

Extraction and characterisation of gelatine from yellowfin tuna skin pretreated with a eutectic solvent

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ABSTRACT

The accumulation and management of waste generated during the processing of seafood products is one of the major current challenges in the fishery industry. Among these residues, fish skin stands out for its high collagen content, from which high-quality gelatine can be obtained. However, conventional methods for gelatine extraction are time-consuming and complex processes that involve the use of strong acidic and alkaline solutions, leading to serious environmental concerns. This research studied the use of a eutectic solvent, formed by two safe and environmentally friendly compounds such as sodium acetate trihydrate and urea, to pretreat the skin of yellowfin tuna before the extraction of gelatine. Two different times of pretreatment were tested, and the subsequent extraction was carried out in warm water, resulting in much higher yields of gelatine (0.3 g/g wet fish skin) than previous studies. Additionally, using FT-IR analysis and amino acid profiling, it was observed that the pretreatment time did not significantly affect the structure or composition of the gelatine. However, some variation was found in its molecular weight distribution and therefore in its rheological properties: with an increase in the pretreatment time, both the molecular weight and the storage modulus of the gel also increased, as well as the gelation and melting temperatures. This contribution offers a simplified process that reduces environmental impact and provides the opportunity to tailor the final product.

1. Introduction

The fishery industry generates unavoidably many residues during fish and shellfish processing, accounting for as much as 50–70% of the total mass of the feedstock, depending on the species (Ideia et al., 2020; Muhammad et al., 2017). Together with an increase >60% in fisheries and aquaculture production in the last three decades (FAO, 2022), this has led to the generation of a vast amount of fish meal but also waste. Although the proper management of these by-products is essential to ensure the sustainability of the industry, they have been traditionally considered as waste and discarded either at the sea (properly done, it returns nutrients to the environment) or in landfills. This strategy represents not only a threat to the environment but also economic losses and resource underutilisation if considering that these marine by-products have been pointed out as, e.g., a source of added value biomolecules (Cooney et al., 2023; Fraga-Corral et al., 2022).

Fish skin is a good example of an underutilised residue with potential for valorisation, since it can constitute up to 30% of the total residues after fish filleting and usually contains large amounts of protein

(Gómez-Guillén et al., 2002), essentially collagen.

Collagen is the major structural protein in an animal's body, accounting for ~30% of the total protein composition (Chen et al., 2019; Song et al., 2021). The chemical structure of collagen consists of three α chains, each containing the repeating Gly-X-Y amino acid sequence (where "Gly" is glycine and the most common X and Y amino acids are proline and hydroxyproline, respectively), twisted around each other to form a right-handed triple helix. This helical structure is stabilised primarily by hydrogen bonds between the peptide amine of glycine and peptide carbonyl groups in adjacent chains, with hydrophobic interactions playing a minor role. Furthermore, in mammalian species, collagen molecules are cross-linked to one another through covalent bonds, which contributes to the strength and stability of tendons, skins or bones (Bhowmick & Fields, 2013; Tang et al., 2022; Vate, Undeland, & Abdollahi, 2022). This highly organised and tightly packed structure, essential for the functionality and performance of these tissues, resists fluid penetration, rendering native collagen insoluble in water. Partial hydrolysis of collagen leads to the unfolding of the triple helical structure along with the breakage of some polypeptide bonds, thus resulting

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in gelatine, a more soluble material with wider applicability (Duconseille, Astruc, Quintana, Meersman, & Sante-Lhoutellier, 2015; Liu, Nikoo, Boran, Zhou, & Regenstein, 2015). Due to its physical and mechanical properties, especially its capacity to form thermo-reversible gels in water, gelatine is largely used in food, cosmeceutical, pharmaceutical, and photographic industries (Cao, Nguyen, Nguyen, Le, & Razumovskaya, 2017; Regenstein & Zhou, 2006). Currently, skin and bones from bovine or porcine species serve as the main sources of gelatine for human consumption. Nevertheless, collagen and gelatine from fish and seafood are of interest, in part because of religious and cultural constraints combined with risks associated with livestock, e.g., bovine spongiform encephalopathy outbreaks (Alves et al., 2022; Regenstein & Zhou, 2006), although gelatine has not been found to contain the virus.

Traditional methods to obtain gelatine from fish skin include acid and alkaline pretreatments as previous stages to the extraction in hot water, or enzymatic hydrolysis (Gómez-Guillén et al., 2002; Grossman & Gan, 1992; Gudmundsson & Hafsteinsson, 1997). The first method involves multiple neutralisation steps, a large amount of water for impurity removal and tissue washing, and the generation of high amounts of effluents that must be adequately treated to comply with the principles of green chemistry and the circular economy. Such complex processes often lead to low quality products and have large chemical, energy, and time requirements.

This study aims to use eutectic solvents as alternatives to harsh chemicals, alkalis and acids, thereby improving on the traditional methods of extraction. Eutectic solvents result from the liquefying effect associated with a eutectic behaviour in the solid-liquid equilibrium of mixtures of two or more compounds that, individually, would not be liquid (and therefore not useable as solvents) at the operating temperature. A particular characteristic of eutectic solvents is their tunability, as it is possible to select parent compounds based on the specific application requirements (Martins, Pinho, & Coutinho, 2019; Smith, Abbott, & Ryder, 2014). For example, in the extraction of gelatine for potential utilisation in cosmeceutical or food industries, parent compounds from the food additives or the GRAS ("generally regarded as safe") list would be attractive. Such is the case of the eutectic configured by urea and sodium acetate trihydrate, which has previously been studied (Gallego, Rodríguez, & Soto, 2023). At the eutectic composition (urea mole fraction of 0.60) its melting temperature is 31 °C, thus enabling its utilisation as a solvent at temperatures above that value. Moreover, the low/negligible vapour pressure of the eutectic, together with its good thermal stability, enables its utilisation as a solvent in a broad temperature range with little vaporisation problems.

About the use of eutectic solvents for the extraction of biomolecules from fish by-products, previous studies have proposed a method that consists of solubilising the fish residue in the eutectic solvent, leading to the need to subsequently recover the value-added compound from the dissolution medium (Bai, Wei, & Ren, 2017; Batista, Fernández, Gaspar, Bronze, & Duarte, 2022; Bisht, Martins, Dias, Ventura, & Coutinho, 2021; Liu et al., 2020). On the other hand, a maceration stage with the eutectic solvent, followed by extraction with water, is proposed in this work.

2. Experimental

2.1. Materials

Skin offcuts of yellowfin tuna (*Thunnus albacares*), obtained as by-products during the fish filleting process, were kindly supplied by Jealsa Foods (Boiro, Galicia, Spain). The tuna was caught in the Atlantic Ocean (FAO area 34) using purse seine and longline fishing methods. The fishing season starts on April 1st and is closed on October 31st. On the boat, the tuna is stored by freezing it in brine at −18 °C. Samples were brought to the laboratory frozen and stored at −20 °C for a maximum of 4 wk. The composition of the skin, provided by the

supplier, is shown in Table 1.

Urea (Bioultra grade, >99.5 wt%) and sodium acetate trihydrate (>99 wt%) were purchased from Sigma Aldrich (Steinheim, Germany) and Scharlau (Sentmenat, Barcelona, Spain), respectively, and they were used as received. The eutectic solvent was prepared by mixing both chemicals (urea mole fraction of 0.60) at 60 °C and stirring. Because the melting temperature of the eutectic mixture is 31 °C (Gallego, Rodríguez, & Soto, 2023), a homogeneous liquid was formed from the two solids. A commercial gelatine (Sigma-Aldrich) with an average molecular weight (Mw) of 60 kDa, obtained from cold water fish skin, was used as a control sample for comparison.

2.2. Extraction

After defrosting at room temperature (22 ± 2 °C), skin was manually separated from meat and scales. Then the cleaned skin was cut into pieces of ~0.5 × ~0.5 cm², and 4.0 g of these pieces were processed per batch. In the first step, the skin pieces were pretreated by maceration. That is, the solid skin was soaked in the liquid eutectic solvent at a solid-to-liquid ratio of 1:3 (w/v) at 35 °C with mechanical stirring. Two pretreatment times were tested: 30 min (0.5 h) and 3.0 h. After pretreatment, the samples were drained and rinsed twice with distilled water at room temperature to remove any traces of the eutectic solution (as verified by the measurement of a neutral pH in the washing waters, given that both components of the eutectic lead to basic pH). The extraction stage with warm water was carried out overnight with a solid-to-liquid ratio of 1:3 (w/v) at 45 °C. Centrifugation (Ortoalresa -Madrid, Spain- Digicen 21R centrifuge) at 18,000×g was applied for 15 min to eliminate small fragments of skin. The clear supernatant was dried using a JP Selecta Conterm 2000209 oven (Selecta, Abrera, Barcelona) at 40 °C (a temperature selected to prevent protein thermal degradation and to preserve the structural, functional, and rheological properties of the gelatine) for 48 h. Each extraction test was done in triplicate. As a control sample, for comparison, an extraction without pretreatment was also done.

2.3. Characterisation

2.3.1. Extraction yield

The extraction yield was determined considering the weight of wet skin before extraction and the weight of dried extracted product (later proven to be gelatine), according to the following equation (Latimer, 2023):

$$\text{Extraction yield (\%)} = \frac{\text{Weight of dried gelatine (g)}}{\text{Weight of wet skin (g)}} \times 100 \quad [1]$$

An equivalent expression was also used with the weight of dry skin in the denominator, to calculate a yield not sensitive to fluctuations in the moisture content of the skin. All analyses were done in triplicate.

2.3.2. Proximate composition

The moisture and ash of extracted gelatines were determined according to AOAC standard methods (950.46 and 900.2A, respectively) using gravimetry (Latimer, 2023). Organic matter was calculated by difference. Total protein content in the sample was determined using the

Table 1
Composition of yellowfin tuna skin, as provided by the supplier.

Composition	Content (g/100 g of wet skin)
Moisture	47.4 ± 0.2
Protein ^a	34 ± 2
Lipid	8.5 ± 0.7
Ash	9.8 ± 0.9

^a Determined by the Kjeldahl method. The protein content was calculated as the product of total nitrogen times the factor 5.6.

Kjeldahl method (International Organization for Standardization, 2023) with a Kjeldatherm Digestor and Vapodest 50s distillation system (C. Gerhardt & Co., Königswinter, Germany). The protein content was calculated as total nitrogen times 5.6 (Mariotti, Tomé, & Mirand, 2008). All analyses were done in duplicate.

2.3.3. Fourier-transform infrared (FT-IR) spectroscopy

The groups and the interactions between the bonds of the gelatines were analysed using FT-IR spectroscopy. Measurements were done using a Varian (Palo Alto, California) 670 IR spectrometer, scanning from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} . All spectra were obtained as 32 scans (110 scans/s) at 25 °C. Analysis of the spectral data was carried out using the Bruker Opus 7.8 data collection software (Bruker Optik, 2015).

2.3.4. Amino acid profile

The amino acid composition of the gelatines was determined by quantitative analysis at Centro Tecnológico da Carne (San Cibrao das Viñas, Spain). The samples were hydrolysed with aqueous HCl (6 N) for 24 h at 110 °C. Then, the extracts were derivatised using the AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) and subsequently analysed using HPLC-FL with a Waters 2695 Separations module equipped with a Waters 2475 Multi Fluorescence detector and a Waters AccQ-Tag Amino Acids C18 analysis column. Quantification was carried out using the external standard method with the Amino Acid Standard H (Thermo Scientific, Rockford, IL, USA). As a result of the hydrolysis, asparagine and glutamine are deamidated resulting in aspartic acid and glutamic acid, respectively. Therefore, the composition detected corresponds to the sum of both components. Two independent measurements were done for each sample.

2.3.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The Mw pattern was determined according to a modified version of the method by Laemmli (1970). Gelatines were dissolved in distilled water at 5 μg powder/ μL and subsequently mixed with NuPAGE LDS sample buffer 4X (Life Technologies, Carlsbad, CA, USA) in the presence of NuPAGE sample reducing agent 10X (Life Technologies) to get a final concentration of 2 μg powder/ μL . The mixtures were heated for 10 min at 70 °C (following the manufacturer's instructions) for total protein denaturation. An aliquot of 20 μL of each sample was loaded onto a precast 4% stacking and 8% resolving polyacrylamide gel (Life Technologies). Also, 5 μL of PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) with Mw ranging from 10 to 250 kDa were loaded along with the samples. The gel was immersed in MES SDS Running Buffer (Life Technologies), and electrophoresis was run at a constant voltage of 60 V for the stacking gel and at 90 V for the resolving gel using a PowerEase 300W power supply (Life Technologies). Protein bands were stained with Coomassie brilliant blue R250 (Thermo Fisher, Rockford, IL, USA) for 1 h shaking using a JP Selecta Rotabit Shaker 3000974 (Selecta, Abrera, Barcelona). Finally, the gel was destained, using distilled water, shaking overnight. Each sample was prepared and run in duplicate.

2.3.6. Gel permeation chromatography – size exclusion chromatography (GPC-SEC)

The Mw distribution of the gelatines was analysed using gel permeation chromatography at the Instituto de Investigaciones Marinas (IIM-CSIC, Vigo, Spain) with an Agilent 1260 LC system (Agilent Technologies, Santa Clara, CA, USA) consisting of a quaternary pump (G1311B), injector (G1329B), column oven (G1316A), diode array (G1315C), refractive index (G1362A), and dual angle static light scattering (G7800A) detectors. Standard and samples were dissolved at 2 g/L in a 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH = 4.5) and pumped through four columns: Proteoma precolumn (5 μm , 8 $\times$ 500 mm), Proteoma 100 Å (5 μm , 8 $\times$ 300 mm), Proteoma 300 Å (5 μm , 8 $\times$

300 mm) and Proteoma 1000 Å (5 μm , 8 $\times$ 300 mm) (PSS GmbH, Mainz, Germany). The column oven and the light scattering detector were kept at 30 °C, and the refractive index detector was maintained at 40 °C. Detectors were calibrated with a polyethylene oxide standard (PSS, Mainz, Germany) of 106 kDa (Mw) and polydispersity index 1.05. Mw values were estimated with refractive index increments of 0.19 (Meyer & Morgenstern, 2003).

The number average molecular weight (M_n), the weight average molecular weight (M_w) and the polydispersity index (PDI) were calculated with the following equations, respectively:

$$M_n = \frac{\sum M_{wi} N_i}{\sum N_i} \quad [2]$$

$$M_w = \frac{\sum M_{wi}^2 N_i}{\sum M_{wi} N_i} \quad [3]$$

$$PDI = \frac{M_w}{M_n} \quad [4]$$

where N_i is the number of protein molecules and M_{wi} is their molecular weight.

2.3.7. Thermal characterisation

Thermal stability and decomposition behaviour of gelatines was studied using thermogravimetric analysis (TGA). Dried gelatine (~5–20 mg) was placed in an open platinum pan and loaded into the measuring chamber of a TA Instruments Q500 thermogravimetric analyser (TA Instruments, New Castle, DE, USA). Samples were heated from room temperature to 800 °C at 5 °C/min in an inert nitrogen gas (99.999%, Nippon Gases Iberia, Madrid, Spain) atmosphere. To avoid contamination, nitrogen gas was also used as the balance purge gas and as the sample purge gas (flow rates of 40 and 60 mL/min, respectively). The recorded thermograms were analysed using the Universal Analysis 2000 software of TA Instruments.

2.3.8. Rheological behaviour

Rheological properties of the extracted gelatines were studied in an Anton Paar MCR 102 (Anton Paar, Graz, Austria) modular compact rheometer, using a cone-plate geometry (1° cone angle, 50 mm cone diameter, 0.098 mm gap) conforming to the small amplitude oscillatory shear (SAOS) methodology (Steffe, 1996). The experiments were done following the protocol by Alves et al. (2022) with slight modifications. Solutions at 6.67 wt% were prepared at 45 °C to facilitate dissolution, and then they were allowed to cool to room temperature before starting the measurements. The dynamic rheological properties were studied at a fixed frequency and strain of 1 Hz and 10%, respectively, which were within the linear viscoelastic region previously assessed using strain sweep tests. The protocol consisted of a heating ramp from 2 to 30 °C followed by a cooling ramp from 30 to 2 °C, both at a rate of 1 °C/min. The storage modulus (G') and the loss modulus (G'') were measured as a function of temperature. Additionally, the mechanical properties of the gels were evaluated using frequency sweeps from 10^{-1} to 10^2 Hz at 2 °C (lowest working temperature).

3. Results and discussion

3.1. Extraction yield

The pretreatment applied to the raw material prior to the extraction step has a major effect on the yield, and also on the properties of the final product (Alves et al., 2022; Gómez-Guillén et al., 2002; Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011; Milovanovic & Hayes, 2018). The effect of the contact time between the skin and the eutectic solvent on both the yield and the properties of the extracted product was studied. Although the extraction yield is often based on the total mass of

wet skin, the results obtained in this way cannot be compared since a variation in the moisture of raw material will distort the results. Therefore, the extraction yield was also calculated on a dry basis for yellowfin tuna skin. As shown in Table 2, the recovery in the case of the pretreated samples was 31–33 g gelatine/100 g wet skin, which represents 60–62 g gelatine/100 g dry skin; and compares favourably to the yield obtained with the non-pretreated sample (row labelled as pretreatment time of 0 h in Table 2). These values were higher than the values of 6–19 g gelatine/100 g wet skin that have been reported (Karim & Bhat, 2009). No significant difference was observed between the yield at 0.5 h pretreatment and that at 3 h pretreatment.

3.2. Proximate composition

Proximate composition is shown in Table 3. The ideal moisture content of powdered gelatine is in the range 8–12% since levels of moisture >16% carry the risk of lumping and microbiological deterioration, while values < 6–8% may lead to hygroscopic gelatines (Işık et al., 2024). These gelatines showed a suitable moisture content. The raw material and its preservation method affect the ash content, ranging from 1.1 to 3.7% in the case of gelatines from yellowfin tuna skin (Pranoto, Marseno, & Rahmawati, 2011; Sousa, Vázquez, Pérez-Martín, Carvalho, & Gomes, 2017). The ash values of the samples extracted with pretreatment in Table 3 are on the higher end of that range. Further studies will be required to ascertain whether it is due to a favoured solubilisation of minerals from the skin in the eutectic solvent or to a minimum presence of sodium coming from the eutectic parent compounds. The sample with the shortest pretreatment time had 88.8% organic matter, compared to 87.2% after 3 h of pretreatment. However, the protein content was statistically equivalent in both cases within the uncertainty: 83 ± 1 % and 84 ± 1 %, respectively. All the values, including those for the sample extracted without pretreatment and for the commercial gelatine, were within the range of values obtained when applying traditional extraction systems.

3.3. FT-IR spectra

For the fish gelatines obtained after pretreatment with the eutectic solvent and subsequent warm water extraction, the most characteristic peaks of gelatine structure could be identified (Fig. 1), namely those peaks identified as amide A, amide B, amide I, amide II, and amide III.

Starting at the highest wavenumber, the first intense and broad band is seen at 3286 cm^{-1} , corresponding to the amide-A signal (Kong & Yu, 2007) associated with the O-H stretching and the N-H stretching coupled with hydrogen bonding (Merina, Suguna, Karpuram, Vijaylakshmi, & Renuka, 2017; Shahvalizadeh et al., 2021). This band is somewhat overlapping the amide-B band, observed at 2937 cm^{-1} and associated with C-H stretching vibrations. Previous studies (Muyonga, Cole, & Duodu, 2004; Silva, Bandeira, & Pinto, 2014) reported that the wide amplitude of this band may be associated with the increased presence of peptide chains, suggesting more degradation of the protein during the process of extraction. The existence of these low Mw fragments implies the presence of more peptide bonds, thus facilitating the possibility of a network interaction that may lead to the formation of gels.

Representative bands for amide-I and amide-II were found, respectively, at 1635 and 1529 cm^{-1} , in agreement with previous results (Kong

Table 2

Gelatine extraction yields (values are average of three replicates $\pm$ standard deviation).

Pretreatment time (h)	Yield (g gelatine/100 g skin)	
	Basis: wet skin	Basis: dry skin
0	27 ± 1	51 ± 1
0.5	33 ± 1	62 ± 2
3	31 ± 1	60 ± 2

Table 3

Composition of gelatine samples (values are the average of two replicates $\pm$ standard deviation).

Pretreatment time (h)	Content (g/100 g sample)			
	Moisture	Ash	Organic matter	Crude protein
0	8.3 ± 0.3	2.1 ± 0.2	90 ± 1	82 ± 1
0.5	8.1 ± 0.2	3.1 ± 0.1	88.8 ± 0.5	83 ± 1
3	9.2 ± 0.2	3.7 ± 0.1	87.2 ± 0.5	84 ± 1
Commercial sample	9.6 ± 0.6	0.2 ± 0.0	90 ± 1	88 ± 1

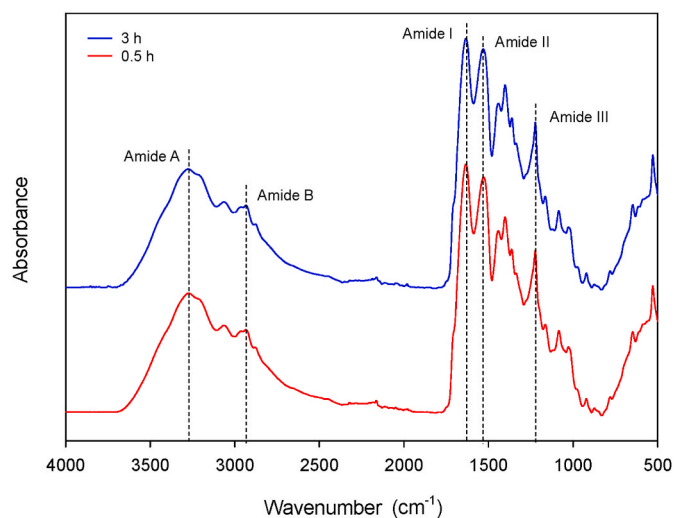


Fig. 1. FT-IR spectra of fish gelatines obtained using warm water extraction from the pretreated raw material. From top to bottom: 3 h pretreatment (blue line), 0.5 h pretreatment (red line). Vertical dashed lines correspond to the wavenumber of the characteristic peaks of proteins and polypeptides.

& Yu, 2007; Merina et al., 2017; Silva et al., 2014; Valcarcel, Hermida-Merino, Piñeiro, Hermida-Merino, & Vázquez, 2021). The absorption zone of the amide-I corresponds to the stretching vibrations of the C=O bond within the peptide backbone of the protein. On the other hand, the peak of the amide-II is associated mainly with the C-N stretching and the N-H bending (Abdollahi & Undeland, 2018; Kong & Yu, 2007). Amide III is associated with the stretching vibration of C-N and N-H deformation from amide bonds, and in this case is seen at 1225 cm^{-1} . It has been associated with the presence of a molecular disorder, probably related to the loss of the triple helix structure (Kumar, Chandra, Elavarasan, & Shamasundar, 2018; Sinthusamran, Benjakul & Kishimura, 2014).

As shown in Fig. 1, no significant differences were found between the spectra of gelatines obtained after the two different pretreatment times. This suggests that, between the two times studied, the contact time does not have an important influence on the observable conformation of the extracted gelatine. Moreover, these key characteristic bands of gelatines are also observed in the FT-IR spectra of the sample extracted with no pretreatment and of the commercial gelatine sample (Figs. S1 and S2 in the Supporting Information).

3.4. Amino acid profile

The amino acid composition of gelatines has an important role in determining their quality and characteristics. The amino acid profiles are shown in Table 4.

Table 4

Amino acid content (expressed in g of amino acid/100 g of total amino acids) of the gelatines obtained from the fish skin, using different times for the pretreatment with the eutectic solvent. Values are represented as the average of two replicates $\pm$ standard deviation.^a

Amino acid	Concentration (g/100 g total amino acids)			Commercial
	0 h	0.5 h pretreatment	3 h pretreatment	
Alanine	9.0 $\pm$ 0.7	7.0 $\pm$ 0.8	7.0 $\pm$ 0.1	9.1 $\pm$ 0.3
Arginine	9.0 $\pm$ 0.2	7.6 $\pm$ 0.7	7.9 $\pm$ 0.1	8.9 $\pm$ 0.2
Aspartic acid ^b	5.0 $\pm$ 0.7	4.8 $\pm$ 0.5	4.7 $\pm$ 0.1	5.4 $\pm$ 0.9
Cysteine	n. d.	n. d.	n. d.	n.d.
Glutamic acid ^c	9.0 $\pm$ 0.3	9 $\pm$ 1	8.8 $\pm$ 0.2	9.4 $\pm$ 0.7
Glycine	23 $\pm$ 1	24.6 $\pm$ 0.3	24.9 $\pm$ 0.4	23 $\pm$ 1
Histidine	2.00 $\pm$ 0.03	5.0 $\pm$ 0.5	4.79 $\pm$ 0.03	2.0 $\pm$ 0.1
Isoleucine	1.27 $\pm$ 0.06	3.4 $\pm$ 0.3	3.4 $\pm$ 0.1	1.3 $\pm$ 0.1
Leucine	2.6 $\pm$ 0.1	0.9 $\pm$ 0.1	0.92 $\pm$ 0.03	2.1 $\pm$ 0.1
Lysine	3.29 $\pm$ 0.04	2.7 $\pm$ 0.3	2.5 $\pm$ 0.1	3.4 $\pm$ 0.2
Methionine	2.1 $\pm$ 0.1	2.2 $\pm$ 0.2	2.3 $\pm$ 0.1	2.1 $\pm$ 0.1
Hydroxyproline	9 $\pm$ 1	7.5 $\pm$ 0.6	7.4 $\pm$ 0.1	8 $\pm$ 1
Phenylalanine	2.17 $\pm$ 0.01	2.0 $\pm$ 0.1	2.2 $\pm$ 0.1	2.09 $\pm$ 0.01
Proline	11.3 $\pm$ 0.8	12.4 $\pm$ 0.1	12.5 $\pm$ 0.2	11.2 $\pm$ 0.6
Serine	5 $\pm$ 2	3.9 $\pm$ 0.4	3.9 $\pm$ 0.1	5 $\pm$ 2
Threonine	3.06 $\pm$ 0.06	4.3 $\pm$ 0.4	4.3 $\pm$ 0.1	3.05 $\pm$ 0.04
Tyrosine	0.40 $\pm$ 0.09	0.32 $\pm$ 0.04	0.36 $\pm$ 0.04	0.38 $\pm$ 0.07
Valine	2.4 $\pm$ 0.3	2.5 $\pm$ 0.3	2.46 $\pm$ 0.02	2.29 $\pm$ 0.05

^a n.d.: not detected.

^b Including asparagine, which is deamidated during the hydrolysis process to aspartic acid.

^c Including glutamine, which is deamidated during the hydrolysis process to glutamic acid.

The most prominent amino acids to consider for fish gelatine are glycine, proline and hydroxyproline. The composition of these three amino acids in the samples obtained after pretreatment was, respectively, around 25, 12.5 and 7.5%, with no significant influence of the pretreatment time. The results were consistent with previous research where the range was 22–35% for glycine, 9–15% for proline and 5–10% for hydroxyproline, and similar gelatine contents were obtained (Alves et al., 2022; Derkach, Voron'ko, Kuchina, & Kolotova, 2020; Gómez-Guillén et al., 2002; Gudmundsson & Hafsteinsson, 1997; Nurilmala, Suryamarevita, Husein Hizbullah, Jacob, & Ochiai, 2022). Gelatine content can be estimated through hydroxyproline content by the method of Sato, Ohashi, Ohtsuki, and Kawabata (1991). Using a conversion factor of 11.42 (according to the proportion of hydroxyproline in the total amino acid content in collagen), the gelatine of the samples extracted with pretreatment was $\sim$ 85%, lower than the value of $\sim$ 97% obtained for the sample with no pretreatment or for the commercial gelatine, and therefore meaning that non-collagenous proteins were also extracted. Proline and hydroxyproline have an important role in the stabilisation of the triple helix, especially hydroxyproline because of its capacity to form hydrogen bonds using the hydroxy group (Piez & Gross, 1960). When the temperature drops below the coil-to-helix transition temperature, the collagen structure undergoes partial regeneration. Segments of polypeptide chains rich in pyrrolidine amino acids adopt a helical conformation and form junction zones capable of retaining water, thereby facilitating gel formation (Haug, Draget, & Smidsrød, 2004; Kasankala, Xue, Weilong, Hong, & He, 2007). Therefore, the combined proline and hydroxyproline content of gelatine affects its physical properties, both in terms of rheological behaviour and thermal stability (Gómez-Guillén et al., 2002; Lin, Regenstein, Lv, Lu, & Jiang, 2017). The combined content represents $\sim$ 20% in all the gelatines included in Table 4, which is consistent with previous values for the skin of warm water fish such as yellowfin tuna (higher than values obtained for cold water species but lower than the 30% found in mammalian gelatines) (Farris, Schaich, Liu, Piergiovanni, & Yam, 2009).

3.5. SDS-PAGE

Mw distribution of gelatines affects their physical, chemical, and biological properties. Fig. 2 shows the result of SDS-PAGE, where A and B represent the two replicates of the same sample, and 1 and 2 denote the gelatines extracted after 3 and 0.5 h of pretreatment, respectively. It can be observed that both samples show very similar patterns: a heterogeneous distribution of bands ranging from 15 to 130 kDa, particularly concentrated around 55–70 kDa. Some bands appear in the 100–130 kDa range, which could be indicative of the presence of the residual α_1 and α_2 chains of collagen. However, there is also evidence of the total absence of any β -dimer (composed of two α chains linked by covalent bonds) or γ -chain (trimer composed of three cross-linked α chains) (Alves et al., 2022). This interpretation is somewhat consistent with the FT-IR spectra: the triple helix structure of collagen has been completely unfolded during the extraction procedure. Furthermore, the presence of lower Mw polypeptide chains confirms the partial degradation of the gelatine. These results are consistent with those of Yu et al. (2023), who characterised gelatines extracted from the skin of other warm water fish species after their pretreatment with sodium hydroxide and acetic acid, and subsequent extraction with hot water at 50 °C for 6 h. A similar pattern was also observed for the sample obtained without pretreatment; whereas for the commercial gelatine, with an average Mw of 60 kDa, very blurred bands were observed (Fig. S3 in the Supporting Information).

3.6. GPC-SEC

A quantitative analysis of the Mw distribution of the gelatines obtained with pretreatment was done using GPC-SEC. The results are shown in Table 5, obtained from the corresponding eluograms shown in

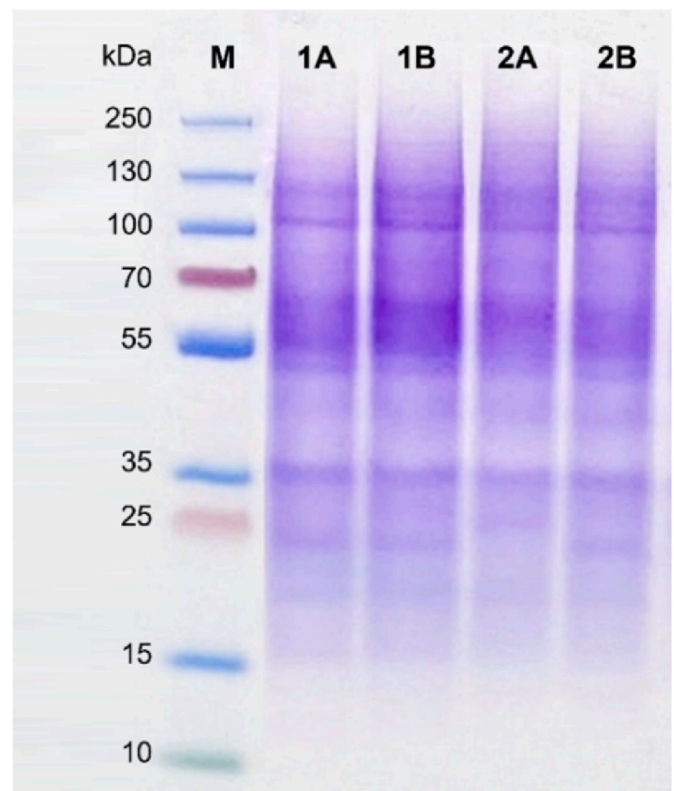


Fig. 2. SDS-PAGE patterns of gelatines. The code M refers to a prestained protein ladder marker (10–250 kDa); the codes 1A and 1B correspond to replicates of the sample extracted after 3 h of pretreatment; and the codes 2A and 2B correspond to replicates of the sample extracted after 0.5 h of pretreatment.

Table 5

Molecular weight distribution of gelatines obtained after two different pretreatment times. Values are represented as the average of two replicates $\pm$ standard deviation.

Pretreatment time (h)	Region ^a	M_n^b (kDa)	M_w^c (kDa)	PDI ^d	Peak area (%)
0.5	1	160 $\pm$ 10	171 $\pm$ 7	1.077	2.4 $\pm$ 0.6
	2	80 $\pm$ 6	83 $\pm$ 4	1.040	16 $\pm$ 2
	3	17 $\pm$ 1	26 $\pm$ 3	1.526	82 $\pm$ 5
3	1	183 $\pm$ 19	193 $\pm$ 14	1.056	7.8 $\pm$ 0.9
	2	83 $\pm$ 9	88 $\pm$ 6	1.053	29 $\pm$ 3
	3	21 $\pm$ 2	30 $\pm$ 2	1.396	64 $\pm$ 7

^a See Fig. 3 for identification of the different regions on the basis of their elution time.

^b M_n : number average molecular weight.

^c M_w : weight average molecular weight.

^d PDI: polydispersity index.

Fig. 3.

Similar to SDS-PAGE, GPC results showed a heterogeneous distribution of the M_w of gelatines regardless of the pretreatment time. In both cases, three different regions could be identified: the first region corresponds to chains with M_w of 171–193 kDa for the gelatines obtained from both treatments. This region, which is associated with the presence of β -dimers (Rigueto et al., 2023), represents a small part of the peak in both cases (2.4 and 7.8%). The second region corresponds to molecules with M_w of 83–88 kDa. It is compatible with the M_w of α chains (Rigueto et al., 2023). In this case, the peak area increases up to 16 and 29%, respectively. The largest region for both gelatines corresponds to a M_w of 26–30 kDa and represents 82 and 64% of the total peak area. The polydispersity index (PDI) of this region was 30–50% higher than in the other regions. Since PDI is a measure of the broadness of the M_w distribution, high values are often related to intramolecular cleavage and some degree of selective hydrolysis (Eysturskard, Haug, Elharfaoui, Djabourov, & Draget, 2009; Rbii, Surel, Brambati, Buchert, & Violleau, 2011), which is consistent with the low values of M_w in this region.

By comparing the results with both gelatines, the M_w of all regions and the peak areas of the first and the second regions (high M_w) are higher in the gelatine obtained after the longer pretreatment time. This

is consistent with Cui et al. (2021), who investigated the solubility and interactions between the same eutectic solvent and commercial porcine gelatine. They found that the M_w of the gelatine increased after being dissolved in the sodium acetate trihydrate-urea eutectic solvent and subsequently regenerated, probably due to a random re-forming of larger molecules using hydrogen bonding.

3.7. Thermal characterisation

Thermal stability and decomposition behaviour of gelatines are affected by their internal structure, M_w distribution and amino acid composition (Lin et al., 2017). The TGA curve (variation of sample weight with temperature, expressed as a percentage of the initial sample mass) is shown in Fig. 4 together with its derivative, for the gelatines obtained with pretreatment. Two main decomposition steps were observed: the first one, with a maximum rate of decomposition at ~ 140 °C and representing a mass loss of $\sim 20\%$, was probably associated with the evaporation of adsorbed and bound water (Correia et al., 2013); and the second one was related to the decomposition of protein and the subsequent loss of amino acids (Martins et al., 2018), with a maximum

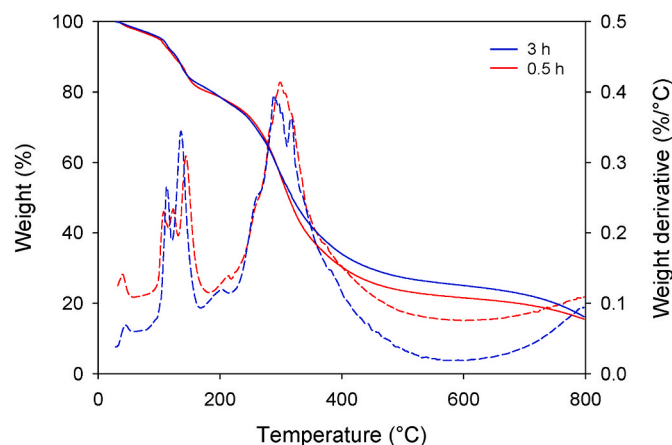


Fig. 4. TGA curves (solid lines), and their derivatives with temperature (dashed lines), for the gelatines extracted from yellowfin tuna skin with two different pretreatment times.

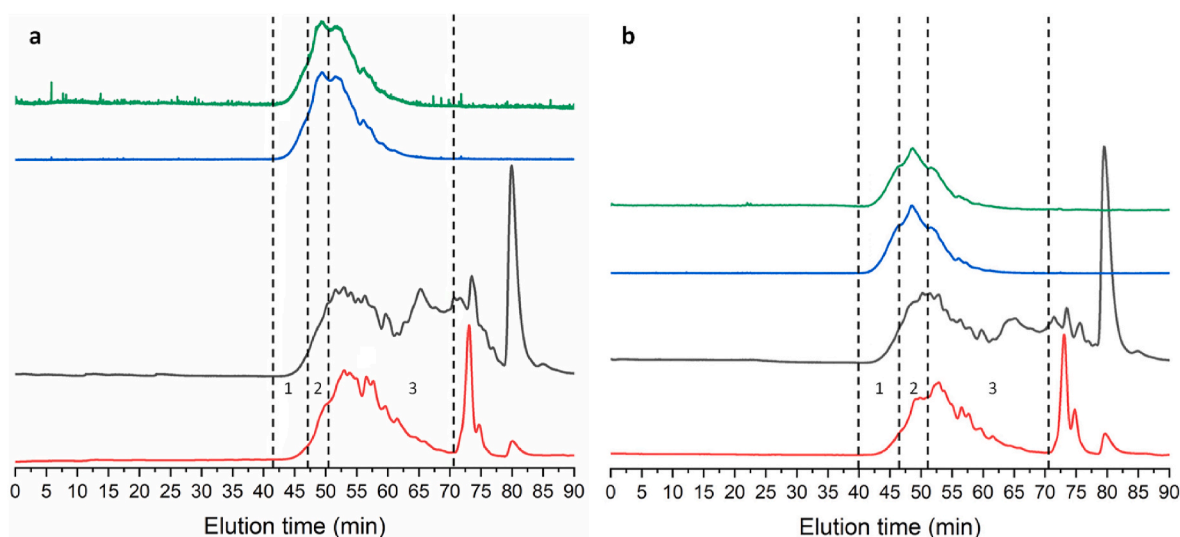


Fig. 3. GPC eluograms of yellowfin tuna gelatines obtained after (a) 0.5 h or (b) 3 h of pretreatment with the eutectic solvent. Signals for the different detectors (from top to bottom): low angle light scattering (green line); right angle light scattering (blue line); ultraviolet (232 nm) (black line); and refractive index (red line). Vertical dashed lines define three ranges (labelled with the cardinals 1, 2, and 3) of elution time for easier interpretation of the results – see Table 5 and associated discussion in the main text.

decomposition rate around 300 °C and increasing the decomposed percentage up to ~75%. Onset decomposition temperature (T_{onset}), temperature of the maximum decomposition rate (T_{max}), and mass loss associated with each of these decomposition steps are shown in Table 6. Minima in the derivative curves at ~600 °C suggest the beginning of a third decomposition step. Since the TGA runs were stopped at 800 °C, the temperature of the maximum decomposition rate for this additional decomposition step was not ascertained. In any case, the residue remaining at the final point of each run is also included in Table 6.

Previous studies reported a similar pattern for thermal decomposition of fish gelatines, although some differences in specific temperature values are worth mentioning. Martins et al. (2018) did the TGA of tilapia scale gelatine, while Valcarcel et al. (2021) investigated the thermal decomposition of gelatines from the skin of seabream, seabass, and rainbow trout. In both cases it was observed that, as in the present study, thermal decomposition occurred in two distinct stages. The T_{max} of the first stage in the case of tilapia scale gelatine was found at 65.4 °C, substantially lower than the current values (116 °C and 111 °C for the gelatines obtained with the two pretreatments, respectively). As shown by Valcarcel et al. (2021), this parameter varies depending on the species from which the gelatine is obtained and on the extraction process. The control sample extracted with no pretreatment showed a previous decomposition step (see Fig. S4 in the Supporting Information and Table 6) at low temperatures (<50 °C), associated with the evaporation of adsorbed water. Regarding the second decomposition step, experimental and literature values are more homogeneous: the current T_{max} values are close to those previously obtained: ~325 °C (tilapia) by Martins et al. (2018) and 301–316 °C (other species) by Valcarcel et al. (2021). Despite the differences in T_{max} values, the overall mass losses after each of the steps reported by Martins et al. (2018) were consistent with this study: 17–21 and 74–78%, respectively. The residue at 800 °C of 15–17% of the initial mass was close to the 19% obtained by Martins et al. (2018), and slightly lower than the 27–31% obtained by Valcarcel et al. (2021). In the case of the commercial gelatine sample, the absence of decomposition steps around 100–150 °C suggests the absence of water association within the gelatine structure (Fig. S5 in the Supporting Information), which could affect the gelling properties. Considering the broadness of the ranges and the strong influence of the origin of the gelatine in its thermal decomposition behaviour, it can be assumed that the current results are consistent with previous reports.

3.8. Rheological behaviour

The viscoelastic properties of gelatine are useful to assess the quality of the product and determine its potential applications. Upon cooling an aqueous solution of gelatine below its sol-gel transition temperature, gelatines undergo conformational changes, forming triple chain helices

Table 6

Onset decomposition temperature (T_{onset}), temperature of maximum decomposition rate (T_{max}), and mass loss (with respect to the initial sample mass) associated with each of the two main decomposition steps identified using TGA in the thermal characterisation of the gelatines. The final residue at 800 °C, as a percentage of the initial sample weight, is also included.

Pretreatment time (h)	Decomposition step	T_{onset} (°C)	T_{max} (°C)	Mass loss (%)	Residue at 800 °C (%)
0	0 th	34	41	9.7	17.8
	1st	154	161	13.7	
	2nd	263	306	78.9	
0.5	1st	116	144	20.1	15.5
	2nd	261	299	78.4	
3	1st	111	136	18.2	16.2
	2nd	253	289	74.4	
Commercial	1st	41	55	12.1	25.3
	2nd	256	302	71.0	

(junction points) to create the three-dimensional network gel structure. Water remains trapped in this matrix, giving the gel its characteristic texture and consistency. The forces governing this transition are physical (specifically hydrogen bonds and van der Waals bonds), which makes the gelation process thermoreversible (Ahmed, 2017). Therefore, one of the most important outcomes for rheological study of gelatines is the determination of the gelling temperature (T_{gel}) and the melting temperature (T_{melt}), which are determined from the intersection of the storage modulus (G') and the loss modulus (G'') curves during cooling and heating of the sample, respectively. Fig. 5 shows the changes of G' and G'' with temperature in a heating ramp and in a cooling ramp, for aqueous solutions of the gelatines pretreated with the eutectic solvent at 6.67 wt%. Both gelatines showed a qualitatively similar behaviour, with some numerical differences. At the beginning of the heating step, G' is significantly higher than G'' , indicating that the solution shows a predominantly solid-like behaviour at low temperatures. When temperature increases, the three-dimensional network starts weakening and the consequence is a decrease in both G' and G'' . Once above the melting point, G'' becomes higher than G' , marking the transition from gel to liquid state. At higher temperatures, G'' remains higher than G' , suggesting the single strand arrangement of polypeptide chains (Kokol, Pottathara, Mihelčič, & Perše, 2021). During the cooling step, G' and G'' strongly increase as a result of the formation of junction zones and the reinforcement of the gel network through hydrogen bonding, van der Waals forces, self-assembly, and hydrophobic associations (Da Silva, Bode, Grillo, & Dreiss, 2015). At a given temperature during the cooling ramp, G' again becomes greater than G'' . The crossover of both curves determines the gelling point, at which gelatine makes the transition from liquid-dominated phase to solid-dominated phase (Ahmed, 2017; Huang et al., 2017).

Table 7 shows the values of T_{melt} and T_{gel} for the gelatine solutions, including the ones of the sample extracted with no pretreatment and of the commercial gelatine. The melting and gelling temperatures of gelatine are normally different reflecting its thermal hysteresis (Rafe & Razavi, 2017). The gelatine obtained at 3 h pretreatment showed higher melting and gelling temperatures than the gelatine extracted in the procedure with a 0.5 h pretreatment, suggesting that a longer contact between the fish skin and the eutectic solvent resulted in a more structured gel network with higher resistance to temperature, which is consistent with the presence of higher Mw chains as shown using the GPC-SEC. This may be related to the existence of some particular interactions between the eutectic solvent and the gelatine, since some studies have shown that the components of eutectic systems can interact with polypeptide chains and become part of the gelatine structure (Cui et al., 2021; Sanchez-Fernandez et al., 2022; Wan, Zhu, & Sun, 2024). This is consistent with the gelatine obtained without pretreatment (see Fig. S6 and Table 7) showing lower melting and gelling temperatures. Previous studies reported gelling and melting temperatures for fish gelatines in the ranges 8–25 and 11–28 °C, respectively (Huang et al., 2019). However, for yellowfin tuna skin, the results were significantly lower than the gelling temperature of 18.7 °C and the melting temperature of 24.3 °C reported by Cho, Gu, and Kim (2005). This may be due to more partial degradation of gelatine. However, the raw materials cannot be considered fully equivalent: in the study by Cho et al. (2005), the skins were fresh; but the current skins were brined. As shown by Alves et al. (2022), gelatines derived from salted skins showed lower gel strength, which is seen as lower values of G' , T_{melt} , and T_{gel} . The lower Mw distribution resulting from this more effective degradation allows the molecules to entangle and form a network at lower energy levels, directly affecting the viscoelastic behaviour (Enrione et al., 2020). The lower gelling temperatures might represent a technical advantage over mammalian-derived gelatines in applications that require better release of aroma and stronger flavour (Choi & Regenstein, 2000). A similar value was found by Sousa et al. (2017), who reported a T_{melt} of 21.9 °C. On the other hand, as shown in Fig. S7, and although the commercial gelatine originates from a cold-water fish and is soluble in water, it is

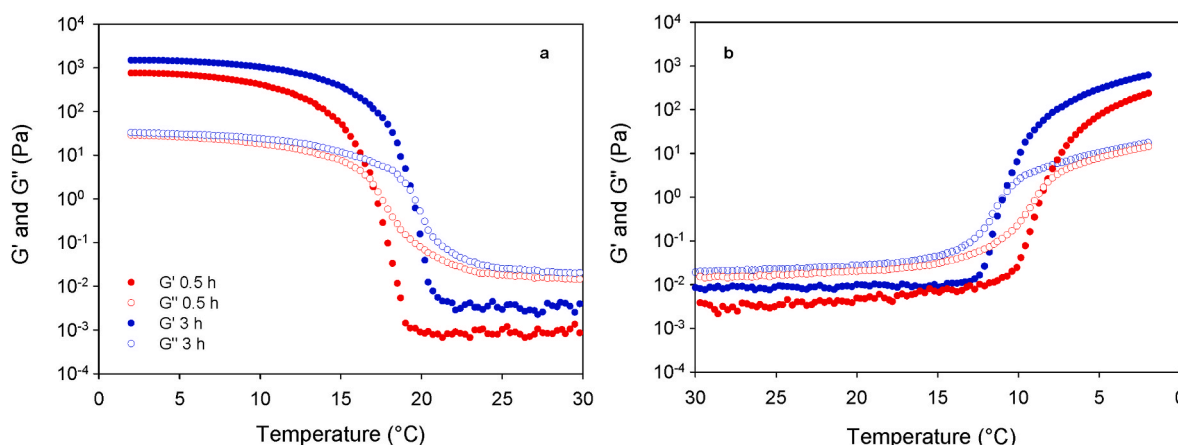


Fig. 5. Variation of storage modulus (G') and loss modulus (G'') of aqueous gelatine solutions with temperature: heating ramp (a) and cooling ramp (b).

Table 7

Melting and gelling temperatures of aqueous solutions of the gelatines.

Pretreatment time (h)	T_{melt} (°C)	T_{gel} (°C)
0	14.6	7.3
0.5	17.0	8.5
3	19.4	11.0
Commercial	5.1	–

unable to gel.

Frequency sweep tests were done to analyse the mechanical properties of the best-quality gels. Fig. 6 shows both G' and G'' as a function of frequency for the gelatines obtained with pretreatment. As expected, the higher values for G' indicated a greater contribution from elasticity than from viscosity, and hence a solid-like behaviour. Moreover, the values of G' are at least one order of magnitude greater than those of G'' for frequencies <10 Hz, indicative of a completely developed and stable gel structure (Morris, Nishinari, & Rinaudo, 2012). Both temperature and frequency sweeps show that the elastic modulus of the gelatine obtained after the longer pretreatment time was higher and, consequently, so is the gel strength.

4. Conclusions

The use of a eutectic solvent as pretreatment agent for the extraction of gelatine from yellowfin tuna skin was studied. This method used, as a

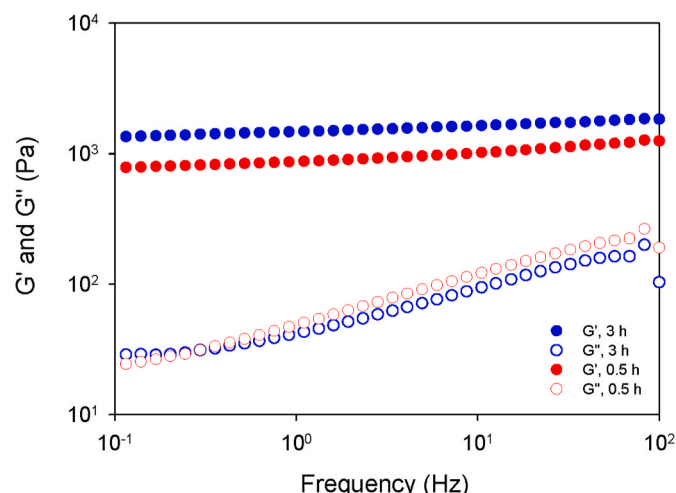


Fig. 6. Frequency sweep tests of the gels at a constant temperature of 2 °C.

pretreatment solvent, a mixture of compounds that in the pure state are solid at the operating temperature. It was tested with two different pretreatment times of 0.5 and 3 h, after which extraction was carried out with warm water as in the traditional procedure. It was confirmed that the eutectic solvent can interact with the skin of yellowfin tuna, such that the subsequent extraction of gelatine is possible.

Comparing the gelatines obtained with the proposed process to a gelatine extracted with water, it can be confirmed that the pretreatment increases the extraction yield. Additionally, the gelatines showed better rheological properties than the one just extracted with water (no pretreatment) and a commercial gelatine obtained from cold-water fish skin.

Regarding the samples extracted with pretreatment, the overall process yields, which exceed literature values by up to 70%, were found to be independent of the pretreatment time. Besides the yield, other parameters related to gelatine characterisation that were not affected by the pretreatment time were: chemical compositions, FT-IR spectra, and amino acid profiles. These were consistent with those of gelatines obtained using the traditional method in previous studies. However, significant differences were found in the Mw distribution: the gelatine obtained after a longer pretreatment time showed a higher Mw, which directly affects its functionality. This increase in Mw is reflected in the rheology studies, where it was observed that this gelatine showed higher values for the storage modulus, as well as higher gelling and melting temperatures. Thus, the pretreatment time could be selected based on the desired application for the gelatine.

In summary, the use of eutectic solvents as an alternative to traditional methods for extracting gelatine from fish waste may result in improvements of various functional properties: it enables the utilisation as pretreatment solvents of compounds that, in the pure state, are solid at the operating temperature; it simplifies the process in comparison with the traditional methods used, reducing the environmental impact; and it allows the tuning of interactions between the fish by-product and the solvent (through judicious selection of the components of the eutectic system) to design the final product with the desired properties for a specific application. This option should be also considered for more challenging targets such as collagen extraction.

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CRediT authorship contribution statement

Cristina Gallego: Writing – original draft, Visualization, Validation,

Methodology, Investigation, Formal analysis. **Eva Rodil**: Writing – review & editing, Methodology, Conceptualization. **Héctor Rodríguez**: Writing – review & editing, Supervision. **Ana Soto**: Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2024.110652>.

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