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ORIGINAL ARTICLE

Valorization of fish by-products: rheological, textural and microstructural properties of mackerel skin gelatins

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Abstract The fish processing industry generates significant amounts of waste which is usually discarded. The present study investigated the recovery of gelatins from Atlantic mackerel (*Scomber scombrus*) skins after pre-treatment with different environmentally friendly organic acids (acetic, citric, lactic, tartaric or malic acid). The chemical composition, the rheological and the textural properties as well as the microstructural characteristics of the extracted gelatins were analysed and compared to commercial bovine hide gelatin. Although the organic acid used in the pre-treatment step did not affect the extraction yield and the chemical composition of the prepared gelatins, differences were observed in terms of rheology and texture. The highest gel strength ($P < 0.05$) was observed with gelatins extracted after pre-treatment with acetic, citric and malic acids (71–80 g). From an industrial point of view, gelatin can be extracted using any of these organic acids with similar yield. However, in order to obtain better rheological and textural properties the use of acetic, citric or malic acid in the pre-treatment step is recommended.

Keywords Mackerel skin · Gelatin · Organic acid · Texture · Rheology · Microstructure

Introduction

Collagen is the most abundant protein in the animal body which represents the major fraction of tendons, skin, bones and connective tissues [1]. The thermal denaturation of collagen produces gelatin, a protein ingredient widely used in the cosmetics, pharmaceutical and food industries, due to its important physical functionality. In the food sector, gelatin is known to improve the elasticity, consistency and stability of the food formulations [2]. Pigskin and cattle bone and hide are generally the main source of commercial gelatins. Fish gelatin gained interest following the outbreak of bovine spongiform encephalopathy (BSE) [3]. In spite of being considered superior to fish gelatins [4], in terms of functional properties, mammalian gelatins may give rise to some dietary concerns. For some cultural, religious and ethnic reasons, pig gelatin is prohibited for use [5]. The increasing demand for halal and kosher foods makes fish gelatin suitable as an alternative to mammalian gelatins.

Fisheries and the fish processing industries are important economic sectors in the world with an estimated global production (both farmed and wild fish catch) of around 148 million tons in 2010 [6]. The fish processing industry generates significant amounts of waste. In general, only the fillets are retained [7] and the bulk of the product consisting of head, guts and frame is usually discarded. This waste represents a potential source for gelatin [8].

The production method significantly affects the physicochemical properties of the gelatin [9]. The industrial process of gelatin manufacture involves either an acid or an alkaline pre-treatment, to break the collagen cross-links,

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followed by solubilisation of collagen fibres by a thermal treatment. For fish materials, acid pre-treatment is usually required to partially cleave the non-covalent bonds in the fish collagen fibres since high levels of hydrogen ions increase the penetration of water around the collagen fibres [9]. Both mineral and organic acids can be used in the extraction of fish gelatins. However, the use of organic acids in the pre-treatment step is preferable.

The main objective of the present work was to extract gelatins from mackerel skins using different organic acids (acetic, citric, lactic, malic or tartaric acids) and to evaluate their effects on the rheological, textural and microstructural properties of the extracted ingredients.

Materials and methods

Materials

Atlantic mackerel (*Scomber scombrus*) were kindly provided by Bord Iascaigh Mhara (BIM, Dublin, Ireland). Mackerel skins were manually removed from the fillet using a knife and cut into small squares (4 cm^2) using a scissor. The processed skins were divided into batches and kept in the freezer at −20 °C until use in less than a month.

Characterisation of mackerel skin

Proximate analysis

The chemical composition of mackerel skin was carried out according to the procedures of the Association of Official Analytical Chemists [10]. Moisture and ash contents were determined according to the methods number 927.05 and 942.05, respectively [10]. Crude protein was determined by Kjeldahl method following the method number 984.13 [10] and using a nitrogen conversion factor of 6.25. Lipid content was determined according to the method of Bligh and Dyer [11].

Hydroxyproline content

The hydroxyproline content of mackerel skin was determined according to the method of Edwards and O'Brien [12] and the collagen content was estimated using a conversion factor of 7.14.

Extraction of gelatin

Gelatin extraction was carried out according to the method described by Khiari et al. [13]. Briefly, mackerel skins (~1.5 kg) were treated with 0.1 N NaOH (for 30 min at 4 °C, repeated 3 times), followed by an acid treatment. In this study, different organic acids (acetic, citric, lactic, malic or tartaric acid) were used separately at different concentrations (25, 50 or 100 mM). The acid treatment was carried out for 4 h at 4 °C. At the end of this step, mackerel skins were washed with distilled water to remove the acid. Gelatin was extracted at 45 °C for 18 h with distilled water then filtered through Whatman No. 4 filter paper (Whatman, Maidenstone, England). Gelatin was deionized using Rexyn™ I-300 (H-OH) beads (Acros, Geel, Belgium). The ion exchange beads were mixed with the gelatin solution and stirred at 150 rpm until the conductivity value of the gelatin solution was less than 50 µSiemens/cm at room temperature. The deionized gelatin was then concentrated by evaporation at 45 °C under vacuum to prevent possible thermal degradation (Büchi UK Ltd., Oldham, UK), and finally freeze dried (Labconco Corporation, Kansas City, MO, USA). All pre-treatment and extraction procedures were done under continuous agitation at 150 rpm and using a raw material/solvent ratio of 1/3 (w/v).

Gelatin extraction yield

Gelatin extraction yield was calculated on a dry basis according to Du et al. [14] as the amount of gelatin with respect to the amount of collagen in the raw material using the following formula:

$$\text{Gelatin extraction yield } (\%) = 100 \times \frac{\text{Dry weight of extracted gelatin (g)}}{\text{Dry weight of collagen in mackerel skin (g)}}$$

Characterisation of gelatins

Proximate analysis of gelatin

The chemical composition of gelatins was analysed according to the methods [10, 11] previously described in Sect. "Proximate analysis". The protein content of gelatin was calculated using a nitrogen conversion factor of 5.4 [15].

Protein profile of gelatins

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedure was carried out according to Khiari et al. [16]. Briefly, 10 µL of gelatin samples (2 mg/mL) and molecular weight markers were loaded onto SDS-polyacrylamide gel having a 4 % stacking gel and 10 % resolving gel (prepared according to the method of Laemmli [17]). The analysis was run for 55 min at a constant current of 25 mA in an Atto Dual Mini-slab Size Electrophoresis Systems AE-6450 (Atto Corporation, Tokyo, Japan). The gel was stained with Coomassie

Brilliant Blue R250 and de-stained using a mixture of isopropanol, acetic acid and distilled water (12:10:78, v/v/v). The molecular weight markers (Sigma, Dublin, Ireland) contained a lyophilized mixture of proteins with molecular weight ranging from 30,000 to 200,000 Da.

Amino acids analysis

The amino acid analysis was performed in the Service of Protein Chemistry at the Centro de Investigaciones Biologicas (CSIC, Madrid) following the method described by Khiari et al. [16]. A known amount of gelatin sample was hydrolysed with 6 M HCl containing 0.1 % phenol for 24 h at 110 °C in vacuum-sealed hydrolysis vials. Norleucine (Sigma–Aldrich, Inc., Madrid, Spain) was incorporated as an internal standard. The amino acid composition was analysed by a cation exchange Biochrom 20 amino acid analyser (Pharmacia Biotech, Ltd., Cambridge, England) using a postcolumn derivatisation technique with ninhydrin. All amino acids were determined at an absorbance of 570 nm, except for proline and hydroxyproline which were measured at 440 nm. Cysteine was determined as cysteic acid according to the method of Hirs [18]. Results were presented as mol% (i.e. residues per 100 residues).

Solubility

The solubility of bovine and mackerel skin gelatin was measured in the pH range of 2–12 according to the method described by Khiari et al. [16]. Briefly, gelatin solutions were first prepared in distilled water to a protein concentration of 0.3 % (w/v). The pH of 8 mL gelatin solution was then adjusted to the desired pH value using either 1 M HCl or 1 M NaOH (Orion pH meter Model 420A, Orion Research Inc, Beverly, MA. USA). The final volume was adjusted to 10 mL by adding distilled water having same pH as the sample. Gelatin solutions were centrifuged at $9000\times g$ for 15 min at 5 °C (SIGMA 2K15 centrifuge, SIGMA Laborzentrifugen GmbH, Osterode, Germany). The protein content of the sample before and after centrifugation was determined using the Biuret assay [19] and considering bovine serum albumin (BSA, Sigma–Aldrich, Inc.) as a reference protein. The solubility was calculated as follows:

$$\text{Solubility}\,(\%) = 100 \times \frac{\text{Protein content in the supernatnat after centrifugation}}{\text{Protein content in the sample before centrifugation}}$$

Rheological characterisation

A preliminary stress amplitude sweep test was performed to determine the linear viscoelastic range (LVE). The LVE is defined as the domain below a strain threshold value where the sample structure is preserved and the elastic (G') and viscous (G'') moduli show a constant high plateau (region insensitive to strain changes). Once the amplitude of the deformation exceeds the threshold value, the structure of the sample is irreversibly destroyed [20].

Dynamic viscoelastic behaviour (DVB)

The dynamic viscoelastic behaviour (DVB) of gelatin samples was performed according to the method described by Khiari et al. [21]. A controlled stress rheometer (Bohlin C-VOR, Malvern Instruments Ltd., Malvern, UK) was set to perform small oscillations with a stress of 1.0 Pa and a frequency of 1 Hz, using a 5.5 cm parallel plate geometry with a gap of 0.2 mm. The viscoelastic properties of gelatin solutions (0.4 mL; 6.67 %, w/v) were measured in the temperature range of 30–5 °C and 5–30 °C, with a heating/cooling rate at 1 °C/min. After completing the cooling process, gelatins were kept at 5 °C for 10 min before starting the heating process. The elastic modulus (G'), viscous modulus (G'') and Tan δ (G''/G') values were obtained as a function of temperature. For comparison purposes, commercial bovine hide gelatin (Gelatin powder 104078, Merck Chemicals Ltd. Nottingham, UK), with a bloom value of 110 g and a particle size of 99 µm, was used at the same concentration as the extracted fish skin gelatin samples.

Frequency sweep

The frequency sweep test was performed according to the method described by Khiari et al. [21]. Gelatin (0.4 mL; 6.67 %, w/v) was placed on the lower plate of a Bohlin C-VOR rheometer (Malvern Instruments Ltd., Malvern, UK) and kept at 5 °C for 10 min before starting the analysis. The assay was performed using 5.5 cm parallel plate measuring geometry and 0.2 mm as a gap. The elastic modulus (G') was obtained as a function of frequency (varying from 0.2 to 5.2 Hz). Commercial bovine hide gelatin was used, at the same concentration as the extracted fish skin gelatin samples, for comparison purposes.

Textural properties

Gel strength

The gel strength was determined according to the AOAC method number 948.21 [10]. Gelatin solutions (6.67 %, w/v) were prepared in distilled water (at 60 °C) then matured at 10 °C for 16–18 h to form the gel. The strength of gelatin gels was determined using samples having 3.5 cm diameter and 1.5 cm height on an Instron Universal Testing Machine model 3300 (Instron Ltd., High Wycombe,

England) fitted with a static load cell of 500 N and equipped with a flat-faced cylindrical probe (diameter of 1.27 cm). The test was run at a penetration speed of 1 mm/s. Gel strength was expressed as the maximum force (g) obtained at 4 mm penetration depth (corresponding to about 27 % displacement) on the gelatin gels. Commercial bovine hide gelatin was used, at the same concentration as the extracted fish skin gelatin samples, for comparison purposes.

Cryo-scanning electron microscopy (Cryo-SEM)

Cryo-scanning electron microscopy (Cryo-SEM) was used to observe the effects of the pre-treatment on the microstructure of the extracted gelatin gels. Cryo-SEM analysis was performed according to the method described by Khiari et al. [21] with minor modifications.

Gelatin was dissolved in distilled water to a final concentration of 6.67 % (w/v) then poured into a mini Petri dish and stored at 4 °C for 2 h to form the gel. Cubic samples ($\sim$0.5 × 0.5 × 0.5 cm^3) were cut from random areas of the gelatin gel and placed on the specimen holder. After freezing by immersion in Slush Nitrogen (−210 °C), the gelatin gel samples were fractured, warmed (at −94.5 °C, 10–5 Torr vacuum, for 15 min to sublime the water), gold coated and viewed in the cold-stage scanning electron microscopy (JEOL JSM-5410, Tokyo, Japan) at an acceleration voltage of 15 kV. Commercial bovine gelatin was used, at the same concentration as the extracted fish skin gelatin samples, for comparison purposes.

Statistical analyses

The entire experiment was repeated three times for three different independent batches. All the analytical analyses were performed in triplicate, except for the amino acid analysis which was performed in duplicate. Analysis of variance (ANOVA) was used to find differences between treatments. Means were compared by the least significant difference (LSD) test, at a significance level of $P < 0.05$ using the PROC MIXED procedure of SAS (v. 9.1.3, SAS Institute Inc., Cary, NC).

Results and discussion

Characterisation of mackerel skin

The average weight of the whole mackerel used in this study was 277.0 g. Average weight of 326 g was reported by Toppe et al. [22] for Atlantic mackerel (*S. scombrus*). The distribution of different components of mackerel was as follows: heads constituted 16.6 %; bones, fins and tails constituted 8.5 %; skins constituted 17.0 %; and viscera constituted 10.5 % of the whole mackerel. According to Leu et al. [23], the average edible portion of mackerel is about 53.5 % (w/w), the rest constituted the inedible parts (heads 17.1 %; bones, fins and tails 8.4 %; skins 10.3 %; and viscera 10.9 %).

In this research study, a significant amount of waste was obtained (52.2 %). This waste was mainly constituted by skins (32.6 %), heads (31.8 %), viscera (20.1 %) and bones (16.3 %). Since skin constituted the most abundant waste from mackerel processing, it was chosen for further investigation.

The proximate composition indicated that mackerel skin comprised 64.6 % water, 2.3 % ash, 18.6 % protein and 13.7 % fat. The hydroxyproline content of mackerel skin (on a dry weight basis) was found to be 3.5 % which corresponded to 24.8 % collagen content. Fish waste are typically discarded overboard or dumped to landfill. However, the European Directive 1999/31/EC on the landfill of waste [24] forbids and restricts the disposal of untreated organic waste not untended for human consumption. The development of new sustainable processes for optimal use of fish waste may represent a new approach to lower the disposal cost and increase profit. Hence, the abundant low quality collagen ($\sim$25 %) in mackerel skins can be converted into potential value added products such as gelatin.

Effect of organic acid concentration on the yield of gelatin extraction

Figure 1 shows the effect of the organic acid concentration (25, 50 and 100 mM) on the extraction yield (expressed on a dry basis as amount of gelatin per total amount of collagen in raw material [14]).

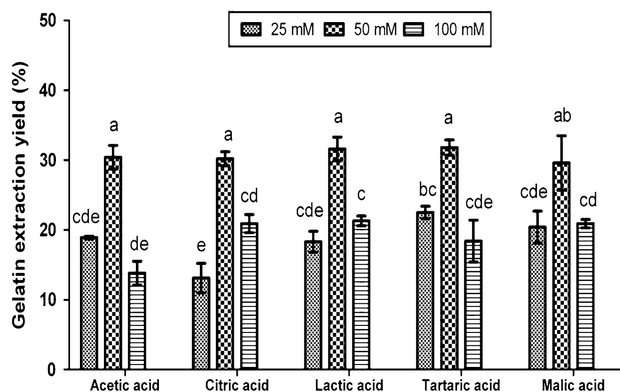

Fig. 1 Effect of the organic acid concentration on gelatin extraction yield. **a–e** Means sharing a common letter are not significantly different from each other, $P > 0.05$

For all organic acid used in the present study (acetic, citric, lactic, malic and tartaric), the extraction yield was found to be significantly higher when using a concentration of 50 mM compared to either 25 or 100 mM. At 50 mM, the extraction yield varied between 29.6 and 31.8 %, but no significant differences were observed among the various treatments.

Regardless of the organic acid, the extraction yields obtained with concentrations of 25 and 100 mM ranged between 13.1–22.5 % and 13.8–21.3 %, respectively. It is known that the acid pre-treatment in gelatin preparation results in the swelling of the skin and the removal of non-collagenous proteins (i.e. sarcoplasmic and myofibrillar proteins) [25]. The low extraction yield at lower concentration (i.e. 25 mM) may therefore be due to incomplete swelling of collagen fibres and limited cleavage of collagen cross-links. On the other hand, the reduced extraction yield at higher concentration (i.e. 100 mM) could be due to greater solubilisation collagen and consequently greater loss during the pre-treatment step. This may be due to the fact that higher concentration of acid increases the amount of hydrogen ions and leads to the cleavage of cross-links and enhanced solubilisation of collagen [26].

Characterisation of extracted gelatins

Proximate analysis and gelatin extraction yield

The proximate composition of mackerel skin gelatins is presented in Table 1. High protein contents (>85 %) were observed in all gelatins regardless of the organic acid used. All mackerel skin gelatins had low moisture content and both ash and lipid contents were less than 1 %. The low fat and ash contents of the extracted gelatins may indicate the efficacy of the pre-treatment operations in eliminating fat and other impurities from mackerel skins. No significant differences were observed among the moisture, ash, protein and fat content of all the extracted mackerel skin gelatins which indicate that the organic acid did not affect the chemical composition of the extracted ingredients.

Bovine hide gelatin had similar moisture and protein contents as the extracted fish gelatins. However, significant differences were observed between the ash and the fat contents (Table 1). The use of a mixture of strong acid cation and base anion exchangers for the deionization of mackerel fish gelatins may have resulted in low ash content (0.7–1.0 %) in these gelatins. Unlike bovine gelatin, residual fat (0.7–0.9 %) was present in the extracted gelatin. Since mackerel is a fatty fish, it would be recommended to pre-treat the skins with dilute organic solvents, such as butyl alcohol [27], in order to remove fat and obtain fat-free gelatins.

Protein patterns of mackerel skin gelatin

The protein patterns in gelatin samples were examined using SDS-PAGE (Fig. 2). Both gelatins obtained from mackerel skins after pre-treatment with acetic, citric and malic acids and bovine hide gelatin comprised one β chain and two different α_1 and α_2 chains (Fig. 2, Lane 2, 3, 4 and 7, respectively). These chains are characteristic of type I gelatins [28]. Similar protein patterns were observed for other fish species such as megrim (*Lepidorhombus boscii*), Dover sole (*Solea vulgaris*), cod (*Gadus morhua*), hake (*Merluccius merluccius*) [29], cuttlefish (*Sepia lycidas*) [30] and Nile perch (*Lates niloticus*) [1].

Lactic and tartaric acids, on the other hand, resulted in gelatins with less β-chain but greater α-chains as observed by their higher intensities (Fig. 2, Lane 5 and 6, respectively). This may be due to the dissociation of the dimeric β chain into monomeric α_1 and α_2 chains. In addition, low molecular size peptides were also observed for these gelatins. The organic acids used in the pre-treatment step hydrolyze some of cross-links causing the loss of rigidity and insolubility of the collagen fibrils [31]. The hydroxyl groups in tartaric and lactic acid (two and one alcohol

Table 1 Proximate analysis and gel strength of commercial bovine hide gelatin and mackerel skin gelatins extracted after pre-treatment with different organic acids

Gelatin	Pre-treatment	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)	Gel strength (g)
Mackerel	AA	10.3 ± 1.8	0.8 ± 0.1^{b}	86.2 ± 1.3	0.7 ± 0.1^{a}	80.2 ± 1.4^{b}
	CA	9.0 ± 1.9	0.7 ± 0.2^{b}	87.2 ± 2.8	0.9 ± 0.2^{a}	76.4 ± 0.7^{b}
	LA	10.2 ± 1.7	0.8 ± 0.1^{b}	85.0 ± 2.7	0.7 ± 0.2^{a}	43.3 ± 0.7^{c}
	MA	8.7 ± 1.6	0.7 ± 0.2^{b}	87.2 ± 2.0	0.9 ± 0.3^{a}	71.1 ± 3.6^{b}
	TA	8.9 ± 1.2	1.0 ± 0.2^{b}	85.9 ± 1.9	0.8 ± 0.1^{a}	49.4 ± 1.4^{c}
Bovine	–	9.9 ± 0.0	1.5 ± 0.1^{a}	88.7 ± 0.2	0.0 ± 0.0^{b}	244.7 ± 14.4^{a}
	P value	0.3502	0.0209	0.3872	0.0135	<0.0001

Values are given as mean ± standard deviation. Means within the same column sharing a common letter are not significantly different from each other, $P > 0.05$

AA acetic acid-extracted gelatin, *CA* citric acid-extracted gelatin, *LA* lactic acid-extracted gelatin, *MA* malic acid-extracted gelatin, *TA* tartaric acid-extracted gelatin

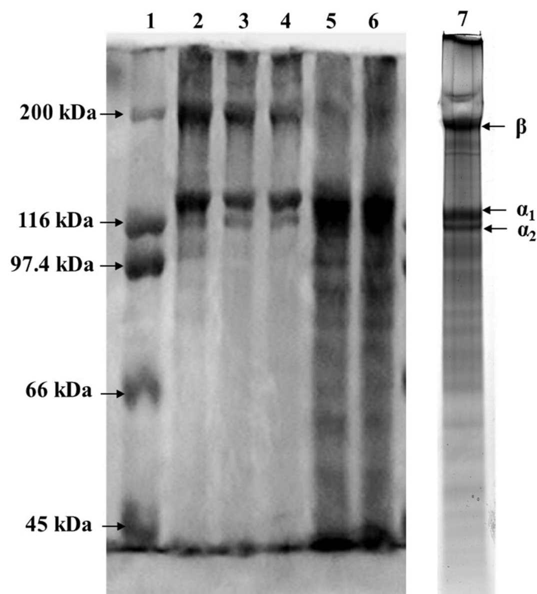

Fig. 2 SDS-PAGE patterns of commercial bovine hide gelatin and mackerel skin gelatins extracted after pre-treatment with different organic acids. *Lane 1* Molecular weight markers (M.W. 30,000–200,000 Da), *lane 2* acetic acid-extracted gelatin, *lane 3* citric acid-extracted gelatin, *lane 4* malic acid-extracted gelatin, *lane 5* lactic acid-extracted gelatin, *lane 6* tartaric acid-extracted gelatin, *lane 7* commercial bovine hide gelatin

group, respectively) may have exhibited lyotropic properties which subsequently improved the dissociation of these two organic acids upon collagen molecules [31] and led to an enhanced degradation of collagen chains. The dissociation of β chain and the presence of low molecular weight components in mackerel skin gelatins pre-treated with lactic and tartaric acids are an indication of their lower molecular weight distributions. According to Muyonga et al. [1], the presence of low molecular weight fragments in gelatin is associated with inferior viscoelastic properties and lower gel strength.

Amino acid composition

The amino acid composition (expressed as mol%) of commercial bovine hide gelatin and the extracted mackerel skin gelatins is presented in Table 2. Minor differences were observed between mackerel skin gelatins. Glycine, the main amino acid in collagen, was present at high content (ranged from 34.2 to 36.0 mol%) in all mackerel gelatin samples. Regardless of the organic acid used in the pre-treatment, alanine, proline and hydroxyproline were relatively high in all gelatins. The imino acid contents (proline + hydroxyproline) of mackerel skin gelatins varied depending on the organic acid used in the pre-treatment step. Acetic acid pre-treatment of mackerel skins resulted in gelatin with greater proline and hydroxyproline content

Table 2 Average amino acid composition (mol%) of commercial bovine hide gelatin and mackerel skin gelatins extracted after pre-treatment with different organic acids

Amino acid	Content (mol%)					
	Mackerel gelatin					Bovine gelatin
	AA	CA	LA	MA	TA	
Asx[a]	4.8	4.8	5.2	4.9	4.7	3.7
Thr	2.4	2.4	2.5	2.4	2.5	1.5
Ser	5.0	5.5	5.4	5.0	5.4	2.1
Glx[b]	7.1	6.9	6.7	6.7	7.3	7.0
Gly	36.0	34.2	34.7	35.6	35.1	33.6
Ala	11.6	11.9	11.9	11.8	11.8	11.3
Cys	0.6	0.8	1.1	1.0	1.2	0.0
Trp	ND	ND	ND	ND	ND	ND
Val	1.6	1.7	1.9	1.7	1.8	2.3
Met	1.4	1.3	1.4	1.4	1.3	0.7
Ile	0.8	0.9	0.9	0.9	0.9	1.3
Leu	2.4	2.5	2.7	2.6	2.6	2.6
Tyr	0.4	0.5	0.5	0.4	0.5	0.6
Phe	1.5	1.4	1.5	1.6	1.5	1.3
His	0.4	0.6	0.6	0.6	0.5	0.9
Lys	2.3	2.6	2.4	2.5	2.6	2.8
Arg	4.9	5	5.1	5.1	5.1	5.2
Pro	9.5	10.8	10.1	9.7	10	13.3
Hyp	7.4	5.9	5.3	6.3	5.4	9.7
Total	100.0	100.0	100.0	100.0	100.0	100.0
Pro + Hyp	16.9	16.8	15.4	15.9	15.4	23.0

The amino acid composition was performed in duplicate and data correspond to mean values

AA acetic acid-extracted gelatin, *CA* citric acid-extracted gelatin, *LA* lactic acid-extracted gelatin, *MA* malic acid-extracted gelatin, *TA* tartaric acid-extracted gelatin, *ND* not detected

[a] Represents the sum of aspartic acid (asp) and asparagine (asn)

[b] Represents the sum of glutamic acid (glu) and glutamine (gln)

(16.9 mol%), followed by gelatins extracted after pre-treatment with citric and malic (16.8 and 15.9 mol%, respectively). Tartaric and lactic acid pre-treatment of mackerel skins generated gelatins with the lowest imino acids contents (15.4 mol%). Bovine gelatin showed lower asparagine/aspartic acid, threonine, serine and methionine contents (3.7, 1.5, 2.1 and 0.7 mol%, respectively) but higher valine, isoleucine, histidine, proline and hydroxyproline contents (2.3, 1.3, 0.9, 13.3 and 9.7 mol%, respectively) compared to all mackerel skin gelatins (Table 2) which can be mainly attributed to the intrinsic differences between the raw materials. It is known that gelatin does not contain tryptophan while cysteine is absent in type I gelatin [32]. Tryptophan was not present in mackerel and bovine skin gelatins. However, low contents in cysteine (0.6–1.2 mol%) were observed in all extracted

gelatins. According to Sukkwai et al. [25], the acid pre-treatment in gelatin preparation results in swelling of the raw material and the removal of non-collagenous proteins, mainly sarcoplasmic and myofibrillar proteins. The presence of cysteine in type I gelatin may indicate a possible contamination by non-collagenous proteins during the extraction process [33]. Protein contamination might be the result of insufficient removal of these proteins by the organic acid. The degree of protein contamination can give an idea about the purity of gelatins and may therefore explain the difference among the imino acid contents. In fact, a strong significant negative correlation was observed between the imino acids and cysteine contents ($R = -0.96$; $P = 0.01$) indicating that the reduced amounts of proline and hydroxyproline were compensated with greater cysteine content. The differences among the imino acid contents of mackerel skin gelatins may affect their rheological properties. Hydroxyproline is known to stabilise the triple-helix strands of collagen. The hydroxyl groups in hydroxyproline are usually involved in this process by forming hydrogen bonds [34].

The nature of the organic acid (i.e. mono, di or tri-carboxylic) plays an important role in the swelling (i.e. uptake of water) of collagen fibres. In acid solutions, the swelling of fibrous proteins, such as collagen, is due to the osmotic pressure arising from the salt formation between the free amino groups of collagen molecules and the organic acid through the Donnan effect [35]. It is known that citric acid is more efficient than tartaric acid in terms of swelling capacity of fish skin [36]. In addition, the swelling of fish skin collagen by lactic acid has been found to be three times greater than that produced by acetic and tartaric acid [36]. The strength of the organic acid may also have resulted in the differences observed among the amino acid content of mackerel skin gelatins. Among all the organic acid used in this study, acetic acid had the lowest pKa value (4.76 at 25 °C). According to Bowes and Kenten [37] at pH 3 or lower, the weaker the acid the greater the swelling. Hence, the use of acetic acid in the pre-treatment may have favoured the disruption of collagen cross-links and resulted in efficient extraction of gelatin.

Gelatin solubility

The protein solubility is an important functional property which provides a prediction of the potential application of proteins. The solubility of mackerel skin gelatin and commercial bovine hide gelatin was measured in the pH range of 2–12 and is depicted in Fig. 3.

All mackerel skin gelatins, regardless of the pre-treatment, showed similar pH behaviours. The solubility was higher at low and high pH values, with maximum at alkaline pH values. Commercial bovine gelatin had better solubility than mackerel gelatin with highest solubility value obtained at pH 2. For both fish and bovine gelatins, the least solubility was observed close to neutral pH. Similar solubility results were reported for bigeye snapper skin collagen [38]. The differences in solubility between mackerel and bovine gelatins might be due to the differences in amino acid compositions mainly the content of polar and non-polar groups in amino acids.

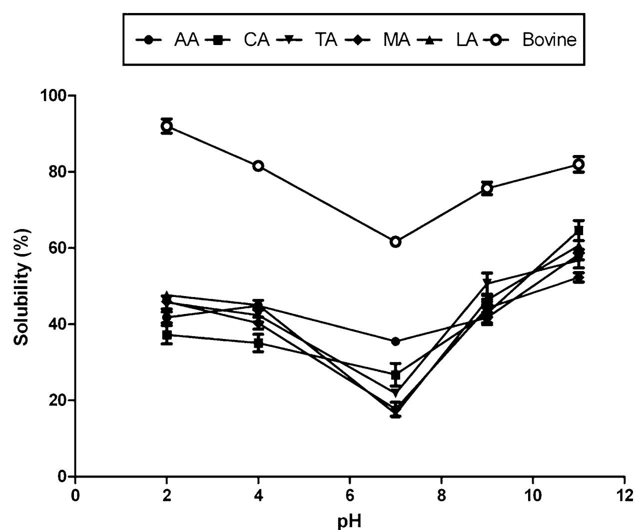

Fig. 3 Solubility of mackerel skin gelatins, extracted using different organic acids, in the pH range 2–12. *AA* acetic acid-extracted gelatin, *CA* citric acid-extracted gelatin, *LA* lactic acid-extracted gelatin, *MA* malic acid-extracted gelatin, *TA* tartaric acid-extracted gelatin, *Bovine* commercial bovine hide gelatin. Each point represents the average of three measurements

Rheological properties of extracted gelatins

Dynamic viscoelastic behaviour

Figure 4 shows the viscoelastic properties, including the storage and loss moduli as well as the phase angle during the cooling (Fig. 4a, c, e) and heating ramps (Fig. 4b, d, f) of commercial bovine gelatin and mackerel skin gelatins extracted after pre-treatment with acetic, citric, lactic, malic and tartaric acids.

In the cooling ramp (i.e. from 30 to 5 °C), the elastic modulus (G') of all mackerel skin gelatins increased rapidly between 17 and 10 °C, representing the transition from solution to gel state (Fig. 4a). Slight differences on the increase rate were observed. Similar behaviour was observed for the viscous modulus (G'') with a gradual increase (Fig. 4c).

As observed from the heating ramp (i.e. from 5 to 30 °C), the elastic modulus (G') of all mackerel skin gelatins decreased slowly between 5 and 15 °C then a rapid decrease was observed between 15 and 23 °C representing

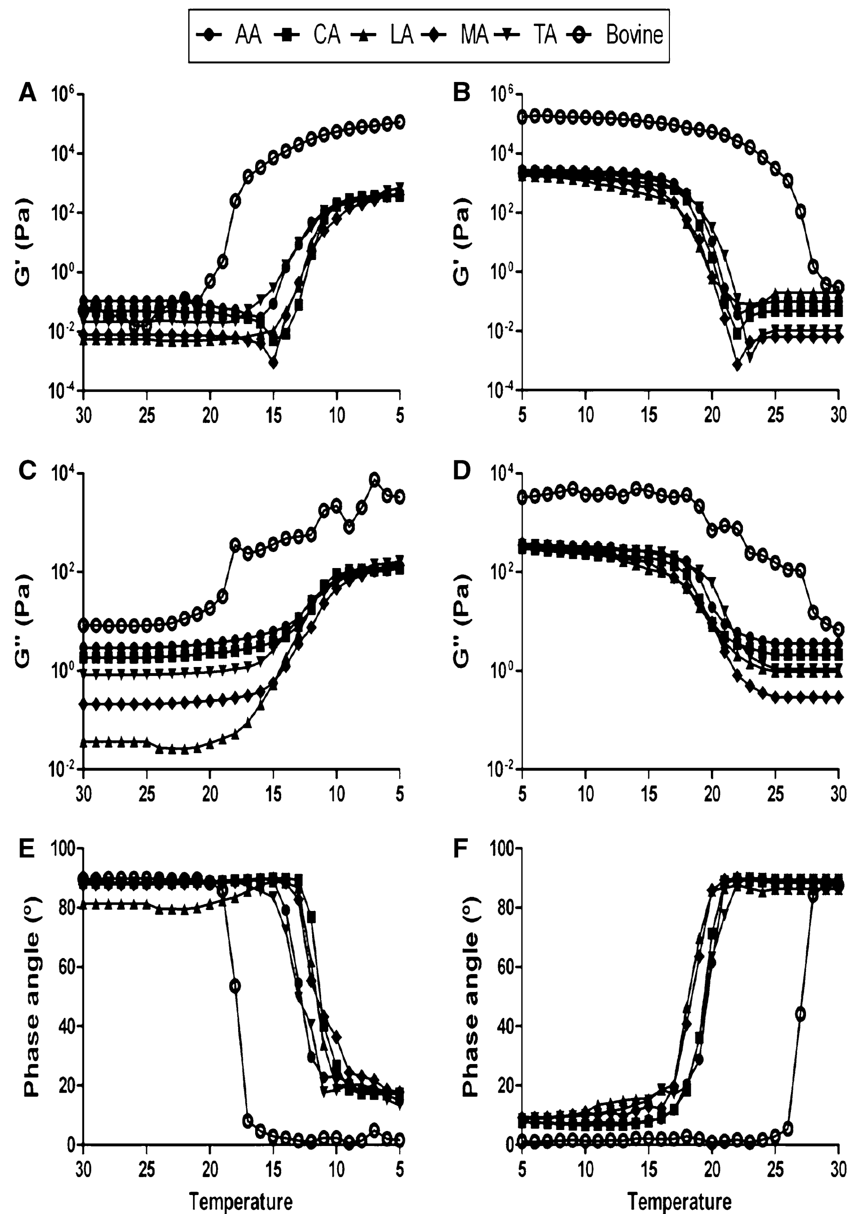

Fig. 4 Viscoelastic properties of commercial bovine hide gelatin and mackerel skin gelatins extracted after pre-treatment with different organic acids. Changes in the elastic modulus G' (**a**, **b**), viscous modulus G'' (**c**, **d**) and phase angle (**e**, **f**) were analysed during cooling from 30 to 5 °C (**a**, **c**, **e**) and subsequent heating from 5 to 30 °C (**b**, **d**, **f**). *AA* acetic acid-extracted gelatin, *CA* citric acid-extracted gelatin, *LA* lactic acid-extracted gelatin, *MA* malic acid-extracted gelatin, *TA* tartaric acid-extracted gelatin, *Bovine* commercial bovine hide gelatin. Each point represents the average of three measurements

the transition from gel to solution (Fig. 4b). The differences among the values of G' at 5 °C during the cooling and heating process could due to the maturation of gelatins at 5 °C for 10 min before starting the heating process. The viscous modulus (G'') showed similar behaviour, with the exception that the decrease was gradual (Fig. 4d).

In both processes (cooling and heating) the phase angle showed similar profiles. All mackerel skin gelatins had a low phase angle at low temperature (Fig. 4e, f) which indicates good gelling ability [39]. The slight differences in the transition curves during the melting and gelling processes among gelatins resulted in slight differences in gelling and melting temperatures of these gelatins. In this study, the gelling temperatures varied from 11.8 to 12.9 °C, while the melting temperatures ranged from 18.4 to 20.4 °C. Previous studies showed that the melting temperatures for fish gelatins vary from 15 to 32 °C [40–42]. The melting temperatures in the present study were lower than that of common mackerel (26.1 °C) as reported by Kimura et al. [42]. This could be due to the variation among the species, the temperature of the habitat, the extraction procedure, the pH and the concentration of gelatins [40].

The amino acid result (Table 2) indicated that mackerel skin gelatins extracted after pre-treatment with acetic acid had the highest imino acid (proline and hydroxyproline) content, followed by gelatins extracted after pre-treatment with citric, malic, tartaric and lactic acid. These differences

may explain the slight difference in gelling and melting temperatures of these gelatins. Commercial bovine hide gelatin had higher viscoelastic properties (i.e. greater elastic and viscous moduli) than all the extracted mackerel skin gelatins (Fig. 4), which resulted in considerably higher gelling and melting points (18 and 27 °C, respectively) compared to the extracted fish gelatins. The better viscoelastic properties of bovine skin gelatin compared to mackerel skin gelatins may be due to the difference in proline and hydroxyproline contents (Table 2). According to Gilsenan and Ross-Murphy [43], the poor rheological properties of fish gelatins compared to mammalian gelatins may be attributed to the difference in imino acid content. It was also suggested that gelatins with higher imino acid content have better rheological properties with higher ability to regain triple-helix structures leading to stabilisation of gelatin gels [29].

Frequency sweep analyses

The effect of the frequency on the elastic (G') modulus was studied (Fig. 5). These analyses were carried out to verify the rheological behaviour of the gelatins and to assess the strength of the gel network. For all mackerel skin gelatin samples, a slight dependence of G' values on the frequency was observed. These results were similar to those observed for cod gelatins [43]. The slopes of G' values as a function of frequency varied slightly among the gelatins depending on the organic acids used. All gelatin gels from mackerel skins showed relatively good textural stability as proven by the low slope of regression lines for G' versus frequency (varying from 0.3 to 0.6). Low slope is an indication of good gel networks and better stability of gelatin gels when subjected to shear forces [44].

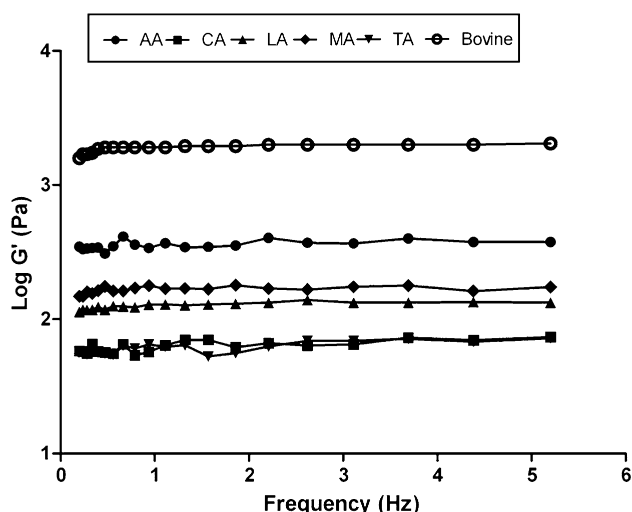

Fig. 5 Frequency sweep test of commercial bovine hide gelatin and mackerel skin gelatin gels at 5 °C. *AA* acetic acid-extracted gelatin, *CA* citric acid-extracted gelatin, *LA* lactic acid-extracted gelatin, *MA* malic acid-extracted gelatin, *TA* tartaric acid-extracted gelatin, *Bovine* commercial bovine hide gelatin. Each point represents the average of three measurements

Commercial bovine hide gelatin gel, on the other hand, was very stable, less affected by the change in frequency and stronger than all mackerel skin gelatin gels (Fig. 5).

Textural and microstructural properties of extracted gelatins

Gel strength

Gel strength is one of the most important physical properties of gelatin [45]. The gel strength of the various gelatin preparations, after overnight maturation at 10 °C, is presented in Table 1.

The gel strength of mackerel skin gelatins was affected by the organic acid. Gelatins extracted from mackerel skins after pre-treatment with acetic, citric and malic showed significantly ($P < 0.05$) high gel strength (71.1–80.2 g) corresponding with gelatin having the highest imino acid contents. As previously discussed (Table 2), tartaric and lactic acid pre-treated gelatins had the least proline and hydroxyproline levels (15.4 mol%), which resulted in lower gel strength (49.4 and 43.3 g, respectively). Gelatins with a bloom value of 108 g for salmon and 71 g for cod skins were reported by Arnesen and Gildberg [3]. Commercial bovine hide gelatin had significantly greater gel strength (244.7 g) compared to all mackerel skin gelatins which could be due to its higher content of imino acids. It is known that the low hydroxyproline content in fish gelatin is responsible for the low gel strength [46]. Other factors affecting the gel strength could be the pH of gelatins. The gel strength might be dependent on the isoelectric point and could also be controlled by adjusting the pH [47]. In this study, all mackerel skin gelatins had similar solubility behaviour in the pH range of 2–12 (Sect. "Effect of organic acid concentration on the yield of gelatin extraction"). The greatest solubility percentages were observed at low and high pH values (Fig. 3). The least solubility percentages were obtained at neutral pH corresponding to the isoelectric point of these gelatins.

Microstructural analysis of gelatins texture by cryo-scanning electron microscopy (cryo-SEM)

Microscopy/Cryo-SEM of various gelatin gels was carried out to investigate the gel microstructure. Cryo-SEM images (Fig. 6) showed that commercial bovine hide gelatin had a honeycomb structure with thin stranded protein network and large number of interconnected pores. These pores were very small and uniform (Fig. 6a). However, all

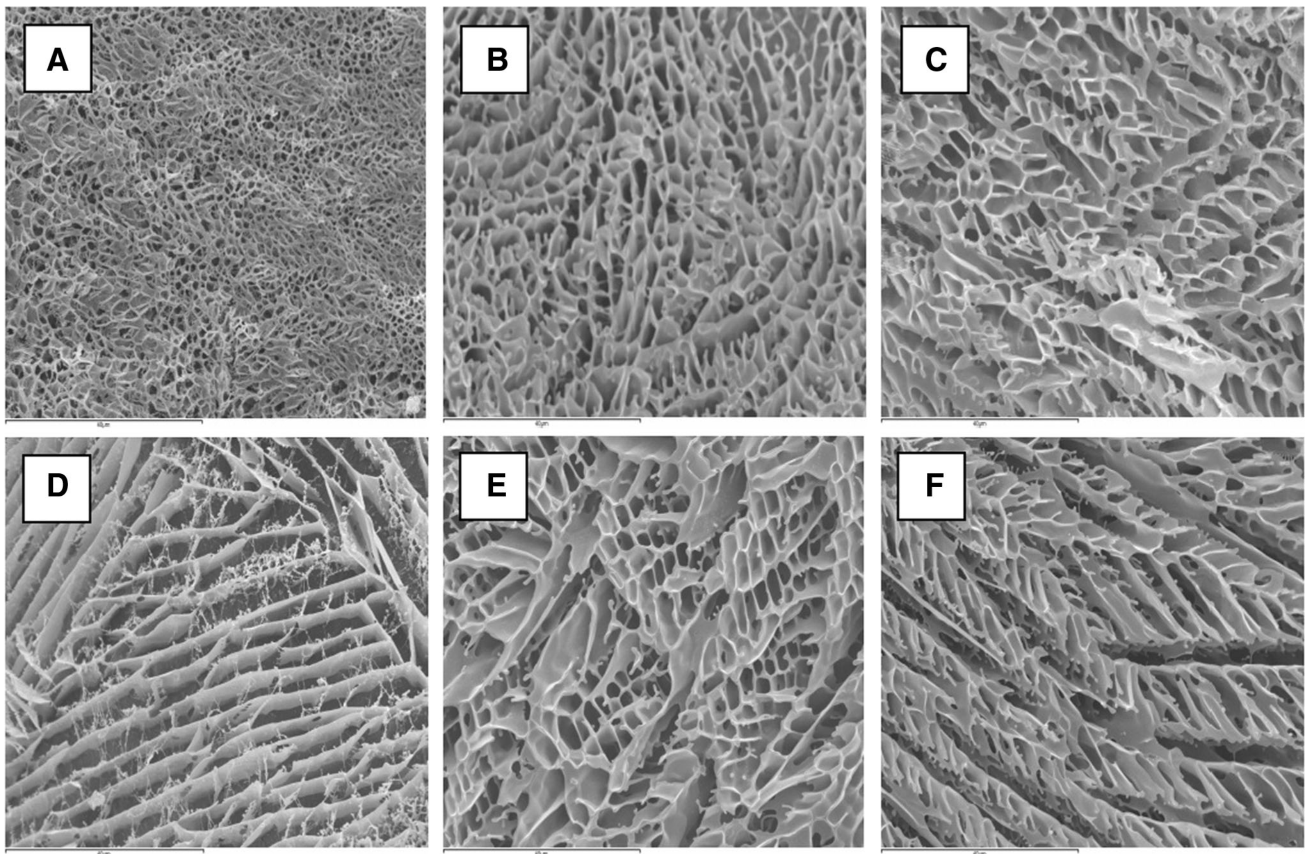

Fig. 6 Representative cryo-SEM micrographs (×1500) of gelatin gels. Commercial bovine hide gelatin (**a**), gelatin from mackerel skin pre-treated with acetic acid (**b**), citric acid (**c**), lactic acid (**d**), malic acid (**e**) and tartaric acid (**f**)

extracted mackerel skin gelatins showed larger voids indicating a relatively weaker gel nature since the higher the number of small interconnected pores the stronger the gel [48].

Mackerel skin gelatins extracted after pre-treatment with acetic, citric and malic acid (Fig. 6b, c, e, respectively) showed a higher number of interconnected small pores than mackerel skin gelatins extracted after pre-treatment with tartaric and lactic acid (Fig. 6d, f, respectively). The Cryo-SEM results showed that the microstructures were highly related to the gel strength values, where denser strands (gelatins extracted from mackerel skins after pre-treatment with acetic, citric and malic acid) represented higher gel strength than the looser strands such as in the case of mackerel skin gelatins pre-treated with tartaric and lactic acid.

Conclusion

The rheological, textural and microstructural properties of mackerel skin gelatins were affected by the organic acid used in the extraction process. The differences among gelatins were related to the imino acids (proline and hydroxyproline) level of gelatins. The use of tartaric and lactic acids resulted in gels with poor rheological properties and weak network structure. Acetic, citric and malic acid pre-treatment of mackerel skins produced stronger and more stable gels making them possibly useful in various food applications.

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