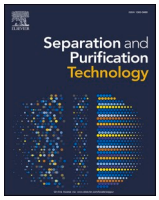
Separation and Purification Technology 363 (2025) 132144

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur



Tuna skin as bioresource for gelatine: Extraction with ionic liquid☆

Paula Souto-Montero [a], Cristina Gallego [a], José Antonio Vázquez [b], Eva Rodil [a], Ana Soto [a,*]

[a] CRETUS. Department of Chemical Engineering, Universidade de Santiago de Compostela E-15782 Santiago de Compostela, Spain
[b] Group of Recycling and Valorisation of Waste Materials (REVAL), Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain

ARTICLE INFO

Editor: Dr. B. Van der Bruggen

Keywords:
Gelatine
Fish skin
Maceration
Ionic liquid

ABSTRACT

Unlocking the ocean's full potential requires converting fish waste into materials and products of interest, thus improving the circularity and sustainability of the fish processing industry. Fish skin is an excellent bioresource for obtaining gelatine, a product of high interest in many industries due to its multifunctionality. The current extraction methods involve several stages with harsh chemicals, which pose environmental concerns. In this work, an innovative method consisting of maceration with ionic liquid and extraction with water is proposed. Unlike other methods based on ionic liquids or deep eutectic solvents that focus on dissolving the fish by-product, and therefore require an energy-intensive stage to recover the protein, a non-dissolving treatment is proposed to facilitate protein extraction with water. The gelatine is precipitated from the aqueous solution by adding acetone. The method was tested obtaining gelatine from tuna skin and using 1-ethyl-3-methylimidazolium acetate, a low toxicity ionic liquid able to disrupt the skin through hydrogen bonding. Optimised conditions led to a recovery yield of 0.188 g gelatine/g wet skin. FTIR showed the characteristic bands corresponding to molecular vibrations and functional groups of the gelatine structure. α- and β-chains constitute approximately the 43 % of the product, with the remainder being peptides with an average molecular weight of 44 kDa. The pyrrolidine amino acids content was 21 %. The gelatine showed good thermal stability. Melting and gelling temperature (20.6 and 12.9 °C, respectively) fall within the typical range for warm water fish. As an advantage in comparison to classical methods, the proposed method avoids the need of multiple treatment steps with strong acids and alkalis.

1. Introduction

The solid residues from the fish industry are significant by-products that include skin, scales, bones, meat portions, viscera, and other unwanted parts of the fish. This matter is typically generated in large quantities, ranging from 20 to 80 % of the original raw material, depending on the level of processing. The disposal of fish industry waste poses environmental and economic challenges, as it can lead to increased energy consumption, financial costs, and environmental impact [1]. The blue economy promotes the sustainable use of ocean resources for economic growth while also preserving the health of marine ecosystems [2]. Unlocking the ocean's full potential requires converting fish by-products into materials and products of interest, thus improving the circularity and sustainability of the fish processing industry [3–5].

Certain valuable products that can be obtained from fish residues must be highlighted. Fish viscera constitute a good source of enzymes, such as pepsin and trypsin [6,7]. Moreover, these can be used in the generation of bioactive fish hydrolysates with applications in the food, pharmaceutical and cosmetic industries, among others. Hydroxyapatite can be obtained from bones and scales, a promising alternative to obtaining it via chemical synthesis. Calcination is the most common method to obtain this calcium phosphate compound. Fish skin has a significant proportion of collagen, a structural protein that constitutes the backbone of tissues and shows unique functional and biocompatible properties. This fibrous protein can also be found in bones, fins and scales. Gelatine is the result of the partial hydrolysation of the triple helix of collagen. It forms colloidal solutions with water [8]. Besides its nutritional and physiological properties, the main advantage of gelatine is its multifunctionality. It is widely used in the formation of gels and foams, as emulsifier, stabiliser, etc. The extraction of collagen/gelatine from fish skin typically involves a series of stages including pretreatment, extraction, and purification. The traditional methods of extraction rely on alkaline and acid treatments followed by extraction with water. Enzymatic hydrolysis is another alternative widely used [7]. Alkaline or acid extraction can lead to serious environmental concerns

☆ This article is part of a special issue entitled: 'ILs-ass adv sep tech' published in Separation and Purification Technology.

* Corresponding author.
E-mail address: ana.soto@usc.es (A. Soto).

https://doi.org/10.1016/j.seppur.2025.132144
Received 5 November 2024; Received in revised form 30 January 2025; Accepted 18 February 2025
Available online 19 February 2025

and are characterised by low yields and high water consumption. Both collagen and gelatine can be further processed into hydrolysates through enzymatic hydrolysis. These peptides show advantages in terms of solubility and digestibility, making them particularly valuable in various applications such as nutraceuticals, cosmetics, and biomedical materials [1,9]. Enzymatic processes can be costly and time-consuming.

Research plays a central role in supporting the transition to a blue economy. Advancements in extraction techniques can lead to new methods that involve the possibility of reusing previously discarded products or improving the efficiency and sustainability of present methods. In this vein, the application of ionic liquids (ILs) and deep eutectic solvents (DESs) for extracting added-value components from fish residues is becoming increasingly important. The advantages that these separation agents could entail are mainly due to their design versatility. The possibility to combine different types of cations and anions in the case of ILs, and to choose the components to mix in DESs, greatly increases the number of possible extraction solvents and also allows their optimisation for the intended application. Their practically null volatility at room conditions enables the solvent to be recycled and reused a quasi-unlimited number of times, and also prevents atmospheric contamination.

Choline chloride/glycerol (1/2) DES was used to extract hydroxyapatite from bighead carp scales [10]. To that aim, the fish residue was dissolved in the DES at 70 °C. The mineral was obtained as precipitate by centrifugation and purified using a NaOH solution. Preliminary tests with several DESs were carried out by the same research group [11], finally selecting choline chloride/1,4-butanediol (1/2) to obtain hydroxyapatite from crucian carp scales. The methodology was previously proposed by Muhammad et al. in 2016 [12] who dissolved carp scales in the IL 1-butyl-3-methylimidazolium. After the dissolution process, water and NaOH solution were added, and a biocompatible hydroxyapatite was obtained as precipitate. Collagen type I was obtained from codfish using aqueous solutions of urea/lactic acid (1/2), demonstrating an improvement of the traditional method with acetic acid [13]. The extraction process followed the classical stages: non-collagenous proteins removal with sodium hydroxide solution, deproteinized skins were treated with a butyl alcohol solution, extraction with the aqueous solution of DES at 4 °C, collagen precipitation by NaCl, and recovery by centrifugation. Collagen type I was extracted [14] from blue shark skins using a mixture of three compounds: citric acid/xylitol/water (1/1/10). In this work, skin powder was directly mixed with the DES at 40 °C. The solution was then centrifuged, and the supernatant dialysed to obtain a pure collagen. Collagen peptides were also extracted from cod skins [15] with choline chloride-oxalic acid DES at 65 °C, being ethanol selected as precipitation reagent. Lactic acid-based DES were used [16] to extract proteins and lipids from marine by-products (codfish bones, mussel meat, and tuna vitreous humour). In this case, solubilisation was carried out at 50 °C, and ethanol was used for the precipitation of the bioactive compounds. Sardine residues, resulting from one of the first steps of the canning process, were submitted to a solid–liquid extraction process with betaine/polyol-based DESs [17]. Results were comparable to those achieved with water in terms of total protein yield. However, an interesting synergistic antimicrobial effect was found. Collagen hydrolysates were obtained [18] from codfish skin by means of urea/propanoic acid (1:2). Treatments with sodium hydroxide solution, butyl alcohol, and DES (all at 4 °C) were required. NaCl was used to extract collagen that was submitted to dialysis and finally to enzymatic hydrolysis with alcalase. COSMO-RS (COnductor-like Screening MOdel for Realistic Solvents) was used by Muhammad et al. in 2017 [19] to select the best IL for collagen extraction from fish scales. 1-Ethyl-3-methylimidazoliumacetate ([C_2mim][OAc]) was found to be the best candidate. The IL was used for dissolution of fish scales at 100 °C and NaCl to recover it as a thin film. No SDS-Page nor molecular weights distribution were presented to confirm the integrity of collagen.

The current extraction methods involve several stages, with harsh chemicals and extensive energy requirements, which pose environmental concerns. Moreover, the extraction of gelatine is an overlooked topic in spite of its multifunctionality and interest. This work focuses on the extraction of gelatine from tuna (*Thunnini*) skin. As a differentiating and innovative factor, all the studies carried out up to now with ILs and DESs are based on the dissolution of the fish material. This proposal is based on a non-dissolution process that we have recently tested with the sodium acetate trihydrate/urea DES [20]. The use of that mixture required a relatively high temperature of work. This limitation is addressed through the use of an IL ([C_2mim][OAc]), with this work aiming to optimise the operation conditions.

2. Design of experiments

The extraction of gelatine from fish skin was carried out, as in commercial processes, in two sequential steps: maceration and extraction with warm water. Usually, maceration is carried out with alkaline or acid solutions. The use of an IL in this stage would avoid the manipulation of strong acids or alkalis, and also atmospheric contamination. To optimise the maceration step with [C_2mim][OAc], a design of experiments was carried out. A Doehlert design [21] was selected due to its relevance in food applications [22]. Table 1 shows the key factors considered in the design: the concentration of IL in water [x_1, wt%], ranging from pure water to pure IL; time [x_2, min], ranging from 0 to 120 min; solvent/skin ratio [x_3, wt/wt] in a proportion from 1 to 3; and temperature [x_4, °C], from 10 to 18 °C.

The extraction yield of fish gelatine [Y, wt/wt], calculated according to Eq. (1), was selected as response variable.

$$Yield(Y) = \frac{\text{weight of dry gelatine}}{\text{weight of wet fish skin}} \quad (1)$$

This four-factor design required a total of 21 experiments, including one central point, as detailed in Table 2. The concentration of IL in water was studied at five levels, maceration time and solvent/skin ratio (wt/wt) at seven levels, and temperature at three levels. All experiments were conducted in duplicate and randomised to minimise the effects of unexpected variations in the observed responses.

The Doehlert design is a type of response surface methodology that considers a quadratic model to describe the process behaviour. The generalised form of the equation is:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j>i}^{4} \beta_{ij} x_i x_j \quad (2)$$

where Y represents the response variable (yield, wt/wt), β_0 is the constant coefficient, β_i, β_{ii} and β_{ij} are the coefficients estimated by regression for the main effect, quadratic and interactions terms of x_1, x_2, x_3 and x_4, respectively. The statistical analysis to determine the optimal conditions of the experimental design was conducted using the Statgraphics version 18.1.16 software program.

Table 1
Experimental domain of the Doehlert design of the maceration process of tuna skin with [C_2mim][OAc]. Variables to be optimised (percentage of IL in an aqueous solution, maceration time, solvent skin ratio and temperature) and range of values.

Independent variables	Symbol	Level	
		−1	+1
% IL (wt%)	x_1	0	100
Maceration time (min)	x_2	0	120
Solvent/Skin ratio (wt/wt)	x_3	1	3
Temperature (°C)	x_4	10	18

Table 2
Doehlert design for independent variables with experimental responses. x_1: percentage of IL in an aqueous solution, x_2: maceration time, x_3: solvent/skin ratio, x_4: temperature, Y_1: extraction yield in first test, Y_2: extraction yield in second test.

Run n°	Variables				Response (Yield, wt/wt)	
	x_1	x_2	x_3	x_4	Y_1	Y_2
1	50	60	2.0	14	0.126	0.106
2	100	60	2.0	14	0.158	0.142
3	75	110	2.0	14	0.073	0.108
4	75	75	2.8	14	0.163	0.188
5	75	75	2.2	17	0.143	0.154
6	0	60	2.0	14	0.132	0.154
7	25	10	2.0	14	0.131	0.138
8	25	45	1.2	14	0.129	0.127
9	25	45	1.8	11	0.120	0.109
10	75	10	2.0	14	0.166	0.149
11	75	45	1.2	14	0.159	0.178
12	75	45	1.8	11	0.140	0.124
13	25	110	2.0	14	0.126	0.147
14	50	95	1.2	14	0.136	0.148
15	50	95	1.8	11	0.129	0.123
16	25	75	2.8	14	0.094	0.121
17	50	25	2.8	14	0.145	0.165
18	50	60	2.6	11	0.163	0.148
19	25	75	2.2	17	0.125	0.145
20	50	25	2.2	17	0.171	0.171
21	50	60	1.4	17	0.161	0.125

3. Experimental section

3.1. Materials

Yellowfin (Thunnus Albacares) tuna skin was provided, in frozen conditions, by Jealsa Foods S.A.U. (Boiro, Spain). The tuna was caught in the Atlantic Ocean and then stored on the boat by being frozen in brine at −18 °C. According to the manufacturer, the content of the wet fish skin was 34.3 % protein, 8.5 % lipid, 9.8 % ash and 47.4 % water (on a wet weight basis). A commercial gelatine (Sigma-Aldrich), derived from cold-water fish skin and with an average molecular weight of 60 kDa, was used as a control sample. The IL [C_2mim][OAc] was purchased from Iolitec with a nominal purity greater than 95 %. It was vacuumed (~1 Pa) at moderate temperature (ca. 333–343 K) for 48 h to remove water or possible volatile impurities. The acetone was purchased from Scharlab with a nominal purity 99.5 % and the water used was bi-distilled.

3.2. Extraction procedure

The unfrozen tuna skin was prepared by manually removing the remaining flesh, leaving the skin completely clean, and then cutting it into small squares of approximately 5 mm × 5 mm. The gelatine production process was carried by maceration with the IL and extraction with water.

In the first step, maceration, 2 g of tuna skin were treated with the solvent in a thermostated (Ultratherm-200P Selecta bath) glass cell mechanically stirred (30 rpm) with an IKA RW 16 basic overhead stirrer. The solvent/skin ratio, and temperature and time of maceration, were fixed through a *design* of experiments (see section 2). Afterwards, the treated skin was separated from the solution, and it underwent five washes with cold water at 5 °C to remove any potentially absorbed IL. The absence of this compound was verified by FTIR and neutral pH of the washing waters.

The extraction step was carried out similarly to traditional processes that use alkaline or acid solutions in the maceration process. That is, the macerated skin was stirred (40 rpm) with water at 50 °C during 1 h. The skin/water ratio used was 1/3 (wt/wt). Next, the mixture was centrifuged (Centrifuge Ortoalresa Digicen 21R) at 18000x g for 5 min, the solid was discarded, and the extract was centrifuged again for 5 min to remove any small pieces of skin remaining. Finally, the gelatine was precipitated from the aqueous solution by adding acetone. An acetone/extract ratio of 1/1 (wt/wt) was required (additional acetone didn't improve the gelatine yield). The gelatine was filtered and dried in an oven at 50 °C for 72 h.

3.3. Gelatine characterisation

All measurements were carried out at least in duplicate, to ensure the repeatability of the tests.

3.3.1. Proximate composition

The Association of Official Analytical Chemists standard methods [23] were used to determine moisture (method 950.10), ash (method 900.2A), and organic matter (method 2.7.08) in extracted gelatines. The Kjeldahl method was used to determine the total nitrogen content. It is based on the conversion of nitrogen into ammonium sulphate, followed by distillation and titration to determine the amount of ammonia by spectrophotometric measurement at 667 nm [23]. The protein content was calculated by multiplying the nitrogen content by a conversion factor of 5.6. Total soluble protein was determined using the Folin-Ciocalteu method and spectrophotometric measurement at 750 nm [24].

3.3.2. FTIR spectroscopy

FTIR spectra of the dried gelatine was carried out using a Varian 670-R FTIR spectrophotometer. The spectra were recorded over a 400 to 4000 cm^{-1} wavenumber range using transmittance mode with 32 scans at 25 °C. The measurement resolution was set at 4 cm^{-1}. Analysis of spectral data was carried out using the BRUKER OPUS 7.8 data collection software.

3.3.3. Electrophoretic analysis (SDS-PAGE)

SDS-PAGE was performed according to Invitrogen NuPAGE® specifications. An aqueous solution of gelatine was prepared at a concentration of 5.0 g/L. Then, 4 μL of the solution was mixed with 2.5 μL of NuPage LDS sample buffer 4X (Invitrogen) and 1 μL of Nupage reducing sample buffer 10X (Invitrogen), completed with water to a total volume of 10 μL (2 μg gelatine/μL), and incubated at 70 °C for 10 min. The sample was then loaded onto an electrophoresis system. 10 μL were loaded onto a precast 4 % stacking and 8 % resolving Bis-Tris plus polyacrylamide gel (Invitrogen) with 1.0 mm thickness. Also, 5 μL of PageRuler Plus Prestained Protein Ladder (10–250 kDa, Thermo Fisher Scientific) was loaded for comparison. Electrophoresis was performed at constant voltage (60 V for the stacking gel and 90 V for the resolving gel), for approximately 45 min, in 1X MES SDS running buffer (Invitrogen) until the dye front reached the end of the gel. At the end, gel was rinsed and stained with Coomassie brilliant R250 (Thermo Fisher) for 1 h with low agitation. Finally, the gels were destained with distilled water and mild shaking overnight.

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

The molecular weight distribution of gelatines was determined by gel permeation chromatography with an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, CA, United States) equipped with quaternary pump (G1311B), injector (G1329B), column oven (G1316A), refractive index (G1362A), diode array (G1315C), and dual-angle static light scattering (G7800A) detectors. Gelatine samples were dissolved at 2 g/L in the mobile phase (0.15 M ammonium acetate/0.2 M acetic acid buffer at pH 4.5), and pumped at 1 mL/min through four columns (PSS, Mainz, Germany): Proteema precolumn (5 μm, 8 × 50 mm), Proteema 100 Å (5 μm, 8 × 300 mm), Proteema 300 Å (5 μm, 8 × 300 mm), and Proteema 1000 Å (5 μm, 8 × 300 mm). Column oven and light scattering detector were kept at 30 °C, and refractive index detector was maintained at 40 °C. The injection volume was 100 μL. The detectors were calibrated

with a polyethylene oxide standard (PSS GmbH, Mainz, Germany) of 106 kDa (Mw) and polydispersity index 1.05. Absolute molecular weights were estimated with refractive index increments of 0.190 [25].

3.3.5. Amino acid profile

The amino acid profile of extracted gelatine was quantified using a Biochrom 30 series amino acid analyser system (Biochrom Ltd., Cambridge, UK). First, the samples were hydrolysed and separated through a column of cation-exchange resin according to the method of Moore et al. in 1958 [26]. The column eluent was mixed with ninhydrin reagent (Sigma-Aldrich) at high temperature. This mixture reacted with the amino acids, forming coloured compounds that were analysed at two different wavelengths: 440 and 570 nm. An internal standard of norleucine (Sigma-Aldrich) was used for quantitative analysis.

3.3.6. Thermal stability

Thermogravimetric analysis (TGA) of the samples was performed in a TA Instruments TGA Q500 thermogravimetric analyser, using flow rates of 40 mL/min and 60 mL/min of nitrogen gas (Nippon Gases, 99.999 %) as balance purge gas and sample purge gas, respectively. For each case, an open platinum pan with ca. 10 – 20 mg of sample was automatically introduced by the apparatus into the furnace chamber. The thermal programme consisted of heating the sample up to 800 °C at 5 °C/min. Thermograms were processed with the software Universal Analysis 2000, version 4.5.0.5, by TA Instruments. T_{onset} was obtained with the onset function of this software, using as references a point before the curve and the peak of the derivative. T_{max} (maximum decomposition rate) is the value of the temperature in the peak of the derivative. The mass loss percentage at each step was calculated with respect to the total mass lost.

3.3.7. Rheological properties

Rheological measurements were conducted using an Anton Paar Rheometer (Rheometer Anton Paar MCR 102), with a Peltier temperature control system, connected to a Cryogenic Bath (Julabo F12). A cone-and-plate geometry was used (50 mm cone diameter, 1° cone angle, and 0.098 mm gap).

Dry gelatine was dissolved in distilled water at 45 °C to prepare a solution with a 6.67 wt% concentration, and then cooled to room temperature [23]. Samples were placed on the rheometer-measuring system and were kept at 2 °C for 20 min before measuring, to allow temperature equilibration. Measurements were carried out within the linear viscoelastic range (1 Hz and 1 % strain), previously assessed by strain sweep tests. A temperature ramp was carried out from 2 to 30 °C, at a heating rate of 1 °C/min, to obtain the melting point (T_{Melt}), followed by a temperature ramp from 30 to 2 °C, at a cooling rate of 1 °C/min, to obtain the gel point (T_{Gel}). The elastic modulus (G') and the viscous modulus (G'') were recorded versus temperature, and T_{Melt} and T_{Gel} were calculated at the crossover points ($G' = G''$).

4. Results and discussion

4.1. Design of experiments

The effect of four maceration factors, concentration of IL in water (x_1), time (x_2), solvent/skin ratio (x_3), and temperature (x_4), were studied using the Doehlert experimental design, in order to achieve a maximum yield in the process of gelatine extraction from tuna skin. Table 1 shows the considered variable ranges. The values of the four factors and response, yield, for each run of the experimental design are presented in Table 2.

In order to establish the optimised extraction of gelatine from tuna fish skin, the response surface methodology (RMS) package was used. Linear and quadratic effects, as well as interactions between factors, were assessed for significant differences using the t-statistic test and ANOVA. As can be seen in the pareto chart (Fig. 1) and the ANOVA (Table S1 in Supporting Information), only five effects are statistically significant at a confidence level of 95 % ($p < 0.05$). The temperature was not a statistically significant factor for the maceration process to increase the extraction yield. However, the concentration of IL in water, maceration time and solvent/skin ratio were statistically significant factors. The concentration of IL in water and the solvent/skin ratio showed a positive effect, while time had a negative effect. The quadratic term for the solvent/skin ratio was also statistically significant, pointing out that the effect of solvent/skin ratio is not linear. Furthermore, the statistical significance of the interaction between the concentration of IL in water and time indicated that the effect of both factors is not independent (Fig. 1).

Fig. 2 shows the main effect of individual factors for gelatine yield. The highest yield was found at a high solvent/skin ratio. The optimal conditions were set, according to the Statgraphics software, for the solvent/skin ratio at the highest value (3 wt/wt) and the temperature near the lowest tested value (12 °C).

The response surface of factors that are not independent, the concentration of IL in water and maceration time, for gelatine yield is shown in Fig. 3. The optimal values for these variables were found to be 100 % of IL and 12 min.

To develop the final fitted RMS equation, all non-statistically significant terms ($p > 0.05$) were eliminated. The resulting equation model is shown in Eq. (3):

$$Y = 0.176 + 0.00089x_1 + 0.000231x_2 - 0.0724x_3 + 0.0192x_3^2 - 0.0000105x_1x_2 \quad (3)$$

Extraction tests were carried out with the optimised values resulting

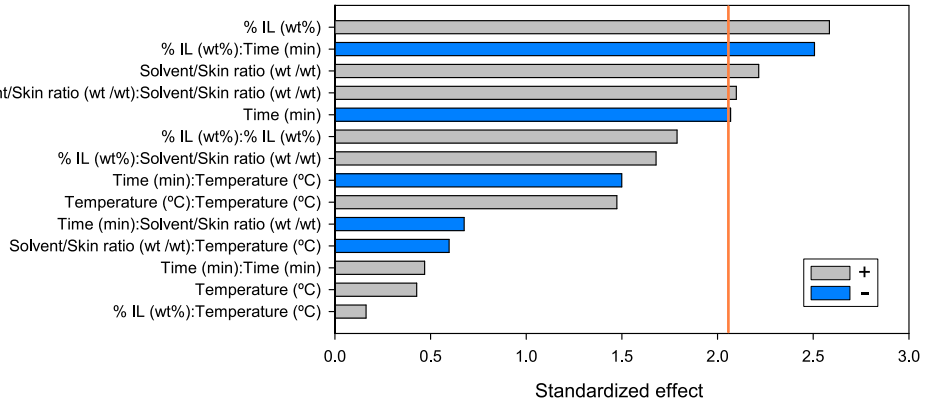


Fig. 1. Pareto chart, obtained from the Doehlert experimental design, showing the effect of considered factors in skin maceration and their interactions for gelatine yield (wt/wt) response.

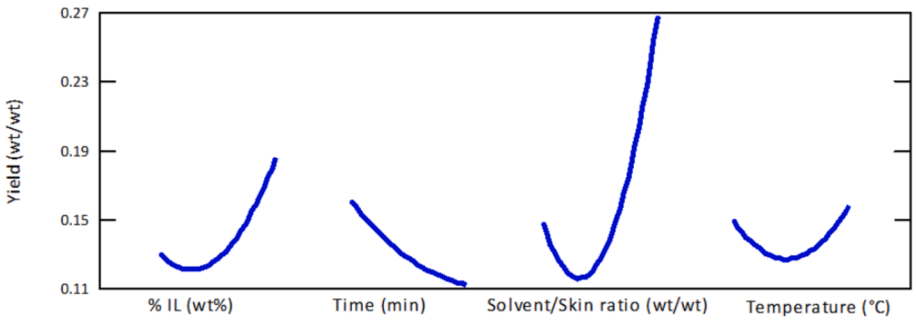


Fig. 2. Main effect, obtained from the Doehlert experimental design, of individual factors in skin maceration for gelatine yield (wt/wt) response.

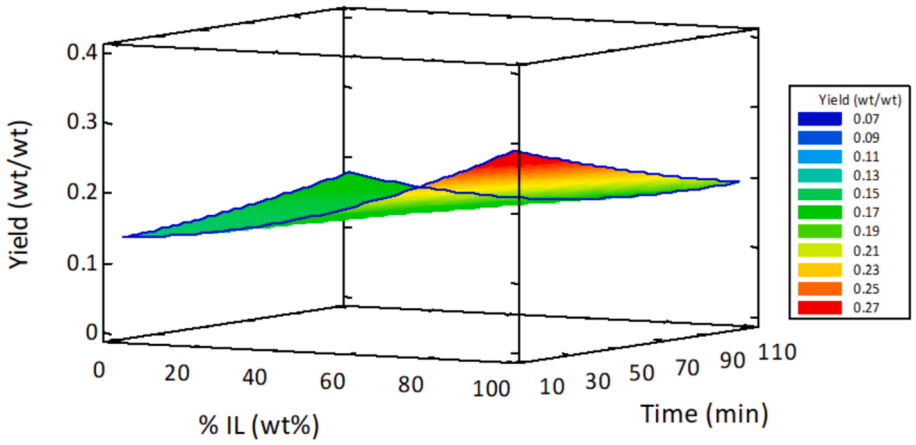


Fig. 3. Response surface plot of the effect of concentration of IL in water (wt%) and maceration time (min) for gelatine yield (wt/wt). Temperature: 12 °C and solvent/skin ratio: 3 (wt/wt).

from the experimental design (Table 3). Under these conditions, the experimental yield achieved was 0.188 g of gelatine/g wet skin, a slightly lower value than that predicted using Eq. (3) (0.21 wt/wt). Thermal solubilisation of collagen (in the presence of acid or alkali) is the usual method to achieve soluble gelatine. Yields between 0.06 and 0.19 (g of gelatine/g wet skin) are usually found depending on the fish, its preservation method and the extraction procedure [27,28]. These values are consistent with the yields reported for gelatine extracted from tuna skin (see Table 4), with the only exception being the value reported by Gallego et al. [20]. So, this simple extraction method is at the high end of the range.

Finally, the adequacy of the model was verified. The predicted values derived from Eq. (3) were plotted against the experimental values, showing good agreement and a random distribution of residuals as depicted in SI. Furthermore, a normal distribution plot was used to investigate the distribution of residuals. If the residuals followed the straight line, it would indicate that the residuals are normally distributed. In this case, the residuals plotted against the predicted gelatine yield (see SI) demonstrate random behaviour and no apparent regular trends.

Table 3
Optimised values obtained in the application of the Doehlert design to the process of maceration of tuna skin with [C_2mim][OAc] and resultant experimental yield.

Variables	Values
% IL (wt%)	100
Time (min)	12
Solvent/Skin ratio (wt/wt)	3
Temperature (°C)	12
Experimental yield (wt/wt)	0.188 ± 0.012

4.2. Proximate composition

The proximate composition of the gelatine obtained is shown in Table 5. The moisture was found to be 8.7 %, a value in the range of commercial gelatines, that is between 9 and 14 % [36]. The ash content (3.8 % in this work) greatly varies depending on the source, and the preservation and extraction methods. Values ranging from 0.7 to 7.8 % were found in the case of gelatines extracted from yellowfin tuna skin (see Table 4). Regarding protein content, Table 4 shows values ranging from 75.3 to 88.4 %, the value obtained in this work (85.9 % obtained from total nitrogen and 86.8 % from amino acids content) being in the range.

4.3. FTIR spectroscopy

The FTIR spectrum displayed in Fig. 4(a) shows various characteristic bands, each corresponding to specific molecular vibrations and functional groups within the gelatine structure. Five major amide bonds can be clearly identified: amide A, amide B, amide I, amide II and amide III. The amide A band (around 3273 cm^{-1}) is associated with the N–H stretching vibration and indicates the presence of hydrogen bonds. Free N–H stretching vibrations typically occur in the range of 3400–3440 cm^{-1} and when the N–H group of a peptide is involved in a hydrogen bond, the position is shifted to a lower wavenumber, usually around 3300 cm^{-1} [37–39].This behaviour is usually found in gelatines extracted at high temperatures [40], with significant presence of peptide chains, and causes an overlapping of Amide A with Amide B region (around 2929 cm^{-1}) associated with C–H stretching vibrations.

The amide I, the most important band in peptide secondary structure analysis, was observed at 1629 cm^{-1}. It is associated with the C=O stretching vibration bonds in the polypeptide chain, thus providing

Table 4
Comparison of results obtained in this work for gelatine extraction from tuna skin using [C_2mim][OAc] with those of previously proposed methods.

Type of Skin	Chemicals	Yield (g/g wet skin)	Mo (wt %)	Ash (wt %)	OM (wt %)	Protein (wt%)	Pyrrolidine amino acids (g/100 g total amino acids)	T_{Melt} (°C)	T_{Gel} (°C)	Reference
Frozen yellowfin	[C_2mim][OAc]	0.188	8.7	3.8	87.5	85.9	21.0	20.6	12.9	This work
Fresh yellowfin	NaOH, HCl							24.3	18.7	[29]
Frozen yellowfin	NaOH, CH_3COOH	0.18	8.3	7.8	83.9	78.1				[30]
Fresh yellowfin	CH_3COOH		10.3	3.7	86.0	81.6		20.3	–	[31]
Frozen skipjack	NaOH, CH_3COOH	0.113	10.9	0.7	89.2	88.4	18.0	24.2	18.7	[32]
Frozen yellowfin	NaOH, HCl, CH_3COOH	0.117	12.0	1.3	86.7	86.5				[33]
Frozen yellowfin	NaOH, H_2SO_4, Citric acid	0.125	10.0	1.1	89.9	90.6	~19.7			[34]
Frozen katsuwonus pelamis	NaOH, CH_3COOH		10.1	1.2	88.7	75.3		30	22	[35]
Frozen yellowfin	Sodium acetate trihydrate/ urea	0.31	9.2	3.7	87.2	84.0	19.9	19.4	11.0	[20]

Mo: Moisture. OM: organic matter. Ash: ashes.

Table 5
Proximate composition (% wt/wt) of gelatine extracted from tuna skin using a process of maceration with [C_2mim][OAc] at 12 °C and extraction with water at 50 °C.

Mo (%)	Ash (%)	OM (%)	Pr-tN (%)	Prt (%)	Prs (%)
8.7 ± 0.3	3.8 ± 0.2	87.5 ± 3.2	85.9 ± 3.0	86.8 ± 3.1	69.8 ± 0.4

Mo: Moisture. OM: organic matter. Ash: ashes Pr-tN: total protein as total nitrogen x 5.6. Prt: total protein content as the sum of amino acids. Prs: soluble protein determined by the method of Lowry.

valuable information about the protein's structural characteristics. The amide II spectral region band is usually found in the range of 1550–1600 cm^{-1} and it is associated with NH bending coupled with CN stretching vibration of protein amide groups. In this case, the signal is found to be shifted to lower absorbance, 1527 cm^{-1}, which indicates the existence of hydrogen bonding in gelatine [41]. The band at 1238 cm^{-1} corresponds to Amide III and represents the vibration of C- N and N–H groups, and a small contribution from the C–C stress and the in-plane bending CO [38,39]. The band around 551 cm^{-1} could be associated to amide IV, and it is due to the bending of O=C-N groups. The spectrum profile of the product extracted using the IL (Fig. 4a) is comparable to those reported in previous studies [37–42] and is identical to that of the commercial gelatine used as reference in this work (Fig. 4b).

4.4. Electrophoretic analysis (SDS-PAGE)

The mechanical properties of gelatine are closely related to its molecular weight distribution. Higher weight average molecular weight gelatines tend to exhibit better gel-forming properties. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a useful tool to qualitatively analyse the protein patterns of the extracted gelatine, allowing the understanding of its composition and quality. The SDS-PAGE of the extracted gelatine is shown in Fig. 5(a). Two bands around 200 and 100 kDa could be associated to β and α-chains, respectively. Also, a heterogeneous distribution of bands at lower molecular weights, concentrated around 50 kDa, can be seen. The presence of low molecular weight polypeptide chains could be due to a partial hydrolysis during the extraction process, which is not unusual in fish gelatines. In fact, the SDS-PAGE of the commercial gelatine used as reference in this work (Fig. 5b), with a Mw of 60 kDa, only showed very blurred bands. Similar patterns were found for gelatines obtained by Sousa et al. in 2017 [34] from the same source (yellowfin tuna skin) but using multiples stages, with several basic and acid treatments. The presence of peptides with molecular weight below 100 kDa was also noticeable in the gelatines obtained by Shyni et al. in 2014 [32]. However, β-chains were not found. Nurilmala et al. in 2020 [43] found that tuna skin collagen and gelatine showed similar patterns with mainly β- and α-chains, lower molecular weights being characteristic of hydrolysates.

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

For a precise analysis of molecular weight distribution, a GPC-SEC analysis was performed. The molecular weight distribution profile for the extracted gelatine is shown in Table 6 and the corresponding eluogram displayed in Fig. 6. As usual in these cases [44], the GPC eluogram exhibits several overlapping polymeric peaks. Three different regions can be identified. Region 1 corresponds to β-chains, since the estimated molecular weight (198 kDa) is within the usual region for dimers of α-chains (160–250 kDa) [45]. A significant 22.3 % of the total area (determined with the refractive index detector) corresponds to these

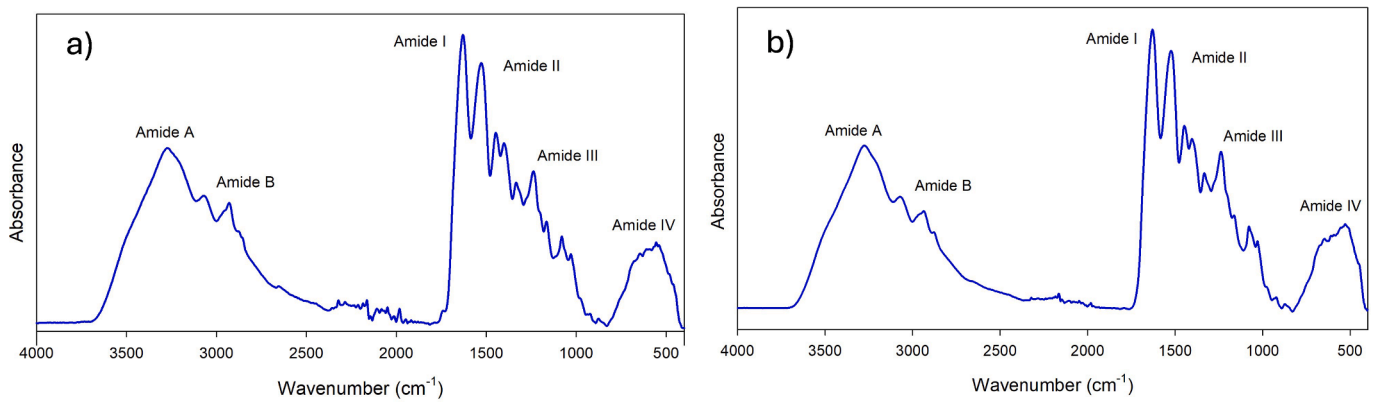


Fig. 4. FTIR analysis of the gelatine extracted in this work (a) and a commercial gelatine (b).

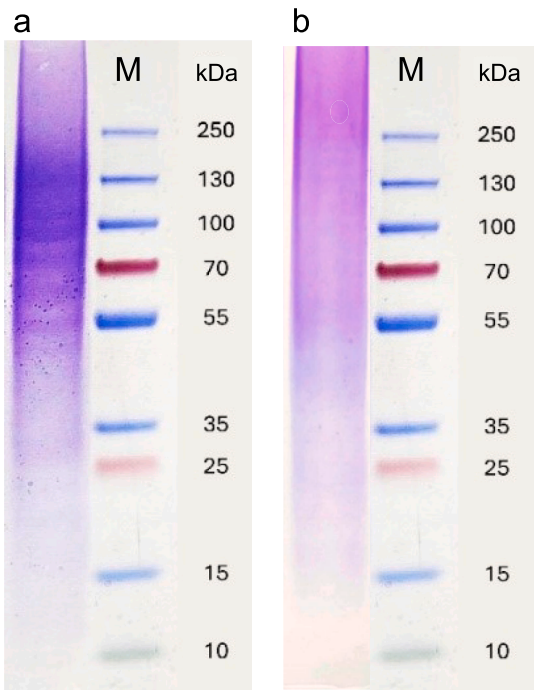


Fig. 5. SDS-PAGE patterns of the gelatine extracted in this work (a) and a commercial gelatine (b). The code M refers to a prestained protein ladder marker (10–250 kDa).

Table 6
Molecular weight (kDa) distribution of gelatine.

Peak number	M_n (kDa)	M_w (kDa)	PDI	Peak Area (%)
1 β-chains	187 ± 9	198 ± 9	1.062	22.3 ± 2.1
2 α-chains	99 ± 4	100 ± 5	1.016	21.4 ± 0.2
3 peptides	37 ± 3	44 ± 6	1.178	54.8 ± 0.2

M_w: weight average molecular weight. M_n: number average molecular weight. PDI: polydispersity index. Peak area (%) corresponds to refractive index detector.

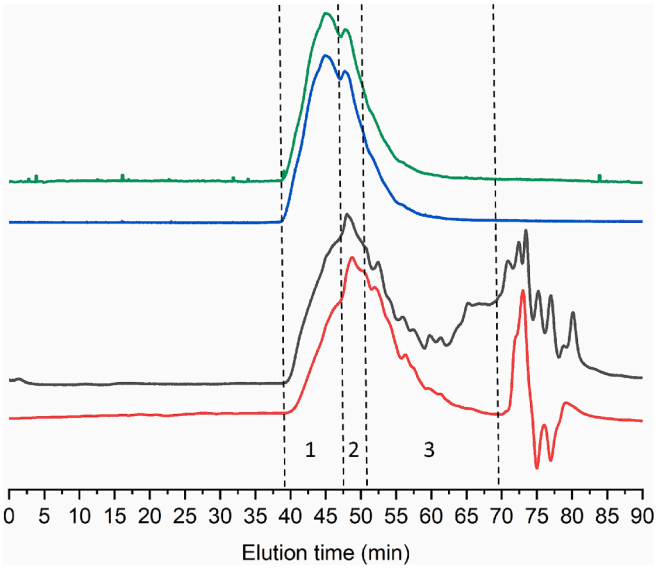


Fig. 6. GPC eluogram of extracted gelatine. 1: β-chains, 2: α-chains, 3: peptides.

gelatine chains. A similar percentage (21.4 %) corresponds to α chains. They are characterised by a molecular weight of 100 kDa, thus being in the range corresponding to these monomeric chains (80–125 kDa). The remaining percentage (54.8 %) corresponds to degraded α-chains and peptides with a weight average molecular weight of 44 kDa. The polydispersity index for α- and β-chains is very close to unity, this meaning that the polymer chains in the sample have similar sizes, resulting in a narrow range of molecular weights [46]. In the case of the small peptides, the polydispersity index is slightly greater than unity due to a mixture of fragments with disperse molecular weight, caused by a partial hydrolysis during the extraction procedure and the existence of peptides. As expected, GPC-SEC studies confirm previous results obtained by SDS-PAGE.

4.6. Amino acid profile

The amino acid profile of gelatine typically includes a variety of amino acids, with high levels of glycine, proline, and hydroxyproline. Hydroxyproline is characteristic of collagen and gelatine, playing a crucial role in their stability and structure. Table 7 shows the amino acids content of the gelatine extracted with the IL. The total protein content, as sum of amino acids, in the dry gelatine is 86.8 % (wt/wt). Glycine is the most relevant amino acid in terms of percentage (22.40 %), followed by proline (11.78 %), alanine (10.33 %) and hydroxyproline (9.24 %). Similar patterns were found for the extraction of fish gelatine from yellowfin tuna skin using traditional methods [32,34]. Multiples stages with several alkaline (NaOH) and acid (H_2SO_4, citric acid) treatments were used by Sousa et al. in 2017 [34] to extract the gelatine. A higher content of glycine was found (32.0–32.4 %, depending on the extraction temperature), with very similar values for the other amino acids (12.3 % alanine, 11.2 % proline and 7.5–8.5 % hydroxyproline). It is known that quality of gelatines, particularly their gel strength, is significantly influenced by the content of proline and hydroxyproline. These amino acids are crucial in the formation of triple helix structures from random coil, which is essential for the gelation properties [27,47]. The typical content of pyrrolidine amino acids (proline and hydroxyproline) in fish gelatine ranges from 16 % to 20 % [45]. Table 4 presents these values for gelatine extracted from tuna skin, with contents ranging from 18 to 21 g per 100 g of total amino acids. The highest value was achieved in this study.

4.7. Thermal stability

Fish gelatine's thermal stability is essential for its performance in various applications. For this reason, a TGA of the extracted gelatine was carried out and it is shown in Fig. 7. The curve exhibits two distinct

Table 7
Amino acids content (% wt/wt) of the gelatine.

Amino acids	% or g/100 g total amino acids
Aspartate	5.24 ± 0.07
Threonine	3.18 ± 0.04
Serine	3.60 ± 0.12
Glutamate	9.60 ± 0.11
Glycine	22.40 ± 0.30
Alanine	10.33 ± 0.04
Cysteine	0.40 ± 0.06
Valine	2.30 ± 0.01
Methionine	2.06 ± 0.05
Isoleucine	1.17 ± 0.03
Leucine	2.70 ± 0.06
Tyrosine	0.43 ± 0.02
Phenylalanine	2.39 ± 0.21
Histidine	1.17 ± 0.22
Lysine	3.60 ± 0.02
Arginine	8.42 ± 0.10
Hydroxyproline	9.24 ± 0.36
Proline	11.78 ± 0.17

weight loss zones, identified by their derivatives. A first decomposition step with an onset decomposition temperature (T_{onset}) of 121 °C and a temperature of maximum decomposition rate (T_{max}) of 125 °C results in approximately a 26 % of mass loss (Table 8). Correia et al. in 2013 [48] associated these changes in mass up to 200 °C to several forms of water association within the gelatine structure: intra-molecular bonds between water and collagen triple helix, water absorbed and bounded to proteins by hydrogen bonds (monomolecular layer), and water absorbed on polymolecular layers. A second degradation step with T_{onset} = 263 °C and T_{max} = 305 °C results in approximately a 74 % of mass loss, and it is associated to protein degradation. Correia et al. in 2013 [48], analysing the thermal degradation of electrospun cross-linked membranes with a 30 % gelatine, found a third degradation step between 400 and 600 °C associated with the decomposition of the gelatine networks. This third step was also found by Hermida-Merino et al. in 2022 [44] who studied the thermal degradation of tuna gelatine. However, depending on molecular weight distribution and amino acids content, fish gelatines are frequently characterised by only the two first decomposition steps [42,49].

4.8. Rheological properties

The ability of gelatine to form thermally reversible gels is a key feature that enables it to transition between a liquid state and a gel state based on temperature changes. This property is particularly advantageous for applications where controlled gelation and melting are required, such as drug delivery systems, food products, and synthesis of bio-materials. Thus, solubility in water and thermo-reversibility are the most noted characteristics of gelatine. According to the official methods of analysis [23], a solution with a 6.67 wt% concentration was prepared in water, and its rheological properties studied.

Fig. 8 shows the thermo-rheological profile of the aqueous solution. A progressive drop of elastic/storage moduli (G') and viscous/loss (G") with increasing temperature from 2 to ~15 °C is observed, followed by a sharp decrease at this temperature and a posterior stabilisation at temperatures higher than 25 °C. At low temperatures the elastic is higher than the viscous modulus indicating a solid-like behaviour. A crossover point around 20.6 °C for heating profiles can be observed. This corresponds to the melting temperature of the gel (T_{melt}). At high temperatures the viscous is higher than the elastic modulus indicating a liquid-like behaviour. During the cooling stage, a new crossover point is observed, corresponding to gelling temperature (T_{gel}), in this case 12.9 °C. Gelatine molecules in water aggregate and undergo a conformational change from a random coil to a triple helix. At the same time, intermolecular hydrogen bonds are formed between gelatine chains. However, the non-covalent associations are easily broken at

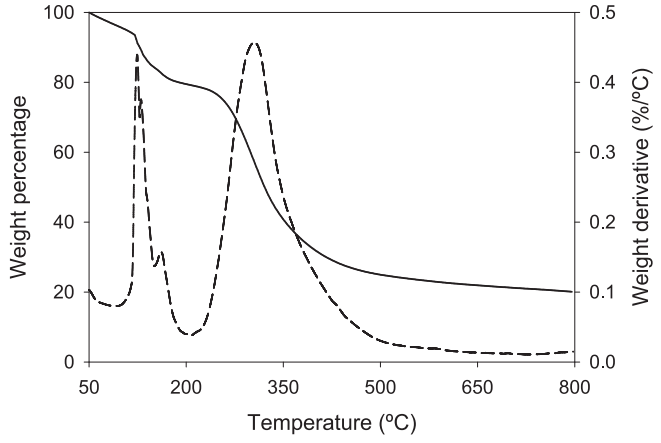


Fig. 7. TGA analysis. Continuous line is the weight percentage and discontinuous trend represents the weight derivative.

Table 8
Thermal characterisation of the extracted gelatine.

Decomposition step	T_{onset} (°C)	T_{max} (°C)	Mass loss (%)	Residue at 800 °C (%)
1st	121	125	26	20
2nd	263	305	74	

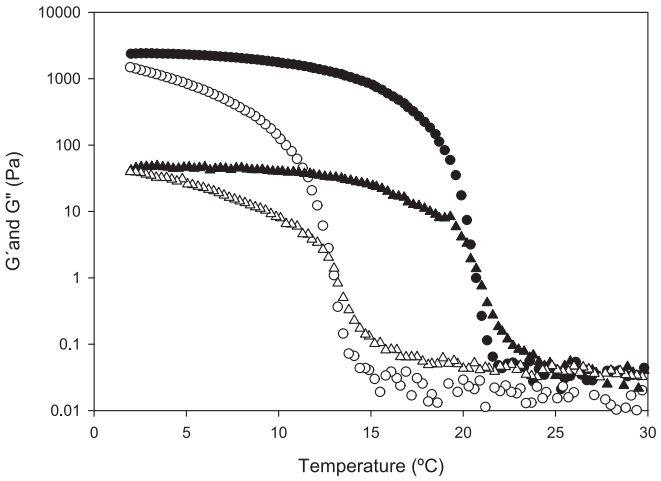


Fig. 8. Thermo-rheology for the extracted gelatine: heating from 2 to 30 °C (● G', ▲ G"), cooling from 30 to 2 °C (○ G', Δ G").

temperatures able to destroy the physical network [50]. This justifies the gelation process and the hysteresis observed in Fig. 8. After heating and cooling cycles, G' and G" practically return to the initial point, thus confirming the thermo-reversible character of the prepared gels is confirmed.

Typical melting and gelling points for fish gelatines range from 11 to 28 °C and 8 to 25 °C, respectively [27]. The gelatine concentration in water, the molecular weight distribution, and the amino acid profile, are variables that affect the rheological properties. Table 4 compares melting and gelling points for gelatines extracted from tuna skin with traditional methods and with this proposal. Even though the values show certain discrepancies (besides the source, the conservation method and the extraction procedure, the measuring method can also affect the values), the obtained temperatures fall within the typical range for warm water. The green advantage over the incumbent technology lies in this new method's absence of harsh chemicals, such as strong acids and alkalis. Furthermore, ILs do not contribute to atmospheric contamination due to their negligible vapor pressure. It is worth mentioning that the method proposed here improves the quality of the gelatine obtained with the sodium acetate trihydrate/urea DES [20], and requires a significantly lower temperature in the maceration step.

5. Extraction process

To design the entire extraction process, the recyclability and loss of the IL must be addressed. After the maceration stage, the treated skin is separated from the IL by filtration and needs to be washed, resulting in some IL being carried away due to its water solubility. To determine the potential loss this might entail, the extraction process was performed in the laboratory three consecutive times, measuring the recovered solvent by weight. The recovery rates were 95.1, 95.4, and 95.3 wt%. Approximately 5 % of the IL exits the process in the aqueous solution. However, the possible separation of [C_2mim][OAc] and water by flash distillation was previously confirmed [51]. Based on this information, the flow diagram for the proposed extraction process is shown in Fig. 9. The proposed stages are shown: trituration of the tuna skin, maceration with

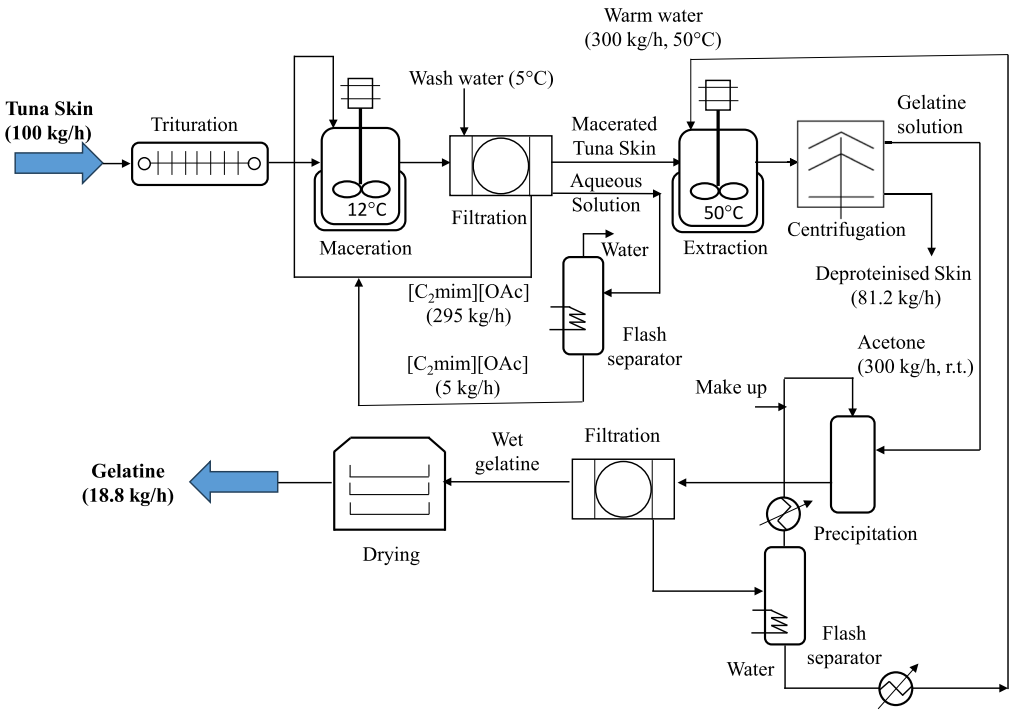


Fig. 9. Flow chart for the process of gelatine production from fish skin using the IL [C_2mim][OAc] as maceration solvent.

[C_2mim][OAc] at 12 °C, separation by filtration with the recycling of the IL, washing of the skin and recovery of the IL from its aqueous solutions by flash distillation, gelatine extraction from the macerated skin with water at 50 °C, protein precipitation by adding acetone and subsequent filtration. Acetone would also be easily separated from water through distillation and recirculated back into the process.

The treatment of 100 kg/h of tuna skin would initially require 300 kg/h of [C_2mim][OAc] (with a large-scale production cost of 30 €/kg [52]) and a similar quantity of acetone. However, after the start-up, there would be no need for additional chemicals (except for make-up acetone to compensate for losses due to its volatility), as both solvents can be recycled. The production of 18.8 kg/h of gelatine (with a current minimum market cost of approximately 30 €/kg) from 100 kg/h of fish skin (costing between 0 and 0.3 €/kg) would be profitable but highly dependent on energy costs.

6. Conclusions

Maceration with [C_2mim][OAc] and water extraction is a valid method to obtain gelatine from fish by-products, namely from tuna skin. As an advantage in comparison to classical methods, it avoids the need of multiple treatment steps with strong acids and alkalis. In comparison with other previously proposed methods with ILs, it avoids the requirement of the complete solubilisation of the skin and its posterior recovery, which requires a complicated separation with high energy costs.

The design of experiments was a useful tool to optimise the maceration step. Higher yields were achieved using pure IL, instead of its mixtures with water, a short maceration time (12 min), low temperature (12 °C) and a solvent/skin (wt/wt) ratio of 3. Extraction with water at 50 °C for 1 h, as commonly carried out in commercial methods, was effective for the recovery of gelatine. The obtained product showed very similar characteristics to gelatine obtained through traditional methods from by-products of warm water fish.

FTIR showed the characteristic bands corresponding to molecular vibrations and functional groups of the gelatine structure. α- and β-chains constitute approximately the 43 % of the product, with the remainder being peptides with an average molecular weight of 44 kDa. The thermal stability, characterised by two decomposition steps, was similar to other fish gelatines and considered adequate for traditional applications. The pyrrolidine amino acids content (21 %) is associated to a gelatine of good quality. Melting and gelling points (20.6 and 12.9 °C, respectively) fall within the typical range for warm water fish.

The proposed strategy not only aligns with principles of circularity and sustainability, but also would be competitive in achieving those goals.

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.

Acknowledgements

The authors are grateful to Rosario Rodil from the Institute of Research on Chemical and Biological Analysis (IAQBUS) of the Universidade de Santiago de Compostela, for his help with the design of experiments. The authors also thank Jealsa Foods S.A.U. (Boiro, Galicia, Spain) for the kind supply of fish skin for the experiments.

Funding Sources

Grant PID2021-123622OB-I00 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe" by the European Union. Author from IIM-CSIC is grateful to Xunta de Galicia (GPC_IN607B 2024/010) for funding.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2025.132144.

REFERENCES

[1] D. Coppola, C. Lauritano, F. Palma-Esposito, G. Riccio, C. Rizzo, D. de Pascale, Fish waste: from problem to valuable resource, Mar. Drugs 19 (2021) N:116, https://doi.org/10.3390/md19020116.

[2] N.V. Veríssimo, C.U. Mussagy, A.A. Oshiro, C.M.N. Mendonça, V.D. Santos-Ebinuma, A. Pessoa, R.P.D. Oliveira, J.F.B. Pereira, From green to blue economy: marine biorefineries for a sustainable ocean-based economy, Green Chem. 23 (2021) 9377–9400, https://doi.org/10.1039/d1gc03191k.

[3] N.V. Salim, B. Madhan, V. Glattauer, J.A.M. Ramshaw, Comprehensive review on collagen extraction from food by-products and waste as a value-added material, Int. J. Biol. Macromol. 278 (2024) 134374, https://doi.org/10.1016/j.ijbiomac.2024.134374.

[4] Y.P. Didion, M. Pinelo, T.G. Tjalsma, Z. Su, M. Malankowska, What is next? the greener future of solid liquid extraction of biobased compounds: novel techniques and solvents overpower traditional ones, Sep. Pur. Technol. 320 (2023) 124147, https://doi.org/10.1016/j.seppur.2023.124147.

[5] M.J. Pereira, C. Brazinha, J.G. Crespo, Pervaporation recovery of valuable aromas from by-products of the seafood industry: modelling of fractionated condensation for off-flavour removal, Sep. Pur. Technol. 286 (2022) 120441, https://doi.org/10.1016/j.seppur.2022.120441.

[6] N. Bhaskar, T. Benila, C. Radha, R.G. Lalitha, Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (Catla catla) for preparing protein hydrolysate using a commercial protease, Bioresource Technol. 99 (2008) 335–343, https://doi.org/10.1016/j.biortech.2006.12.015.

[7] P. Ideia, J. Pinto, R. Ferreira, L. Figueiredo, V. Spínola, P.C. Castilho, Fish processing industry residues: a review of valuable products extraction and characterization methods, Waste Biom. Valor. 11 (2020) 3223–3246, https://doi.org/10.1007/s12649-019-00739-1.

[8] R. Schrieber, H. Gareis, Gelatine Handbook: Theory and Industrial Practice, Wiley-vch Verlag GmbH & Co. Kgaa., (2007), https://doi.org/10.1002/9783527610969.

[9] A. León-López, A. Morales-Peñaloza, V.M. Martínez-Juárez, A. Vargas-Torres, D. I. Zeugolis, G. Aguirre-Álvarez, Hydrolyzed collagen-sources and applications, Molecules 24 (2019) N:4031.

[10] Y. Liu, J. Li, D. Wang, F. Yang, L. Zhang, S. Ji, S. Wang, Enhanced extraction of hydroxyapatite from bighead carp (Aristichthys nobilis) scales using deep eutectic solvent, J. Food Sci. 85 (2020) 150–156, https://doi.org/10.1111/1750-3841.14971.

[11] Y. Liu, M. Liu, S. Ji, L. Zhang, W. Cao, H. Wang, S. Wang, Preparation and application of hydroxyapatite extracted from fish scale waste using deep eutectic solvents, Ceramic. Int. 47 (2021) 9366–9372, https://doi.org/10.1016/j.ceramint.2020.12.067.

[12] N. Muhammad, Y. Gao, F. Iqbal, P. Ahmad, R. Ge, U. Nishan, A. Rahim, G. Gonfa, Z. Ullah, Extraction of biocompatible hydroxyapatite from fish scales using novel approach of ionic liquid pretreatment, Sep. Pur. Technol. 161 (2016) 129–135, https://doi.org/10.1016/j.seppur.2016.01.047.

[13] M. Bisht, M. Martins, A.C.R.V. Dias, S.P.M. Ventura, J.A.P. Coutinho, Uncovering the potential of aqueous solutions of deep eutectic solvents on the extraction and purification of collagen type I from Atlantic codfish (Gadus morhua), Green Chem. 23 (2021) 8940–8948, https://doi.org/10.1039/D1GC01432C.

[14] M.P. Batista, N. Fernández, F.B. Gaspar, M.R. Bronze, A.R. Duarte, Extraction of biocompatible collagen from blue shark skins through the conventional extraction process intensification using natural deep eutectic solvents, Front. Chem. 10 (2022) 937036, https://doi.org/10.3389/fchem.2022.937036.

[15] C. Bai, Q. Wei, X. Ren, Selective extraction of collagen peptides with high purity from cod skins by deep eutectic solvents, ACS Sustain. Chem. Eng. 5 (2017) 7220–7227, https://doi.org/10.1021/acssuschemeng.7b01439.

[16] M.M. Abdallah, M. Cardeira, A.A. Matias, M.R. Bronze, N. Fernández, Lactic acid-based natural deep eutectic solvents to extract bioactives from marine by-products, Molecules 27 (2022) N:4356, https://doi.org/10.3390/molecules27144356.

[17] L.A. Rodrigues, I.C. Leonardo, F.B. Gaspar, L.C. Roseiro, A.R.C. Duarte, A. A. Matias, A. Paiva, Unveiling the potential of betaine/polyol-based deep eutectic systems for the recovery of bioactive protein derivative-rich extracts from sardine processing residues, Sep. Pur. Technol. 276 (2021) 119267, https://doi.org/10.1016/j.seppur.2021.119267.

[18] I. Silva, B.M.C. Vaz, S. Sousa, M.M. Pintado, E.R. Coscueta, S.P.M. Ventura, Gastrointestinal delivery of codfish skin-derived collagen hydrolysates: deep eutectic solvent extraction and bioactivity analysis, Food Res. Int. 175 (2024) 113729, https://doi.org/10.1016/j.foodres.2023.113729.

[19] N. Muhammad, G. Gonfa, A. Rahima, P. Ahmad, F. Iqbal, F. Sharif, A.S. Khan, F. U. Khan, Z.U.H. Khan, F. Rehman, I.U. Rehman, Investigation of ionic liquids as a pretreatment solvent for extraction of collagen biopolymer from waste fish scales using COSMO-RS and experiment, J. Mol. Liquids 232 (2017) 258–264, https://doi.org/10.1016/j.molliq.2017.02.083.

[20] C. Gallego, E. Rodil, H. Rodríguez, A. Soto, Extraction and characterisation of gelatine from fish skin pretreated with a eutectic solvent, Food Hydrocolloid. 159 (2025) 110652, https://doi.org/10.1016/j.foodhyd.2024.110652.

[21] A.D. Doehlert, Uniform shell designs, J.R. Stat. Soc. Series C (Appl. Stat.) 19 (1970) 231–239, https://doi.org/10.2307/2346327.

[22] U.M.F.M. Cerqueira, M.A. Bezerra, S.L.C. Ferreira, R. de Jesus Araújo, B.N. da Silva, C.G. Novaes, Doehlert design in the optimization of procedures aiming food analysis – a review, Food Chem. 364 (2021) N:130429, https://doi.org/10.1016/j.foodchem.2021.130429.

[23] G.W. Jr. Latimer, (ed.). Official Methods of Analysis of AOAC INTERNATIONAL, 22 Ed., Oxford University Press, New York, 2023. Doi: 10.1093/9780197610145.001.0001.

[24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275, https://doi.org/10.1016/S0021-9258(19)52451-6.

[25] M. Meyer, B. Morgenstern, Characterization of gelatine and acid soluble collagen by size exclusion chromatography coupled with multi angle light scattering (SEC-MALS), Biomacromol. 4 (2003) 1727–1732, https://doi.org/10.1021/bm0341531.

[26] S. Moore, D.H. Spackman, W.H. Stein, Chromatography of amino acids on sulphonated polystyrene resins. an improved system, Anal. Chem. 30 (1958) 1185–1190, https://doi.org/10.1021/ac60139a005.

[27] A.A. Karim, R. Bhat, Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins, Food Hydrocol. 23 (2009) 563–576, https://doi.org/10.1016/j.foodhyd.2008.07.002.

[28] A.L. Alves, F.J. Fraguas, A.C. Carvalho, J. Valcárcel, R.I. Pérez-Martín, R.L. Reis, J. A. Vázquez, T.H. Silva, Characterization of codfish gelatin: a comparative study of fresh and salted skins and different extraction methods, Food Hydrocolloid. 124 (2022) 107238, https://doi.org/10.1016/j.foodhyd.2021.107238.

[29] S.M. Cho, Y.S. Gu, S.B. Kim, Extracting optimization and physical properties of yellowfin tuna (Thunnus albacares) skin gelatin compared to mammalian gelatins, Food Hydrocolloid. 19 (2005) 221–229, https://doi.org/10.1016/j.foodhyd.2004.05.005.

[30] M.S. Rahman, G.S. Al-Saidi, N. Guizani, Thermal characterisation of gelatin extracted from yellowfin tuna skin and commercial mammalian gelatin, Food Chem. 108 (2008) 472–481, https://doi.org/10.1016/j.foodchem.2007.10.079.

[31] Y. Pranoto, D.W. Marseno, H. Rahmawati, Characteristics of gelatins extracted from fresh and sun-dried seawater fish skins in Indonesia, Int. Food Research J. 18 (2011) 1335–1341.

[32] K. Shyni, G.S. Hema, G. Ninan, S. Mathew, C.G. Joshy, P.T. Lakshmanan, Isolation and characterization of gelatin from the skins of skipjack tuna (Katsuwonus pelamis), dog shark (Scoliodon sorrakowah), and rohu (Labeo rohita), Food Hydrocolloid. 39 (2014) 68–76, https://doi.org/10.1016/j.foodhyd.2013.12.008.

[33] P.D. Karayannakidis, S.E. Chatziantoniou, A. Zotos, Co-extraction of gelatin and lipids from Yellowfin Tuna (Thunnus albacares) skins: physicochemical characterization, process simulation and economic analysis, J. Food Proc. Preser. 39 (2015) 2361–2370, https://doi.org/10.1111/jfpp.12484.

[34] S.C. Sousa, J.A. Vázquez, R.I. Pérez-Martín, A.P. Carvalho, A.M. Gomes, Valorization of by-products from commercial fish species: extraction and chemical properties of skin gelatins, Molecules 22 (2017) 1545, https://doi.org/10.3390/molecules22091545.

[35] E.T.A. Tümerkan, Ü. Cansu, G. Boran, J.M. Regenstein, F. Özoğula, Physiochemical and functional properties of gelatin obtained from tuna, frog and chicken skins, Food Chem. 287 (2019) 273–279, https://doi.org/10.1016/j.foodchem.2019.02.088.

[36] A.T. Alfaro, E. Balbinot, C.I. Weber, I.B. Tonial, A. Machado-Lunkes, Fish gelatin: characteristics, functional properties, applications and future potentials, Food Eng. Rev. 7 (2015) 33–44, https://doi.org/10.1007/s12393-014-9096-5.

[37] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, H. Kishimura, F. Shahidi, Isolation and Characterisation of collagen from the skin of brownbanded bamboo shark (Chiloscyllium punctatum), Food Chem. 119 (2010) 1519–1526, https://doi.org/10.1016/j.foodchem.2009.09.037.

[38] K.J. Payne, A. Veis, Fourier transform IR spectroscopy of collagen and gelatin solutions: deconvolution of the amide I band for conformational studies, Biopolym. 27 (1988) 1749–1760, https://doi.org/10.1002/bip.360271105.

[39] J. Valcarcel, J. Fraguas, C. Hermida-Merino, D. Hermida-Merino, M.M. Piñeiro, J. A. Vázquez, Production and physicochemical characterization of gelatin and collagen hydrolysates from turbot skin waste generated by aquaculture activities, Mar. Drugs 19 (2021) 491, https://doi.org/10.3390/md19090491.

[40] J.H. Muyonga, C.G.B. Cole, K.G. Duodu, Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (Lates niloticus), Food Chem. 86 (2004) 325–332, https://doi.org/10.1016/j.foodchem.2003.09.038.

[41] F. Pati, B. Adhikari, S. Dhara, Isolation and characterization of fish scale collagen of higher thermal stability, Bioresource Technol. 101 (2010) 3737–3742, https://doi.org/10.1016/j.biortech.2009.12.133.

[42] J. Valcarcel, C. Hermida-Merino, M.M. Piñeiro, D. Hermida-Merino, J.A. Vázquez, Extraction and characterization of gelatin from skin by-products of seabream, seabass and rainbow trout reared in aquaculture, Int. J. Mol. Sci. 22 (2021) N:12104, https://doi.org/10.3390/ijms222212104.

[43] M. Nurilmala, H.H. Hizbullah, E. Karnia, E. Kusumaningtyas, Y. Ochiai, Characterization and Antioxidant Activity of Collagen, Gelatin, and the Derived Peptides from Yellowfin Tuna (Thunnus albacares) Skin, Mar. Drugs 18 (2020) 98, https://doi.org/10.3390/md18020098.

[44] C. Hermida-Merino, D. Cabaleiro, L. Lugo, J. Valcarcel, J.A. Vázquez, I. Bravo, A. Longo, G. Salloum-Abou-Jaoude, E. Solano, C. Gracia-Fernández, M.M. Piñeiro, D. Hermida-Merino, Characterization of Tuna Gelatin-Based Hydrogels as a Matrix for Drug Delivery, Gels 8 (2022) N:237, https://doi.org/10.3390/gels8040237.

[45] V. Gudipati, Fish gelatin: A versatile ingredient for the food and pharmaceutical industries, in: S.-K. Kim (Ed.), Marine Proteins and Peptides: Biological Activities and Applications, John Wiley and Sons, New Jersey, 2013, pp. 271–295, https://doi.org/10.1002/9781118375082.ch13.

[46] I. Suárez, B. Coto, Broadening of polymer chromatographic signals: Analysis, quantification and correction through effective diffusion coefficients, J. Chromatograph. A 1407 (2015) 193–202, https://doi.org/10.1016/j.chroma.2015.06.068.

[47] S.R. Derkach, N.G. Voron'ko, Y.A. Kuchina, D.S. Kolotova, Modified fish gelatin as an alternative to mammalian gelatin in modern food technologies, Polymers 12 (2020) N:3051, https://doi.org/10.3390/polym12123051.

[48] D.M. Correia, J. Padrão, L. Rodrigues, F. Dourado, S. Lanceros-Mendez, V. Sencadas, Thermal and hydrolytic degradation of electrospun fish gelatin membranas, Polym. Test. 32 (2013) 995–1000, https://doi.org/10.1016/j.polymertesting.2013.05.004.

[49] M.E.O. Martins, J.R. Sousa, R.L. Claudino, S.C.O. Lino, D.A. do Vale, A.L.C. Silva, J. P.S. Morais, M.S.M. de Souza Filho, B.W.S.T. de Souza, Thermal and chemical properties of gelatin from Tilapia (Oreochromis niloticus) Scale, J. Aq. Food Product Tech. 27 (2018) 1120–1133, https://doi.org/10.1080/10498850.2018.1535530.

[50] Q. Xing, K. Yates, C. Vogt, Z. Qian, M.C. Frost, F. Zhao, Increasing mechanical strength of gelatin hydrogels by divalent metal ion removal, Sci. Reports 4 (2014) N:4706, https://doi.org/10.1038/srep04706.

[51] N. Caeiro, M.K. Wojtczuk, H. Rodríguez, E. Rodil, A. Soto, Recovery of dialkylimidazolium-based ionic liquids from their mixtures with acetone or water by flash distillation, *J. Mol. Liquids* 346 (2022) 118292, https://doi.org/10.1016/j.molliq.2021.118292.

[52] R. Kalb. Getting closer to Industrialization of Ionic Liquids. Where are we standing?. Plenary lecture presented at 7th Iberoamerican Meeting on Ionic Liquids, Coimbra, November 2024.