of codfish to preserve the meat (a traditional conservation process used in Portugal) weakens gel junction, resulting in gelatins with lower gel strength, lower melting temperatures and consequently, lower gelling ability. Future studies will pass by developing strategies to overcome these limitations, so that the portuguese fish industry by-products can also be valorized by their economically use, which can become a driver to a more responsible society.

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6 Declaration of interest

The authors declare no conflicts of interest.

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