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Influence of extraction variables on the structure and physical properties of salmon gelatin

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ABSTRACT

This work focuses on investigating the effect of extraction conditions (pH and time) on the biochemical and physical properties of salmon gelatin (SG).

SG was extracted from salmon skins under different pH and time conditions at 60 °C. The characterization of the material considered proximate composition, amino acid profile, molecular weight (MW), gel strength, X-Ray diffraction, thermal properties, dynamic mechanical properties and dynamic vapour sorption analysis.

Results showed that higher protein content was obtained with extraction condition pH5/2h, while lower protein content was obtained at condition pH3/5h. Extraction performed at pH5 produced SG with MW > 120 kDa, while processing condition at pH3 resulted in MW bands distributed between 20 and 100 kDa. Higher contents of proline and hydroxyproline were detected in SG with high MW. This behaviour was directly correlated with gel strength and thermo-mechanical properties: higher gel strength and E' modulus were observed in SG with high MW, suggesting higher amount of triple helical structures in gelatin matrix. This was also supported by higher values of tanδ detected as the MW of SG decreased. This may be related with decreasing the crystalline fraction of SG. Thermal properties revealed no significant differences in melting temperature and glass transition temperature values among samples. The melting energy was significantly lower for SG with lower MW. This was confirmed by X-Ray diffraction where the intensity of the diffraction peak at ~$2\theta = 8^{\circ}$ significantly decreased for SG extracted under more aggressive conditions. Finally, gelatin extracted under mild conditions showed higher moisture content, which was in agreement with higher amounts of triple-helix structures.

Our results suggest the possibility to modulate the physical properties of SG by tuning the extraction process to obtain tailored gelatin structures for high-value applications in food technology, tissue engineering and biomedicine.

1. Introduction

Salmon production is one of the most important areas to aquaculture industry. In according with reports published on 2015, Norway and Chile are the main worldwide salmon producers with 1.400.000 and 570.000 tonnes, respectively, of which ~85% corresponded to Atlantic salmon (SalmonChile, 2016; Statistics Norway, 2016). One of the most important by-products generated by the salmon industry is fish skin. It has been estimated to be ~5% of whole mass of the fish (Transparency Market Research, 2013). Currently, this by-product is mainly transformed for the production of fish flour, which is a low value commercial product destined as protein source for animal feeding.

A classical approach to add-value for fish processing by-products is based on the development of extraction procedures to obtain certain molecules with biological relevance, such as collagen and gelatin derivatives. Gelatin is a very versatile soft material with a wide range of industrial applications. Although gelatin has been

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used for many years in the food and pharmaceutical industries, gelatin presents high potential to develop high-value commercial products in fields still not well explored. Owing to the ability of salmon gelatin to flow at room temperature, it is a biomaterial that presents high potential to be used in the development of edible coatings to extend the shelf life of fresh meat products or in the development of capsules to protect labile bioactive nutrients (fatty-acids, vitamins, probiotics and nutraceuticals). But interestingly, a relatively new research area is related to the use of gelatin for biomedical applications, such as the design and development of polymer cell scaffolds for wound healing and tissue engineering, as well for designing novel bio-inks for 3D printing.

Gelatin is obtained through the hydrolysis of collagen fibbers extracted from skin, cartilage, bones and/or hair of animals (Karim & Bhat, 2009). The gelatin extraction protocols typically include the use of acid or alkaline chemicals for hydrolysis at relative high temperature (50-80 °C). This has been found to significantly affect the molecular weight and isoelectric point of gelatin (Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011; Joly-Duhamel, Hellio, & Djabourov, 2002; Karim & Bhat, 2009). The origin or source of the gelatin also defines its properties. Gelatin from warm-blooded animals (eg: cattle, pork) shows different amino acid profile and gel strength in comparison to cold-water fish gelatin (Joly-Duhamel et al., 2002). Cold-water fish gelatin presents lower concentration of imino acids (proline and hydroxyproline) and lower molecular weight distribution compared to gelatin from mammalian origin. As a consequence, cold-water fish gelatin shows marked differences with respect to thermal, rheological, viscoelastic and mechanical properties when it is compared to mammalian and warm-water fish gelatin (Aguirre-Álvarez, Foster, & Hill, 2012; Díaz, López, Matiacevich, Osorio, & Enrione, 2011; Elharfaoui, Djabourov, & Babel, 2007; Eysturskard, Haug, Ulset, Joensen, & Draget, 2010; Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009; Haug, Draget, & Smidsrød, 2004; Joly-Duhamel et al., 2002).

Another reason to consider gelatin from marine sources is due to cultural and religious aspects, as some specific or well-informed customers (e.g. Halal, Kosher, etc) will not consider using or eating products containing pork or cattle gelatin. Moreover, the use of bovine gelatin is still somehow controversial due to the Bovine Espongiform Encephalopathy (BSE) outbreak that occurred in the 90's (Gomez-Guillen et al., 2011; Karim & Bhat, 2008; Karim & Bhat, 2009).

In the literature many studies report different extraction protocols for fish gelatin (Table 1). These studies, however, are mainly focused in the optimization of extraction yield rather than obtaining a gelatin with well-defined and standardized properties. Therefore, thinking about a biomaterial specially designed for high-value industrial applications such as 3D printing, cell scaffolds, soft capsules for labile compounds and edible food coatings, controlling the structure of salmon gelatin is particularly relevant in order to tune its physical properties for each specific application.

Hence, the aim of this work was to study the effect of the extraction conditions (pH and time) of gelatin from salmon skin on its biochemical profile and physical properties. This work is part of comprehensive study oriented to design a biomaterial with tailored properties for high-value industrial applications.

2. Materials and methods

2.1. Salmon gelatin extraction

Salmon gelatin was extracted from Atlantic salmon (*Salmo salar*) skins following the protocol proposed by Zhou & Regenstein (2004) with some modifications (Díaz et al., 2011). The salmon skins were first cleaned in order to eliminate all residues of muscle and scales and then cut into squared pieces of ~2 cm^2. A series of pre-treatments were carried out by submerging the square of skin in a 0.1 M solution NaOH, which were subsequently stirred at a constant speed at 10 °C for 1 h. Then, the skins were washed with distilled water and the process was repeated once again at the same conditions as before. After washing, the pieces of salmon skins were submerged into a 0.05 M acetic acid solution and stirred at a constant speed at 10 °C for 1 h. Then the gelatin extraction process was carried out under different pH and time conditions at a temperature of 60 °C as shown in Table 2. The supernatant liquid was subsequently vacuum filtered using paper filters (22 μm, Whatman, Merck, Darmstadt, Germany) and dried in an oven at 55 °C during 24 h. The dried gelatin obtained was grounded (KN195 Knifetec, FOSS Analytical Co. Ltda., China) and stored at 5 °C until further use.

2.2. Preparation of salmon gelatin films

Salmon gelatin films were prepared by cold casting method from salmon gelatin suspensions (7% w/v) previously held at 60 °C during 40 min. Gelatin solutions were subsequently poured into rectangular teflon moulds and maintained at 5 ± 1 °C for 7 days to obtain flat and transparent films with a final thickness of 0.03 cm. The films were then cut to dimensions of 8.5 cm length and 1 cm width and maintained in P_2O_5 for 7 days. The dried films were then equilibrated at 20 °C in desiccator jars under relative humidity of 44% using saturated solutions of K_2CO_3, until equilibrium was reached (difference between consecutive mass weighing lower than 0.05%). The moisture content of equilibrated samples was determined by oven drying at 105 °C for 24 h.

2.3. Proximate composition of films

Proximate analysis of gelatin samples was assessed according to AOAC methods (AOAC, 2012), which considered moisture content (oven drying at 105 °C, 24 h), fat content (solvent extraction by Soxhlet method), protein concentration (Kjeldhal method, %N x 5.55) and ash content (oven heating at 550 °C). Non-nitrogenous fraction was determined by difference, which is calculated subtracting to 100% the total sum of the other components (in percentage) present in sample.

2.4. Amino acid profile

The concentration of different amino acids present in each salmon gelatin samples was determined by reverse-phase high-performance liquid chromatography (HPLC-RP) as previously reported (Rebane & Herodes, 2010). Briefly, 10 mg of sample was hydrolysed with a solution of 6 N HCl at 110 °C for 24 h. The hydrolysate obtained was derivatised with 20 μL of phenylthiocyanate (10% w/v) to generate phenylthiocarbamyl amino acids, which were separated and quantified by HPLC-RP at 254 nm. A liquid chromatograph (Waters 600 controller, Massachusets, USA) with a diode array detector (Waters 996) and a Phenomenex (Los Angeles, California, USA) Luna RP18 column (150 mm × 4.6 mm, particle size 5 μm) was used. Gradient separation was performed using two solvents: (A) 0.14 M anhydrous sodium acetate (pH 5.9)/acetonitrile (94:6 v/v) solution and (B) HPLC-grade acetonitrile/water (60:40 v/v) solution. The injection volume was 20 μL, the column temperature was 40 °C and the run time was 30 min. Amino acid quantification was carried out using external standards of each analysed amino acid (Sigma-Aldrich, Steinheim, Germany). Amino acid content of salmon gelatin samples was reported as $g/100g_{protein}$.

Table 1
State of the art of fish gelatin extraction protocols reported in the literature during the last ten years.

Reference	Fish specie	Main conclusion
(Badii & Howell, 2006) {Badii & Howell, 2006 #5}	Horse mackerel	Aminoacid content and α-helix structure influence directly on gel strength. Also, a synergistic effect was observed with ovalbumin on gel strength.
(Chiou et al., 2006)	Allaska pollock and Allaska pink salmon	Crosslinking ratio in fish gelatin using genipin and glutaraldehyde is favored at high pH. However, gel strength measured after five days is lower in cross-linked fish gelatins in comparison with pig gelatin modified using the same cross-linkers.
(Arnesen & Gildberg, 2007)	*Salmo salar*	Both salmon and cod gelatin show similar physical properties (viscosity, aminoacid profile and gel strength). Main difference is the lower serine content in salmon gelatin.
(Carvalho et al., 2008)	Atlantic halibut	Gelatin with good film forming capacity. Sorbitol presence allows obtaining more extensible films in gelatin with higher amount of low molecular weight fractions.
(Eysturskard, Haug, Elharfaoui, et al., 2009)	Cod, haddock and pollack	Mechanical properties in gelatin (modulus and Bloom values) are directly related with molecular weight and they can be improved when low molecular weight fractions of gelatin are discarded.
(See et al., 2010)	Warm water fish (snakehead, catfish and red tilapia)	Acid-alkaline treatment allowed obtaining high extraction gelatin yield. The obtained gelatin had a gel strength similar to gelatin obtained from mammals, but higher than gelatin obtained from cold water fish.
(Ahmad & Benjakul, 2011)	Unicorn leatherjacket	Higher gel strength gelatin is obtained when phosphoric acid is used instead of citric acid. Processing time is critical to determine the gelatin chain size.
(Uriarte-Montoya et al., 2011)	Squid	A "cold ripening" process (4 °C, 2 days) increases the gelatin extraction yield. Gelatin obtained may be used as alternative source of functional compound for application in foods.
(Jeya Shakila et al., 2012)	Red snapper and grouper	Gelatin obtained with high viscosity, gel strength and water holding capacity that can be used as an alternative to mammal gelatin.
(Niu et al., 2013)	Tilapia	Different acids were tested to optimize the yield extraction of gelatin. No significant difference was observed among acids.
(Weng et al., 2014)	Tilapia	Gelatin extracted under different pH conditions (3–9). No significant differences in permeability and transparency values were observed. Gelatin with higher mechanical properties were obtained with less aggressive treatments (pH 5).
(Nikoo et al., 2014)	Amur sturgeon	The extraction protocol was optimized in terms of yield extraction and gel strength.
(Jakhar, Basu, Sasidharan, Chouksey, & Gudipati, 2012)	Blackspotted croaker	This fish skin is a good source for obtaining gelatin with higher yield extraction, and with gel strength and melting temperature similar to commercial mammal gelatin.
(Sinthusamran, Benjakul, & Kishimura, 2014)	Seabass	The extraction process was optimized in terms of gel strength, concluding that a process conducted at 50 °C during 3h allows obtaining a gelatin with mechanical properties equivalent to bovine or pig commercial gelatin.
Hanjabam et al. (2015)	Unicorn leatherjacket	An extraction methodology was optimized which allowed obtaining gelatin with gel strength and melting temperature similar to commercial gelatin.
See, Ghassem, Mamot, and Babji (2015)	African catfish	A methodology was optimized in terms of pre-treatment allowing improved protein recovery, gel strength, viscosity and both melting and gelling temperatures.
(Jridi et al., 2015)	Octopus	The use of pepsin in extraction process was tested. It was observed lower gel strength and melting temperature, and emulsion and foaming forming properties were lowered when pepsin was used.
(Tang et al., 2015)	Tilapia, Grass carp and Silver carp	Tilapia showed better physical-chemical properties, higher viscosity and higher film forming properties than other specimens tested, which could be related with differences at conformation and primary structure of tilapia gelatin.
(Abdelmalek et al., 2016)	Squid	An enzymatic process with higher yield extraction was designed. Gelatin obtained showed good emulsion and foam forming properties and good water holding capacity.
(Sae-leaw, Benjakul, & O'Brien, 2016)	Seabass	The use of citric acid, isopropanol and tannic acid were effective to prevent lipid oxidation and volatiles compounds formation, which can modify sensory properties of gelatin.
(Kittiphattanabawon, Benjakul, Sinthusamran, & Kishimura, 2016)	Clown featherback	Properties of gelatin obtained are dependent of temperature and time used. Process at 45 °C during 6–12h allows obtaining gelatin with equivalent properties (or even better) than commercial bovine gelatin.
(Chen et al., 2016)	Tilapia	Sub-units α-1 have important role during formation of helical structures and mechanical properties of gelatin.
(Huang, Kuo, Wu, & Tsai, 2016)	Tilapia	Description of new collagen extraction process based on hydro-extrusion technique which allows obtaining a biomaterial with high physical-chemical functionality.

2.5. Molecular weight

The molecular weight (MW) of salmon gelatin samples was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), performed as reported before by Laemmli (1970) using a 7.5% separating gel and a 4% stacking gel (292 g/L acrylamide, 8 g/L *N,N*-bis-methylene-acrylamide). A broad-range SDS-PAGE MW standard (Bio-Rad, Philadelphia, USA) was used as MW marker. All samples were heated at 99 °C for 6 min prior to loading (200 μg of protein). The electrophoresis (EC 250-90, Equilab, Santiago, Chile) was run at 100 mA (0.75 mm thickness). Gels were stained with 1.25 g/L Coomassie Blue R-250 (Sigma-Aldrich, Steinheim, Germany) in methanol/acetic acid/water (50:10:40 v/v/v) solution (Merck, Darmstadt, Germany) and destained in methanol/acetic acid/water (20:10:70 v/v/v) solution.

Table 2
Extraction conditions (pH and time) used in this study. Treatments are presented from most severe to mildest conditions and this nomenclature is held in all the text.

Treatment	pH	Time (h)
A	3	5
B	3	2
C	4	3.5
D	5	5
E	5	2

2.6. Gel strength

The gel strength of salmon gelatin samples was determined following the method reported by Wainewright (1977). The salmon gelatin gels (6.67% w/v) were prepared in Bloom jars (150 mL,

Stable Micro Systems, UK) by dissolving dry salmon gelatin in distilled water at 60 °C during 20 min and after holding the suspension at 40 °C during 40 min. The prepared suspension was held in an incubator at 4 °C (aging temperature) for 16–18 h. Gel strength was assessed on a texture analyser TA.XTplus (Stable Micro Systems, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, and equipped with a R1.27-cm-diameter cylindrical probe. The maximum force (in g) was determined when the probe penetrated a distance of 4 mm into the gelatin gels.

2.7. Powder X-ray diffraction

The molecular structure of salmon gelatin films was analysed using a D8 Advance powder X-Ray diffractometer (Bruker, UK). The X-ray generator was equipped with a copper tube operating at 40 kV and 30 mA and producing CuKα radiation of 0.156 nm wavelength. The experimental settings were an incident angle 2θ from 5° to 40° at an angle step of 0.02° per 0.1 s. The rotational speed of the sample holder was set to 60 rpm. The obtained patterns were subtracted by the holder spectra and baseline corrected over the measurement scanning angles using the software OriginPro 8 SR0 V8.0724 (BT24, USA). Films with an average thickness of 0.03 cm were selected and analysed to limit the effect of thickness on the powder X-ray diffraction patterns.

The amount of triple helix configuration X_c in percentage in the salmon gelatin films was calculated using equation (1).

$$X_c = \frac{A_c}{A_c + A_a} \times 100 \quad (1)$$

where A_c is the area of the diffraction peak located at ~2θ = 8° corresponding to the triple helix configuration in the salmon gelatin films (Badii, MacNaughtan, Mitchell, & Farhat, 2014; Fadel, Hassan, & Oksman, 2012). A_a is the area under the hump located at ~ 2θ = 21°, corresponding to the fraction of amorphous material in the salmon gelatin films. The integration was performed using OriginPro 8 SR0 V8.0724(BT24, USA). All the samples were analysed in duplicate.

2.8. Thermal properties

~20 mg of salmon gelatin film pieces were loaded into aluminium pans of 40 μL and subjected to thermal scans using a DSC-1 (Mettler-Toledo, Switzerland). Prior to performing the measurements, the DSC was calibrated using indium (melting temperature of 156.6 ± 1.56 °C and melting enthalpy of ΔH = 28.6 ± 1 J/g). The reference used during the analysis was an empty pan. All experiments were performed in triplicate using the following thermal profile: cooling down from 25 °C to −40 °C at 40 °C/min, holding at −40 °C for 5 min, heating up from −40 °C to 120 °C at a heating rate of 10 °C/min, holding at 120 °C for 5 min, cooling down to −40 °C at 40 °C/min, holding at −40 °C for 5 min and heating up again up to 120 °C at 10 °C/min. The transition temperature (Tm) related to melting of gelatin was determined as the onset of the endothermic peak observed in the first heating scan. The energy associated to helix to coil transition (melting energy) defined as the change in enthalpy (ΔH) was calculated from the area under their corresponding endothermic peak and expressed as a function of dry gelatin mass. The glass transition temperature (Tg) value was obtained from the second heating scan and defined as the midpoint of the change in heat capacity.

2.9. Dynamic mechanical properties

The mechanical properties of salmon gelatin films under dynamic conditions were determined using a DMA-1 instrument (Mettler-Toledo, Switzerland). The gelatin film samples were cut into strips with typical size ~2 cm length, ~1 cm width and ~0.03 cm thickness. The strips were covered with silicone oil (Dow Corning, USA) to avoid moisture loss during analysis. The instrument was used in tension mode and a temperature scan from −100 °C to 120 °C at a heating rate of 3 °C/min was used. The experiments were performed using a frequency of 10 Hz. At least five replicates were measured for each sample. Average and standard deviations are reported.

2.10. Sorption properties

Water sorption isotherms were obtained using a dynamic vapour sorption system (Intrinsic DVS, Surface Measurements Systems, USA).

~35 mg of grounded gelatin films with diameter ~150 μm, previously stored over P_2O_5 for one week, was loaded in to the DVS basket. The programmed cycle of equilibrating relative humidities was from 0 to 90%, with 10% increments (10 points) in between. The temperature was set to 20 °C. The samples were considered to be at equilibrium when the value dm/dt (slope of the changing in mass with time) was set to be < 0.0005 mass %/min.

If the sorption equilibrium was not reached within the experiment time-scale (8 h), an exponential function was used to extrapolate the moisture content at time equal to infinity. A detailed description of this equation has been reported before (Roman-Gutierrez, Guilbert, & Cuq, 2002). The accuracy of the fitting was evaluated by the application of a mean relative error as described by Coupland, Shaw, Monahan, O'Riordan, & O'Sullivan (2000).

2.11. Statistical analysis

If pertinent, the statistical significance was assessed by a paired *t*-test (same variances) using the Solver tool in Excel (Office 2010, Microsoft Corp.). Pearson linear correlations ($p < 0.05$) were calculated using the Statgraphics Centurion XVI (StatPoint Inc., Rockville, MD, USA).

3. Results and discussion

3.1. Proximate composition

The results of proximate composition of salmon gelatin samples obtained using different pH and time extraction conditions are reported in Table 3. It is evident that the extraction conditions have a strong effect on the salmon gelatin composition, where the protein content significantly increases when the extraction conditions are milder, in other words the highest protein content was obtained for the salmon gelatin extracted at pH 5 (95.0 g/100 g after 5 h and

Table 3
Proximate composition of salmon gelatin samples obtained through different extraction conditions tested in this study.

Component	$g/100g_{wet\ sample}$				
	pH3/5h	pH3/2h	pH4/3.5h	pH5/5h	pH5/2h
Moisture	6.6	5.2	3.8	4.8	3.0
Protein*	81.1	85.2	92.4	95.0	95.9
Fat**	–	–	–	–	–
Ash	0.4	0.5	0.6	0.2	1.1
Non-nitrogenous fraction	11.9	9.1	3.2	–	–

*%N x 5.5.
**Detection limit <0.52 g/100 g.

95.9 g/100 g after 2 h). The lowest protein content was obtained for the salmon gelatin extracted at pH 3 during 5 h (81.1 g/100 g). These results are in agreement with the study carried out by Weng, Zheng, and Su (2014) who reported a lower protein content (~78%) when the gelatin extraction from tilapia scales was performed at pH 3 compared to gelatin extracted at pH 5 and pH 9 (89–90%). Possibly the use of excess acid during the gelatin extraction over-hydrolyses collagen molecules causing the loss of recoverable protein during the process (Jamilah & Harvinder, 2002; Niu et al., 2013). The protein content reported in our study is in the same range previously reported in the literature for fish gelatin (78–95 g/100 g) (Jeya Shakila, Jeevithan, Varatharajakumar, Jeyasekaran, & Sukumar, 2012).

The amount of non-nitrogenous fraction was higher in the salmon gelatin extracted at pH 3 (11.9 g/100 g after 5 h, and 9.1 g/100 g after 2 h). This fraction could include aldehydes and other carbonyls compounds obtained by deamination of free aminoacids and gelatin peptides generated during the hydrolysis process at low pH (Voet & Voet, 2010). Moreover, in this study both moisture and ash content did not show a clear trend as a function of extraction condition, whilst the results suggest that fat content was under the detection limit of analytical technique used for all samples analysed. These results suggest a significant effect of both pH and time used during the extraction process on the proximate composition of salmon gelatin.

3.2. Aminoacid profile

The results corresponding to the determination of the amino acid profile by HPLC-RP of salmon gelatin samples are reported in Table 4. Although the protein content showed direct correlation with extraction conditions (Table 3), our results showed that extraction method did not modify significantly the amino acid profile among gelatin samples. Similar results have been reported previously by Weng et al. (2014), whom reported only slight differences in the amino acid profile of tilapia gelatin extracted at different pH conditions. Similar results were also reported by Arnesen and Gildberg (2007) when comparing the amino acid profile of gelatin obtained from Atlantic salmon (*Salmo salar*) and cod. For both salmon and cod gelatin, that measurement was carried out after an acid-based extraction process performed at 56 °C or 65 °C. They stated that the amino acid compositions were identical regardless the extraction temperature. According to Arnesen and Gildberg (2007) the extent and thermostability of intermolecular crosslinks must be an essential factor influencing the extractability of gelatin from skin tissues. However, it is not a key factor in terms of amino acid composition.

As expected, the amino acids present in the highest concentrations were glycine, proline and hydroxyproline. Interestingly, it can also be observed a high concentration of charged amino acids (arginine, glutamic and aspartic acid, lysine), representing almost 30% of the total amount of amino acids. The presence of a relatively high amount of charged amino acids may play an important role in the intra and intermolecular interactions that take place during the formation of triple-helix structures after thermal unfolding and further cooling below gelling temperature (Acevedo, Díaz-Calderón, López, & Enrione, 2015).

3.3. Molecular weight

The molecular weight distribution of salmon gelatin samples assessed by SDS-Page electrophoresis is shown in Fig. 1. The extraction conditions showed a strong effect on the molecular weight distribution of gelatin. When extracted at pH 3, the salmon gelatin has a wider distribution in molecular weight in the range of 20–100 kDa, which is closely related to presence of smaller gelatin fragments obtained from the hydrolytic process. In the case of the gelatin extracted at pH 5, it shows polymer fragments mainly distributed above 100 kDa (Fig. 1). Both salmon gelatin extracted at pH5 (2 and 5 h) showed similar molecular weight distribution, with well-defined bands at ~120 kDa and others higher than 245 kDa. Presumably, bands of ~120 kDa correspond to α-chains of the salmon gelatin while bands >245 kDa should correspond to β-chains. Indeed, the molecular weight of α-chains in fish gelatin has been reported to be in the range of 100–120 kDa and for β-chains in the range of 200–250 kDa when it was extracted from cod, tilapia, megrim, red snapper, grouper, grass carp, catfish and Alaska Pollock (Arnesen & Gildberg, 2007; Eysturskard et al., 2010; Gudmundsson, 2002; Jeya Shakila et al., 2012; Weng et al., 2014; Zhang, Xu, & Wang, 2011; Zhou & Regenstein, 2004) under acid conditions. Specifically to *Salmo salar* gelatin Díaz et al. (2011) reported molecular weights of ~95 kDa and ~195 kDa associated to α and β-chains respectively, whereas Arnesen and Gildberg (2007) reported values of ~110 kDa for α-chains and >200 kDa for β-chains.

The extraction process carried out at pH 4 for 3.5 h showed a similar pattern. Less intense bands at ~120 kDa, ~245 kDa and

Table 4
Amino acid content (g/100$g_{protein}$) of salmon gelatin samples obtained through different extraction conditions.

Aminoacid	g/100$g_{protein}$				
	pH3/5h	pH3/2h	pH4/3.5h	pH5/5h	pH5/2h
Alanine	9.0	8.8	8.9	8.9	8.8
Arginine	8.9	9.2	9.2	9.1	8.9
Aspartic acid	7.6	7.7	7.2	7.3	5.2
Glutamic acid	11.9	11.8	11.5	11.6	10.6
Glycine	25.5	25.4	25.4	25.6	27.9
Histidine	0.7	0.8	0.8	0.8	0.7
Hydroxyproline	7.2	8.3	8.2	8.1	8.6
Isoleucine	1.2	1.1	1.1	1.1	0.9
Leucine	2.2	2.1	2.1	2.1	2.2
Lysine	4.5	4.2	4.4	4.5	4.4
Metionine	1.9	1.9	1.8	1.8	1.9
Phenylalanine	1.9	1.8	1.9	1.8	1.8
Proline	9.8	9.6	9.7	9.7	10.6
Serine	4.4	4.1	4.3	4.3	4.4
Threonine	2.0	1.9	2.0	2.0	1.9
Tyrosine	–	–	0.1	–	–
Valine	1.3	1.3	1.4	1.3	1.1

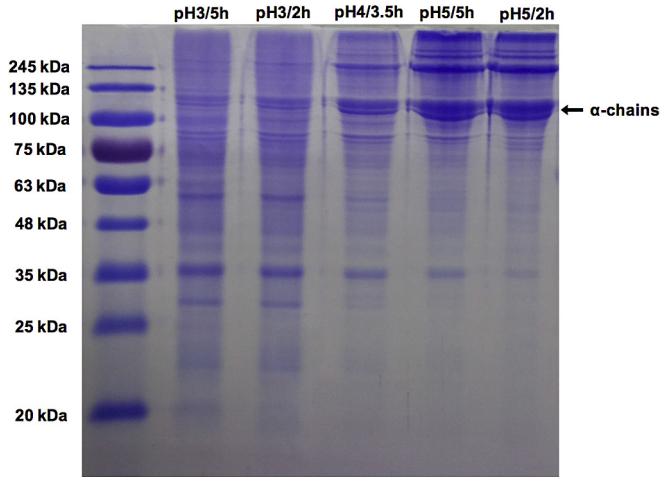

Fig. 1. SDS-Page electrophoresis of salmon gelatin samples obtained through different extraction conditions.

above were observed while some additional bands appeared corresponding to molecular weights of ~55 kDa and ~35 kDa. The gelatin obtained under the most severe extraction condition (pH 3, 2 or 5 h) showed a quite different pattern, lacking bands corresponding to molecular weights >245 kDa. Only very clear bands associated to molecular weight of ~120 kDa were observed. In addition, a series of intense bands corresponding to molecular weights of ~55 kDa, ~35 kDa and ~30 kDa were detected. This is evidence of the hydrolytic effect occurring under more severe extraction conditions. Indeed, it is clear in Fig. 1 that bands corresponding to molecular weights >245 kDa faded away as the extraction process was more aggressive (low pH and longer time) while bands corresponding to low molecular weight (~35 kDa) were more intense. These bands, related to the reference molecular weight of ~35 kDa, would reflect the presence of gelatin oligopeptides or subunits of the α-chains (Eysturskard et al., 2010). These results demonstrate the key role of pH in controlling the molecular weight distribution of the extracted gelatin. In this regard, in order to improve the functional quality of the gelatin produced, Zhang et al. (2011) have stated the importance of controlling the pre-treatment and extracting processes to reduce the production of degraded peptides and higher molecular weight aggregates.

3.4. Gel strength

The effect of the extraction pH and time on the gel strength (Bloom) of salmon gelatin gels is showed in Fig. 2. Low pH and long extraction time significantly decrease the gel strength. Interestingly the results suggest that the gel strength is more influenced by extraction pH than extraction time. The highest value of gel strength was obtained for salmon gelatin extracted at pH 5, whereas the lowest gel strength value ($p < 0.05$) was reached at pH 3. This result highlights the significance of molecular weight of the gelatin chains on gel strength. This observation has been previously reported in the literature, where it has been widely stated that differences in gel strength may be governed by molecular weight distribution (Ockerman & Hansen, 1988) as well as by complex interactions governed by the imino acid composition, the ratio α/β chains present in gelatin (Ahmad & Benjakul, 2011; Gómez-Guillén et al., 2002); Ahmad & Benjakul, 2011; Giménez, Turnay, Lizarbe, Montero, & Gómez-Guillén, 2005; Gómez-Guillén et al., 2002) and the higher content of free hydroxyl group amino acids (Arnesen & Gildberg, 2007; Jeya Shakila et al., 2012). Free hydroxyl groups play an important role in the generation of hydrogen bonds and helical structures during the storage gel strengthening (Arnesen & Gildberg, 2007) and its content depends on factors such as amino acid composition, size of protein chains and gelatin concentration (Muyonga, Cole, & Duodu, 2004b; Muyonga, Cole, & Duodu, 2004a). The triple helix formation takes place by association of the different α-chains during cold maturation of the gel. It has been suggested that longer gelatin chains can participate in longer and/or more frequent triple helices, leading to an overall fractional increase in the helix amount (Eysturskard, Haug, Ulset, & Draget, 2009). This resulted in an increase in mechanical properties, since the gel strengthening has been attributed to the regeneration of helical structures between gelatin peptides chains and formation of hydrogen bonds between hydroxylated amino acids and water molecules (Haug et al., 2004). Indeed, a linear relationship between gel strength and the triple helix content in gelatin has been previously reported (Bigi, Panzavolta, & Rubini, 2004). Therefore, the higher gel strength obtained would be related to molecular weight bands distributed above 100 kDa (Fig. 1), protein concentration and hydroxyproline contents (Tables 3 and 4, respectively). Additionally, Pearson correlation analysis revealed that protein content and both glycine and proline content significantly contributed ($p < 0.05$) to the gel strength ($r = 0.95$, 0.92 and 0.92, for protein, glycine and proline content respectively).

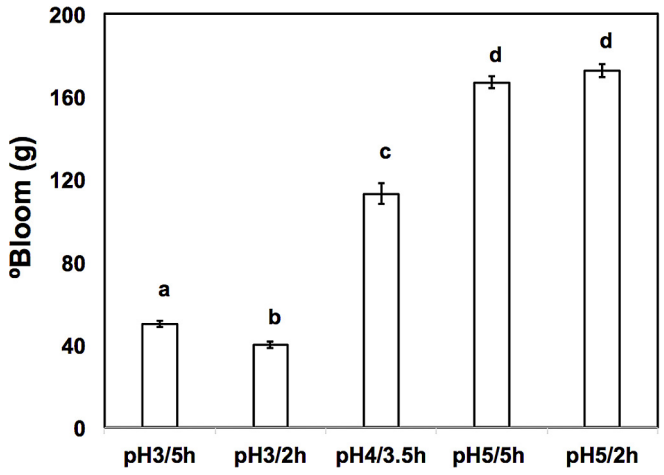

Fig. 2. Gel strength (Bloom) of salmon gelatin samples obtained through different extraction conditions. Different lowercase letters show significant differences between the extraction conditions ($p < 0.05$).

For the salmon gelatin extracted at pH 3, the gel strength was higher in the samples obtained from a 5 h extraction process compared to samples obtained from a 2 h extraction process. This rather unexpected result suggests that the gel strength would not only be governed by molecular weight distribution. Similar considerations have been established in the literature (Eysturskard, Haug, Elharfaoui, Djabourov, & Draget, 2009; Eysturskard, Haug, Ulset, et al., 2009). In those studies, the authors reported that higher polydispersity index (and thus a different molecular weight distribution, as in our work) as well as differences in isoelectric point may lead to a less functional gel network and consequently to lower Bloom values. Also it has been reported that gel strength depends on pH (Gudmundsson & Hafsteinsson, 1997), with more compact and stiffer gels formed by adjusting the pH of the gelatin close to its isoelectric point, where the protein chains will be less electrically charged and thus the gelatin strands would be closer to each other. Therefore, differences in gel strength also could be explained from differences in terms of z-potential and isoelectric point addressed by different extraction conditions.

The wide range of gel strength values reported for similar concentrations of gelatin hydrogels obtained from various animal sources are related to differences in proline and hydroxyproline content (See, Hong, Ng, Wan Aida, & Babji, 2010). The latter has been associated with the temperature of the habitat where the animal develops and live (Karim & Bhat, 2008). A gel strength of 108 g has been reported for salmon gelatin hydrogels (Arnesen & Gildberg, 2007), which is in agreement with the results obtained in this study for the salmon gelatin extracted at pH 4 for 3.5 h. Another study reported lower Bloom values for gelatin from unicorn leatherjacket when the extraction was carried out for 8 h under acid conditions, in comparison with 4 h extraction under the same conditions (Ahmad & Benjakul, 2011).

3.5. Powder X-Ray diffraction

Fig. 3 shows the X-ray patterns for salmon gelatin extracted through different conditions of pH and time. A broad background with a peak intensity located at ~22° is observed, which is typically related to the amorphous fraction of gelatin (Bigi et al., 2004). The peak located at $2\theta = 9°$ corresponds to the triple helix structure

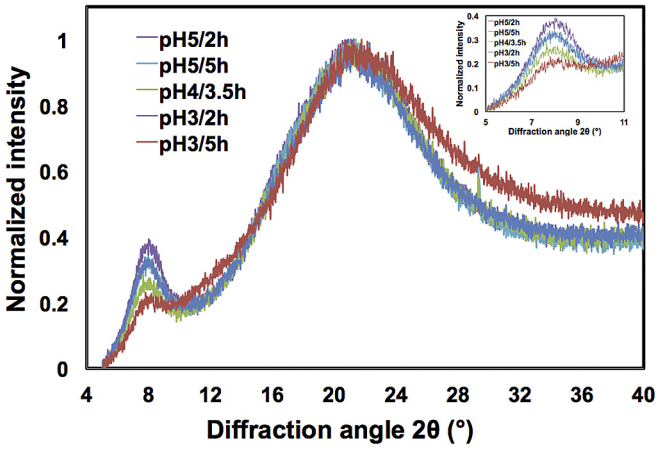

Fig. 3. Powder X-ray diffraction patterns of salmon gelatin samples obtained under different extraction conditions.

present in collagen and renaturated gelatin (Badii et al., 2014; Fadel et al., 2012). Its intensity has been shown to be proportional to the triple helix content of gelatin-based materials (Badii et al., 2014; Bigi et al., 2004; Quero et al., 2015). In the present study, this peak is located at $2\theta = 8°$ as reported in Fig. 3. This slight shift in the diffraction peak could be related to the origin of gelatin or variations in moisture content. A correlation between the intensity of this peak and the extraction conditions is observed. A higher intensity for this diffraction peak ($p < 0.05$) was observed for salmon gelatin samples extracted under the mildest conditions of pH and time. The area under this diffraction peak was also found to be significantly higher ($p < 0.05$) for the gelatin samples extracted under mild conditions. This reflects a higher triple helix content as calculated using Equation (1). As reported in Table 5, the percentage of triple-helix content was found to be higher (8.5 and 9%) for the salmon gelatin extracted at pH 5. Lower percentage of triple helix content (3.1%) were obtained for salmon gelatin obtained under the most aggressive extraction condition (pH 3 for 5 h). However, a rather unexpected result for the gelatin extracted at pH 3 2 h was obtained. A percentage of triple helix configuration of 7.3% in salmon gelatin extracted at pH 3 2 h was estimated. This rather surprising behaviour may result from a local non-homogeneous distribution of ordered and amorphous zones inside the gelatin materials. This result may also reflect that the folding process from random coil to triple helix is influenced not only by the molecular weight distribution but also by other factors including high polydispersity and differences in isoelectric point as previously referenced in Section 3.4. The higher triple helix contents estimated by powder X-ray diffraction analysis in gelatin samples extracted under milder conditions are consistent with results of gel strength previously reported and discussed in our study. Since the samples extracted at pH 5 (2 h and 5 h) have higher triple helix content as reported in Table 5, the gel strength is higher (Fig. 2). The latter would support the importance of the triple helix content on the mechanical properties of gelatin-based materials. Moreover, this data would reflect that triple helix would be strongly influenced by molecular weight distribution and biochemical profile (e.g. proximate composition and amino acid profile). Direct correlation between triple helix content measured by powder X-Ray Diffraction with gel strength have been reported previously in the literature (Badii et al., 2014; Bigi et al., 2004).

3.6. Thermal properties

Table 5 reports the thermal properties of salmon gelatin measured by DSC. This data indicates that the extraction conditions of salmon gelatin have a significant effect on the triple helix melting enthalpy (ΔH, $J/g_{dry\ sample}$), as shown in the first temperature scan. For the most aggressive conditions (pH 3), the melting enthalpy is significantly lower compared to the melting energy observed for gelatin extracted under milder conditions (pH 5). The melting enthalpy is directly proportional to the relative amount of triple helical structures present in the polymer (Achet & He, 1995; Badii et al., 2014). The DSC data is in agreement with both X-ray diffraction data and gel strength results previously discussed in this study. A lower melting enthalpy (ΔH~9–14 $J/g_{dry\ sample}$) reflects a lower triple helix content (Table 5), which results in a gelatin with a reduced gel strength as reported in Fig. 2. Moreover, salmon gelatin extracted under pH 5 for 2 or 5 h show a significantly higher melting enthalpy (ΔH~27 $J/g_{dry\ sample}$), which could be then related to a higher triple helix content and molecular weight (100 kDa) (Fig. 1). Pearson correlation analysis revealed that protein content and gel strength significantly contributed to the melting enthalpy of salmon gelatin ($r = 0.97$ and 0.90, $p < 0.05$, for protein content and gel strength, respectively).

Table 5 also reports values of melting (Tm) and glass transition (Tg) temperatures. The results show that the extraction conditions of salmon gelatin does not significantly affect Tm and Tg. Since the moisture content of all salmon gelatin films was within the same range (11–14%), our results suggest that the thermal properties of salmon gelatin was not affected by its composition or structure, (e.g. proximate composition, amino acid profile, molecular weight distribution, triple helix content). This result is in agreement with a previous study reported in the literature (Bigi et al., 2004). In that work significant differences in melting energy (ΔH) were observed between pigskin gelatin having different Bloom strength, but Tm and Tg remained similar. Marked differences in both Tm and Tg in our samples possibly could be detected at lower moisture contents.

3.7. Dynamic mechanical analysis

Fig. 4 reports the values of storage modulus (E′) obtained from DMA. The results show a similar trend compared with the gel strength values previously discussed. From a temperature of

Table 5
Triple helix content and thermal properties of salmon gelatin films obtained by X-ray Diffraction and DSC analysis, respectively. Values in brackets correspond to standard deviation (n = 3). Within the same column, different superscript lowercase letters show significant differences between the extraction conditions ($p < 0.05$).

Gelatin sample	Moisture content (%, wet basis)	Triple helix content (%)*	Melting temperature (Tm, °C)	Melting energy (ΔH, $J/g_{dry\ sample}$)	Glass transition temperature (Tg, °C)
pH3/5h	11.1 (0.9)^{a}	3.1	76.4 (1.2)^{a}	9.2 (0.5)^{a}	48.4 (0.8)^{a}
pH3/2h	13.8 (0.8)^{b}	7.3	74.4 (0.3)^{a}	13.8 (1.1)^{b}	37.1 (1.1)^{b}
pH4/3.5h	14.3 (1.1)^{b}	5.2	72.5 (0.2)^{b}	27.5 (0.2)^{c}	34.9 (0.2)^{c}
pH5/5h	14.9 (0.9)^{b}	8.5	74.2 (0.3)^{a}	25.6 (0.6)^{c}	38.6 (1.3)^{b}
pH5/2h	14.1 (1.2)^{b}	9.0	75.2 (0.2)^{a}	27.7 (1.4)^{c}	37.1 (0.4)^{b}

*Triple helix content of salmon gelatin films was calculated using Equation (1) from X-ray Diffraction spectra (Fig. 3).

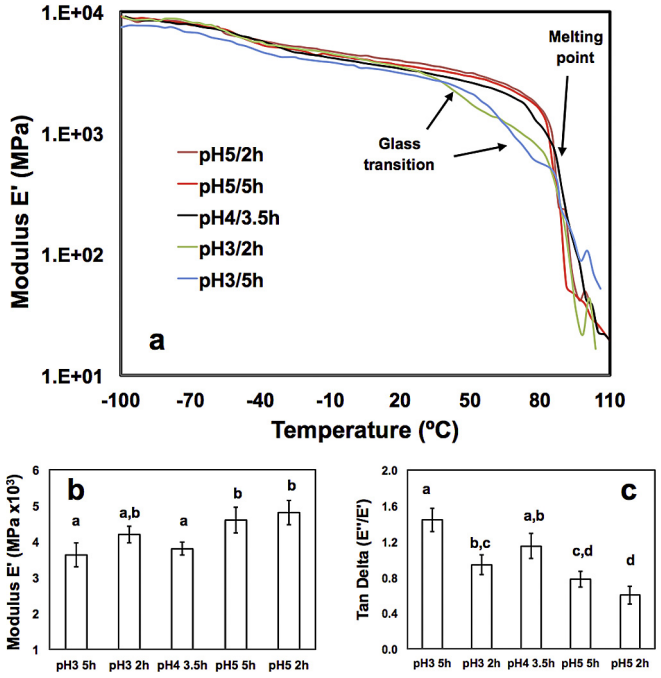

Fig. 4. **(a)** Dynamic mechanical properties (E′, Tan δ, Tg, Tm) of salmon gelatin samples obtained through different extraction conditions. **(b)** Discrete values of Modulus E′ recorded at 4 °C. **(c)** Value of Tan δ peak height associated with melting of gelatin films. In plots **(b)** and **(c)** different letters represent significant differences between extraction conditions ($p < 0.05$).

~−30 °C up to ~80 °C, E′ values were higher in gelatin samples extracted under milder conditions (pH 5) and lower for those obtained under the most aggressive conditions (pH 3). This is more clearly evidenced when value of E′ recorded at a temperature of 4 °C as reported in Fig. 4b. These results are in agreement with previous works reported in the literature, showing that an increase in the triple helix content generate stiffer gelatin films (Chiou et al., 2009; Eysturskard, Haug, Elharfaoui, et al., 2009; Eysturskard, Haug, Ulset, et al., 2009). For example, higher values of elastic modulus were reported for gelatin films cast at low temperatures (4 °C) compared to films cast at 60 °C (Chiou et al., 2009). The authors related this increase to the level of renaturation of gelatin strands during casting process. Another study on fish gelatin described a significant increase in dynamic storage modulus as the average molecular weight increased (Eysturskard, Haug, Elharfaoui, et al., 2009; Eysturskard, Haug, Ulset, et al., 2009). The authors explained this result owing to the ability of longer gelatin chains to participate in more frequent triple helices configuration formation. On the other hand, the unexpected value of the average E′ modulus for the salmon gelatin sample obtained from an extraction process at pH 3 for 2 h could be due to its lower moisture content as reported in Table 6, but also due to differences in the triple helix content (Fig. 3). With respect to thermal transitions, the gelatin extracted at pH 5 and pH 4 showed only one transition detectable from the onset of drop of E′ modulus. This group of samples showed a transition close to 80 °C, which should correspond to the melting point of salmon gelatin films in accordance with the melting temperature previously measured by DSC (Table 5). Interestingly, an additional transition was detected only for the salmon gelatin obtained from more aggressive extraction conditions (pH 3, 2 and 5 h). These transitions correspond to drops of E′ modulus, one of them being close to 40 °C (pH 3, 2 h) and the other close to 50 °C (pH 3, 5 h). Both of them are associated with a peak in tan δ (E''/E'), located at ~42 °C and ~55 °C respectively (data not shown). This result suggests differences in physical state of gelatin matrix between samples due to differences in the extraction conditions. As a result gelatin extracted at pH 5 (2–5 h) and at pH 4 3.5 h only showed the occurrence of a transition related with the melting of the triple helix configuration, presumably because salmon gelatin extracted under these conditions shows higher triple helix content, and hence lower molecular mobility promoted by higher molecular weight of gelatin strands, which is consistent with higher gel strength (Fig. 2), higher triple helix content (Fig. 3) and higher melting energy measured by DSC (Table 5). On the other hand, both gelatin samples extracted under more aggressive conditions (pH 3) display two transitions. The first transition is very likely to be related to the glass transition and the second to the melting of the triple helix configuration present within the gelatin matrix. The Tg and the melting temperatures of the gelatin extracted at pH3/5h were found to be 51.93 °C and 84.47 °C respectively. The Tg and the melting temperatures of gelatin extracted at pH 3 2 h were found to be, 40.02 °C and 79.23 °C respectively. These DMA values are in agreement with the DSC data reported in Table 5. This result suggests the presence of a higher amorphous fraction in the films made with highly hydrolysed salmon gelatin would generate a high degree of molecular mobility, which is consistent with lower melting energy (Table 6) and lower gel strength (Fig. 2). Additionally, a transition close to −50 °C is observed in all salmon gelatin samples, which correspond to thermal transition of silicon oil used to coat the sample during the analysis (data not showed).

Tan δ is also a relevant parameter to assess differences in helical structures between samples. According to the literature, a decrease in tan δ peak value is directly correlated to a reduction in the amorphous fraction present in the system due to possible molecular reordering phenomenon (Romdhane, Price, Miller, Benson, & Wang, 2001) and re-crystallization (Lionetto, Maffezzoli, Ottenhof, Farhat, & Mitchell, 2005). As reported in Fig. 4c, there is a direct correlation between the tan δ peak value associated with the triple helix content and the extraction conditions of salmon gelatin. Consequently, lower values of tan δ peak, corresponding to higher degree of molecular order, are observed in the gelatin extracted under milder conditions (pH 5). This behaviour is in agreement with the gel strength (Fig. 2), triple helix content (Fig. 3) and DSC results reported in Table 6. Pearson correlation analysis showed that E' Modulus is significantly affected by the triple helix content ($r = 0.95$, $p < 0.05$), but also revealed an inverse correlation between tan δ peak value and triple helix content ($r = -0.98$, $p < 0.05$).

Table 6
GAB model parameters.

Gelatin sample	Monolayer moisture content, m_0 (%, wet basis)	C Constant (adimensional)	K Constant (adimensional)	Mean Relative Error, MRE (%)
pH3/5h	5.69	3.50	0.89	2.81
pH3/2h	5.29	2.71	0.88	2.66
pH4/3.5h	6.97	3.12	0.88	2.68
pH5/5h	7.28	4.39	0.89	2.18
pH5/2h	7.34	4.30	0.88	2.20

3.8. Dynamic sorption properties

The sorption analysis of salmon gelatin samples was carried out in order to correlate the physicochemical characterization with a macroscopic property with technological interest such as the interaction polymer-water. Sorption isotherms curves obtained at 20 °C for gelatin extracted under different conditions are presented in Fig. 5. The salmon gelatin isotherms show the typical sigmoidal shape (isotherm type II) belonging to the multilayer molecular adsorption phenomenon in porous surfaces (Anderson, 1946). This pattern is typical for matrices holding small amount of water at low relative humidity and large amounts of water at high relative humidity levels (Figura & Teixeira, 2007; García-Pérez, Cárcel, Clemente, & Mulet, 2008). Moisture sorption analysis of gelatin with different molecular weight distribution is not common in the literature. One can see in Fig. 5, that extraction conditions have a direct effect on the water sorption properties of the gelatin. Lower equilibrium moisture content was reached in samples obtained under the most aggressive extraction conditions (e.g. pH 3, 2 or 5 h), whilst the highest equilibrium moisture content was reached in samples extracted under the mildest conditions (pH 5, 2 or 5 h). A similar behaviour was reported for water sorption isotherm in pigskin gelatin samples for three different molecular weights at 50 °C (Sablani, Kasapis, Al-Rahbi, & Al-Mugheiry, 2002). The investigation showed that the moisture content of pigskin gelatin decreased upon molecular weight decrease, in the water activity range $0.1<a_w<0.8$. Another study performed by Chiou et al. (2009) reported higher sorption values in Alaska pink salmon gelatin films prepared by cold-casting (4 °C) in comparison to gelatin films made by hot-casting (23–60 °C). The same findings were also recently reported by Badii et al. (2014) in bovine skin gelatin. Possibly the higher renaturation levels in gelatin led to greater water sorption due to the ability of triple helical structures to form more hydrogen bonds with water than amorphous gelatin strands (Chiou et al., 2009), which is consistent with the role played by water as stabilizer of gelatin helix described by Brodsky and Ramshaw (1997), whom have proposed that typical poliproline-II-helical (PPII) conformation of gelatin is stabilized by water bridges between groups capable of hydrogen bonding in the triple helix (Bella, Brodsky, & Berman, 1995). These findings are in agreement with our results. In our study, it has been shown that salmon gelatin extracted at pH 5 have higher gel strength (Fig. 2), higher triple helix content (Table 5), higher melting energy (Table 6) as well as higher elastic modulus and lower tan δ peak height value (Fig. 4). All those results are closely related with how the extent of molecular ordering in the matrix. Since the higher renaturation level should modify the gelatin-water interaction, this phenomenon could help to explain the behaviour presented in this section. This may be further supported by data obtained from amino acid profiling (section 3.2.). One can note that salmon gelatin extracted at pH5 shows the highest hydroxyproline content (Table 4), which have shown to play an important role in the promotion of interaction between gelatin chains through the formation of water bridges (Brodsky & Ramshaw, 1997), and therefore allowing higher moisture content in the sample.

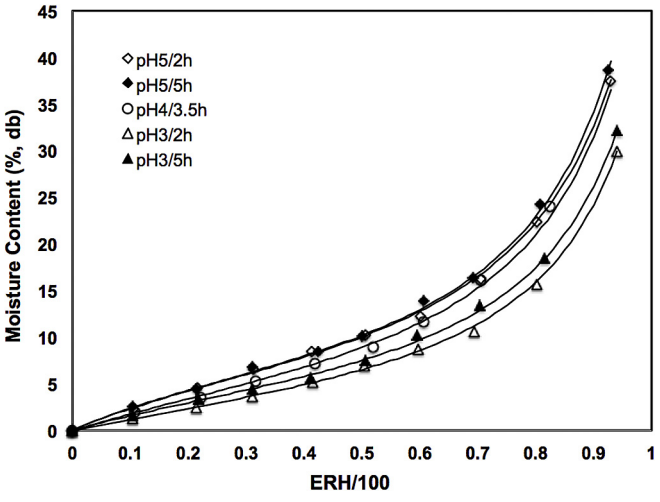

Fig. 5. Moisture isotherms curves obtained by dynamic sorption analysis at 20 °C of salmon gelatin samples obtained through different extraction conditions. Filled lines correspond to modeling by GAB equation.

Salmon gelatin isotherms were well fitted by the GAB equation (Fig. 5). The value of parameters obtained by GAB fitting are presented in Table 6. The fact that gelatin with higher molecular weight distribution showed higher moisture sorption is well reflected by GAB parameters. The monolayer moisture content (m_0) of salmon gelatin extracted under mild conditions is higher than for the salmon gelatin extracted under more aggressive conditions. This suggests less availability of polar sites for bonding with water in samples extracted at pH 3 (2 and 5 h). Another study reported higher values of m_0 in fish gelatin with higher triple-helix content (Chiou et al., 2009). On the other hand, constant C in the GAB model is related to adsorption energies of the monolayer. Therefore, a decrease in this value upon decrease of the extraction pH would suggest that water molecules are less strongly bounded to polar sites of the gelatin matrix (Enrione, Hill, & Mitchell, 2007). With respect to the K parameter, values close to 1 (0.88–0.89 in all gelatin samples) could be related to a smaller difference between the energy associated with the heat of sorption of the multilayer and the heat of condensation of pure water.

4. Conclusions

The physicochemical properties of salmon skin gelatin are strongly and significantly influenced by the extraction conditions. Our results show that pH used to carry out the extraction plays a major role (rather than time) in controlling the triple helix content of salmon gelatin. An extraction process performed under mild conditions (pH 5) generates salmon gelatin with higher triple helix content, which is well reflected by higher gel strength, higher melting energy, higher elastic modulus and higher sorption moisture. This is also directly correlated with higher protein content, higher amount of imino acids (proline and hydroxyproline) and gelatin strands with molecular weight higher than 120 kDa. The opposite behaviour was observed in salmon skin gelatin obtained under more aggressive conditions at pH 3.

These results highlight the relevance of controlling the extraction process variables as a strategy for designing a salmon gelatin-based biomaterial with well-defined physical properties. Thus, a specially designed and controlled extraction process allows obtaining a biomaterial with tailored and standardized biochemical composition, physico-chemical and structural properties, which is oriented to high-value industrial applications such as food coating, nutraceuticals and bioactive encapsulation, tissue engineering and 3D printing. Properties and performance of salmon gelatin to each application can be defined by tuning the extraction process parameters.

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