Effect of pretreatment-assisted extraction on the physicochemical and structural properties of Rhinobatos cemiculus skin gelatin

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Abstract
Different pretreatment methods were applied on *Rhinobatos cemiculus* skin, and their effect on the physicochemical properties of the extracted gelatin was investigated. Prior to extracting gelatin, the skin was pretreated using three methods: bleaching (RCG-BT), ultrasound (RCG-UST) and microwave (RCG-MT). The properties of the resulting gelatins were compared to those of the untreated-skin gelatin (RCG-WT). The pretreatment of *R. cemiculus* skin was found to differently affect the isoelectric point (pH_i), color, structure, thermal stability as well as foam/emulsion-forming capacities of the derived gelatins. In fact, the SDS-PAGE profile revealed the presence of the three gelatin characteristic chains (β and α_1/α_2) as predominant components in all gelatins with a slight decrease in bands intensity for gelatin exposed to microwave radiation. Based on FTIR analysis, gelatin characteristic bands were found in all gelatins with a slight shift for RCG-MT proving its structural re-arrangements confirmed by X-ray diffraction. Furthermore, among the applied pretreatments on RCG, ultrasound and bleaching were found to be the best techniques giving the most thermally stable gelatins. Additionally, functional properties related to gelatin derived from ultrasound pretreated skin were found to exhibit the highest foam expansion and emulsion activity.

Keywords Fish gelatin · Pretreatment · Structural properties · Thermal stability · Functional properties

Introduction

A main waste reduction strategy for fishing industries and local markets is the recovery of byproducts from fish processing residues. Fish processing by-products exploitation creates additional revenue and moderates disposal costs. By virtue of striking advantages such as biocompatibility, nontoxicity, hydrophilicity, low cost and gelation [1], gelatin from marine sources is widely employed as a promising biopolymer. Fish gelatin is considered as a class of biopolymers resulting from the partial hydrolysis of fish collagen, the most abundant protein of skin, connective tissue, cartilage, bones and tendons [2]. Once heated above its transition temperature, collagen generates a mixture of protein and peptides of different sizes [3]. Studies revealed that gelatin is composed of abundant amino acids known for their nutritive uses as dietary products. The natural origin of fish gelatin makes its derivative products relatively harmless to the body renewing then their interest and facilitating their use in clinical therapies and drugs [4].

Gelatin is considered as one of the most frequently used GRAS (Generally Recognized as Safe) biopolymers thanks to its biocompatibility and easy availability [5]. Hence, the dietary intake of fish gelatin can possibly have exceptional benefits for people with chronic illnesses such as diabetes, osteoporosis and hypertension [4]. In fact, bioactive peptides such as fish gelatin are regarded as safer and more economical ACE inhibitors with no side effects which make them excellent antihypertensive constituents in the treatment of people suffering from high blood pressure [6]. Moreover, fish gelatin hydrolysates were found to be beneficial for the attenuation of bone brittleness thanks to their high content in alanine and glycine [7]. The administration of fish gelatin products permits peoples with diabetes to get long-term profits for their health conditions by accelerating the phases of wound healing and reducing the inflammatory response [8].

Apart from its use in biomedical field, fish gelatin exhibits excellent properties related to its gelling behavior through gel formation, water binding and texturizing capacities and others associated with their surface behavior including foam and emulsion formation and stabilization, adhesion, cohesion and film-forming properties [9, 10]. It's well known that collagen cross-links are firm to thermal and acid treatment during the traditional process [11], resulting in a low gelatin yield. Various strategies were applied either to increase gelatin extraction such as proteases for cuttlefish skin [12], varying the employed acids [13] and the thermal treatment temperature [14] or to improve the functional properties through NaOH and NaCl pretreatments [15]. These methods were found to increase the yield to some degrees nevertheless the resulting gelatin presents inferior gelling property [16]. Thus, the extraction conditions and the applied pretreatment are essential parameters to fix in order to guarantee the required properties of the final product [17, 18].

Recently, ultrasonication has become an emerging technology for extraction of biologically interesting molecules in order to increase the extraction efficiency and to reduce the extraction time of gelatin from bovine and golden carp skins, respectively [16–19]. Furthermore, ultrasound has been reported to improve the

functional properties of proteins, triggered by changes in conformation [20, 21]. Additionally, the marine raw material, from which gelatin powder was obtained, was generally a bit darker in color due to the pigments it may contain [22]. Thus, the extracted gelatin may not draw the attention for some industrial practices. However, bleaching could be executed prior to gelatin extraction to minimize this weakness [23] since hydrogen peroxide has been commonly used as bleaching agent in seafood processing [24]. Kori, Rama and Kidwai [25] reported also that bleached striped catfish skin showed improved resulting gelatin color. Furthermore, microwave has been stated to extract natural products such as phenolic compounds [26], polysaccharides [27] and green coffee oil [28, 29].

Up to now, only few researches were dedicated to gelatin extraction using microwave. Binsi, Nayak, Sarkar, Joshy, Ninan and Ravishankar [29] investigated thermal characteristics and gelation properties of microwave-extracted fish scale gelatin combined with natural gums. Park, Choe, Kim, Hwang, Song, Yeo, Kim, Choi, Lee and Kim [30] evaluated the effect of microwave on quality characteristics of duck feet gelatin and proved that microwave treatment could reduce extraction time compared to conventional extraction method.

The current study shows the potential features of the skin of a fish species newly studied, *Rhinobatos cemiculus*, as a marine source which is used for the extraction of gelatin, to exploit the skin as a by-product of a marine biomass widely spread on the Mediterranean coasts. It provided also the physicochemical and the functional properties of the obtained gelatins when using different skin pretreatment methods prior to the extraction step which are bleaching, microwave and ultrasound.

Materials and methods

Preparation of raw material

Fresh blackchin guitarfish (*R. cemiculus*) skin was procured from the local commercial market located in Sfax, Tunisia. Samples were placed in ice and then transported in polyethylene bags to the laboratory. Upon arrival, the remaining meat was trimmed. The obtained skin was rigorously washed using cold water and stored at − 20 °C until further used for gelatin extraction.

Skin pretreatments and gelatin extraction

Pretreatments

Three types of treatments were separately applied on *R. cemiculus* skins, prior to their treatment with NaOH and acetic acid, namely ultrasound-, bleaching- and microwave-assisted treatments. The process is detailed below and shown in Fig. 1.

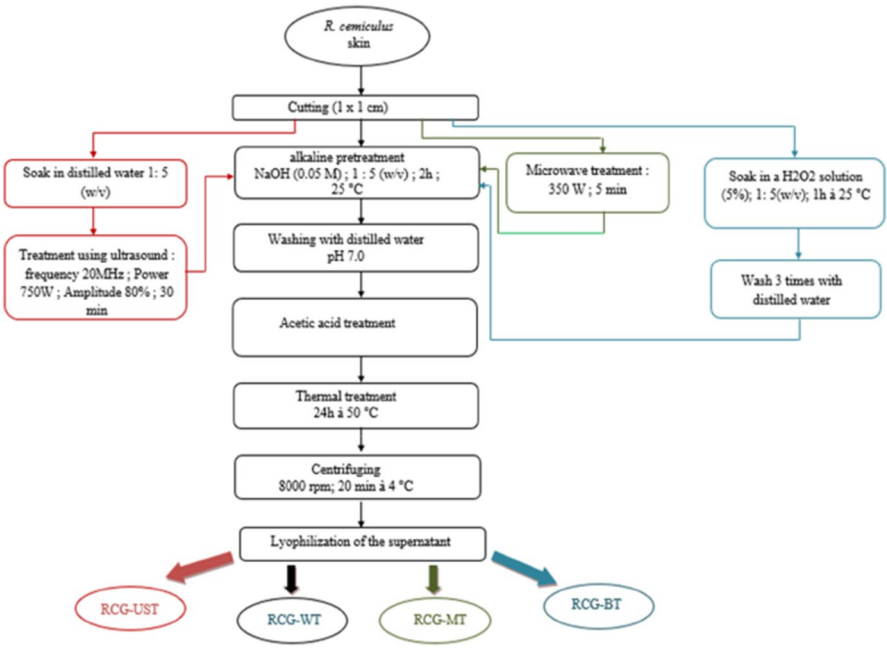

Fig. 1 Skin pretreatments and gelatin extraction process

Ultrasound-assisted pretreatment The extraction of gelatin from *R. cemiculus* skins using ultrasound-assisted treatment (UST) was based on Ali, Kishimura and Benjakul [16] procedure with slight modifications.

In fact, skins previously cut into small pieces were mixed with distilled water at a skin/water ratio of 1:5 (w/v). The obtained mixture was then subjected to ultrasonication via a reactor Vibra–Cell (Sonics & Material, Inc, Newtown, CT, USA), using a 25-mm-diameter probe with a frequency of 20 kHz and a power of 750 W. Amplitude of 80% during 30 min at 5 s acting and 5 s resting time pulse mode was applied. The temperature of the mixture was maintained at 25 ± 2 °C via an iced bath containing 2% NaCl.

Bleaching-assisted treatment The extraction of gelatin from *R. cemiculus* skins using bleaching-assisted treatment (BT) was based on Gómez-Guillén et al. [31] procedure with modifications.

The prepared skin was subjected to bleaching in 5% of H_2O_2 solution with a skin: solution ration of 1:5 (w/v) for 1 h at room temperature. Bleached skins were washed thrice using 5 volumes of distilled water.

Microwave-assisted treatment Gelatin extraction was performed using a microwave (MA-11645D, GoldStar, Korea, frequency 2450 MHz). The microwave treatment (MT) was carried out at 350 W for 5 min as described by Park, Choe, Kim, Hwang, Song, Yeo, Kim, Choi, Lee and Kim [30].

Gelatin extraction

To extract gelatin from the pretreated gelatin, non-collagenous substances, mainly non-collagenous proteins, were removed as follows: *R. cemiculus* skins were cut into small pieces of almost 2×2 cm^2 using an electric carving knife. The resulting pieces were soaked in 0.05 M NaOH solution (1:5, w/v) and kept under continuous stirring for 2 h at 25 °C. A fresh alkaline solution was replaced every 30 min to guarantee better elimination of non-collagenous substances. After that, the pieces of treated skin were kept under a stream of tap water until a neutral or faintly basic pH of the washing water was reached. Besides, an acid medium was used in order to swell and disturb the non-covalent, intra- and inter-molecular bonds of the studied matrix [32]. Thus, the washed alkali-treated skins were soaked in 0.2 M acetic acid solution. The sample/acetic acid solution ratio was fixed at 1:5 (w/v), and the mixture was kept under stirring for 18 h at 25 °C. Subsequently, the pH of the mixture was raised to 7.0 using 10 M NaOH solution and solubilized at 50 °C for 18 h with continuous stirring to achieve the gelatin extraction. A centrifugation at $6000 \times g$ for 30 min at 25 °C was carried out to remove any insoluble material. Finally, the supernatant was collected and then freeze-dried to obtain *R. cemiculus* gelatin powder (RCG).

Yield of extraction and proximate analysis of the extracted gelatins

The extraction yield was determined based on the following formula:

$$\text{Yield } (\%) = \frac{\text{Weight of freeze dried material(g)}}{\text{Wet weight of gelatin (g)}} \times 100$$

The moisture, ash and fat contents of extracted gelatins were determined according to the AOAC methods number 927.05, 942.05 and 920.39 B, respectively [33]. Nitrogen content was determined using Kjeldhal protocol according to the AOAC method number 984.13 [33], and a conversion factor of 6.25 was used to calculate the protein content in gelatin samples. All measurements were carried out in triplicate.

Color determination

A bench-top colorimeter (CR-5; Konica Minolta) was used to measure the color coordinates of extracted gelatins. Gelatin gels of 6.67% (w/v) were prepared and three color parameters, namely a^*(redness/greenness) and b^*(yellowness/blueness) and L^* (lightness/brightness), were determined as described by Tkaczewska, Morawska, Kulawik and Zając [18]. Three readings were taken per sample.

Electrophoretic analysis

Protein patterns of the extracted gelatins were determined using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli [34] with slight modifications. Gelatin samples (10 mg) were dissolved in 1 ml of distilled water at 50 °C. The prepared gelatin samples, 50 µg of each sample, were mixed with loading buffer (5% mercaptoethanol, 2% SDS and 0.002% bromophenol blue) at a ratio of 1:5. The prepared mixtures were heated at 90 °C for 10 min and then loaded onto 5% w/v stacking gel and 7.5% w/v separating gel.

Isoelectric point determination

The determination of the isoelectric point of gelatin samples was carried out according to the method described by Binsi, Nayak, Sarkar, Joshy, Ninan and Ravishankar [29]. Samples of 0.5% (w/w) were dissolved in distilled water at 50 °C and were analyzed using a UV/visible spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd, China) at 600 nm.

Fourier Transform infrared spectra

The Fourier transform infrared (FTIR) analysis was carried out on freeze-dried gelatin samples. A spectrometer (Thermo Fisher Scientific, Model: Nexus) equipped with an attenuated total reflectance (ATR) accessory mounted into the sample compartment was used. The internal reflection crystal made of diamond had an angle of incidence to the IR beam of 45°. A measurement range of 4000–800 cm^{-1} and a resolution of 4 cm^{-1} were chosen to collect automatic signals in 32 scans. The recorded signals were normalized against a background spectrum collected from the clean at 25 °C.

X-ray diffraction analysis

X-ray diffraction (XRD) patterns were collected on Bruker D5000 ray diffractometer using a Cu Kα radiation source. Analyses were operated at 40 kW and 20 mA, and patterns were recorded in the range of $2\Theta = 7–40°$ at scanning rate 1°/min.

Scanning electron microscopy

The microstructure of the extracted gelatins was visualized using a scanning electron microscope (Hitachi S4800). Prior to imaging, lyophilized samples were

fixed on brass stub and sputtered with thin gold layer in order to make the sample conductive. An accelerating voltage of 2.0 kV was used.

Thermal properties

The thermal stabilities of the extracted gelatins were tested using thermogravimetric analysis (TGA 128 Q500 High Resolution, TA Instruments), working under nitrogen flow. All powders were heated at a constant rate of 20 °C/min from 25 to 600 °C. The weight of gelatin powder in each experiment was about 3 mg. Thermograms were obtained showing mass change of the sample as a function of temperature augmentation.

Techno-functional properties

Emulsifying property

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method described by Pearce and Kinsella [35] with slight modification. Gelatin solutions at different concentrations (0.5, 1, 2, 3 and 4%) were prepared by dissolving dry gelatin into distilled water at 50 °C. Six milliliters of each solution was then homogenized with 2 ml of soybean oil using IKA® ULTRA-TURRAX® T18 Homogenizer at a speed of 20.000 rpm for 1 min at room temperature. Aliquots of the obtained emulsion were pipetted out from the bottom of the tube at 0 and 10 min and diluted 100-fold with 0.1% SDS solution. A vortex mixer was then used to thoroughly homogenize the mixtures for 10 s. The absorbance of the resulting mixtures obtained at $t=0$ min and $t=10$ min was measured at 500 nm using a spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd, China) to determine the EAI and the ESI according to the following formulas:

$$\mathrm{EAI}\left(\mathrm{m}^2/\mathrm{g}\right) = \frac{2 \times 2.303 \times A_0}{0.25 \times \text{protein weight(g)}}$$

$$\mathrm{ESI(min)} = \frac{A_0}{\Delta A} \times \Delta t$$

where A_0 is the absorbance of the diluted emulsion immediately after homogenization, ΔA is the variation of the absorbance between 0 and 10 min, Δt is the time interval, in this case 10 min.

Foaming property

Foam expansion (FE) and foam stability (FS) of gelatin samples were determined, as described by Shahidi, Han and Synowiecki [36] with a slight modification. Twenty-five milliliters of gelatin solution with different concentrations (0.5, 1, 2, 3 and

4%) were prepared by dissolving dry gelatin into distilled water at 50 °C, and then mixtures were subjected to whipping in order to incorporate the air using IKA® ULTRA-TURRAX® T18 Homogenizer at a speed of 20.000 rpm for 1 min at room temperature. The mixture was then transferred into a 50-ml cylinder and allowed to stand for 0, 30 and 60 min.

Foam formation capacity and foam stability were expressed as the volume ratio of foam to liquid at 0 min and the ratio of initial volume of foam to volume of foam after the fixed time interval, respectively. These two parameters were calculated according to the following formulas:

$$\mathrm{FE}(\%) = \frac{V_\mathrm{T} - V_0}{V_0} \times 100$$

$$\mathrm{FS}(\%) = \frac{V_\mathrm{t} - V_0}{V_0} \times 100$$

where V_T is the total volume (ml) after whipping, V_0 is the volume (ml) before whipping, V_t is the total volume (ml) after standing for 30 and 60 min.

Textural profile analysis

Textural profile parameters were determined according to the method described by [37] with slight modifications. A gelatin solution of 6.67% was prepared in a beaker with an inner diameter of 3.8 cm using distilled water as a solvent and the mixture was stirred until total dissolution. Prior to measurements, the gelatin solution was kept at 4 °C for 18 h for gel maturation. A TA1 texture analyzer (LLOYD Instruments, England) equipped with P/100 probe was used, and two compression cycles of 50% of gel's original height were applied with 25 mm/min speed. Analysis was carried out in triplicate.

Statistical analysis

Statistical analyses were executed with SPSS version 17.0, professional edition using ANOVA analysis. A standard deviation with a confidence level set at 95% was fixed to compare all parameters analyzed for all extracted gelatins.

Results and discussion

Effect of skin pretreatment on gelatin extraction

The extraction yield is an important parameter to determine, especially when the gelatin is meant to be produced in industries [38]. The yield of the extracted RCG using different treatments (ultrasound-, bleaching- and microwave-assisted treatments) are shown in Table 1. The yield of the extracted gelatin significantly depended on the skin pretreatment method. Results showed that the yield of RCG-WT extracted

Table 1 Proximate composition of *R. cemiculus* skin gelatin without and with pretreatment. Results (%) are expressed based on wet weight matter

	Yield (%)	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
RCG-WT	16.13 ± 0.88^{c}	3.53 ± 0.65^{c}	4.55 ± 0.24^{c}	91.98 ± 0.45^{b}	0.11 ± 0.02^{b}
RCG-MT	14.49 ± 0.94^{d}	4.50 ± 1.77^{b}	14.8 ± 0.07^{b}	80.28 ± 0.31^{c}	0.25 ± 0.06^{a}
RCG-UST	20.35 ± 0.39^{a}	5.34 ± 1.7^{a}	0.41 ± 0.01^{d}	94.06 ± 3.09^{a}	0.21 ± 0.03^{a}
RCG-BT	18.19 ± 0.09^{b}	4.06 ± 0.82^{c}	33.32 ± 0.3^{a}	62.34 ± 1.55^{d}	0.25 ± 0.01^{a}

[a,b,c,d]Letters in the same column within different pretreatments indicate significant differences ($p < 0.05$)

RCG-WT: Untreated-skin gelatin; RCG-MT: Gelatin from the microwave-pretreated skin; RCG-UST: Gelatin from the ultrasound-pretreated skin; RCG-BT: Gelatin from the bleaching-pretreated skin

conventionally, without any treatment, was at $16.13 \pm 0.88\%$, while it increased with ultrasound and bleaching reaching $20.35 \pm 0.39\%$ and $18.19 \pm 0.09\%$, respectively, and decreased with microwave to attain $14.49 \pm 0.94\%$. These findings may be attributed to the fish species and pretreatment method used as mentioned by Gómez-Guillén, Turnay, Fernández-Dıaz, Ulmo, Lizarbe and Montero [31] and Zhou and Regenstein [39]. For instance, the yields of gelatin extracted conventionally from fish skin have been reported for shortfin scad (7.25%) and sin croaker (14.3%) [40], brown stripe red snapper (9.4%) and big eye snapper (6.5%) [41], cod (11.8%) and Atlantic salmon (15.3%) [42].

As for the gelatin extracted from *R. cemiculus* skin without prior-treatment, it presented the lowest yield which may be due to collagen molecules cross links stability when treated thermally as mentioned by Zhou and Regenstein [39]. However, the extraction yield was improved when using ultrasonication, reaching the highest yield among the applied treatments. This rise was suggested to be due to the mechanical effect of ultrasound and the formation of small vapor-filled cavities known as cavitation phenomenon [43, 44]. Actually, the cavitation facilitated the slackening of the treated skin and led to the enhancement of hot water penetration into the slackened matrix during the extraction [21]. This finding proved that prior ultrasonication was able to destabilize collagen structure, hence inducing the transition of collagen to soluble gelatin [45].

Likewise, results in Table 1 showed that bleached samples had a greater yield of 18.19% when compared to non-bleached samples, suggesting that the presence of H_2O_2 might induce the break of collagen molecules in *R. cemiculus* skin, resulting in gelatin extraction efficiency as verified by the yield increase. Hydrogen peroxide was found to disrupt the hydrogen bond of collagen [46]. Donnelly and McGinnis [47] reported that the agitation of a tissue containing collagen in a H_2O_2 solution resulted in its liquefaction.

George, Zynudheen, Anu, Binsi and Joshy [22] noted a noticeable increase in the yield of gelatin obtained with H_2O_2 compared to the yield of the gelatin obtained without H_2O_2 bleaching. In the present study, the yield of gelatin extracted from *R. cemiculus* skin was slightly higher than that reported from cuttlefish skin (17.12%) treated with H_2O_2. Otherwise, the yield of gelatin extracted using microwave was the lowest. This fact may be attributed to the considerable amount of drying of fish

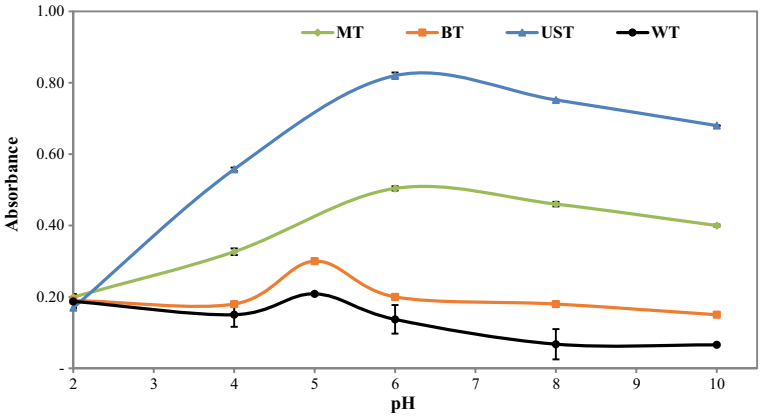

Fig. 2 Absorbance spectra of *R. cemiculus* gelatins as a function of pH variation. WT: without treatment, BT: bleaching treatment, MT: microwave treatment and UST: ultrasound treatment

skin surface caused by the microwave which might have inhibited efficient gelatin extraction [30].

Isoelectric point determination

The isoelectric point of gelatin is considered as an important aesthetic property, depending on the use for which the gelatin is intended. The transmittance of each gelatin solution was represented in Fig. 2. Samples with different treatments appeared visually similar; however, spectrophotometric readings showed different clarities when compared to gelatin solution conventionally extracted. The obtained profile showed that both the pH of the solution and the type of treatment previously applied on gelatin strongly influenced its transmittance. For gelatin obtained from skin treated with microwave (RCG-MT) and ultrasound (RCG-UST), the turbidity profile of their solutions was same. The turbidity of RCG-MT and RCG-UST increases to pH 6.0 and then decreases for alkaline pH. On the other hand, the turbidity profile of conventionally extracted RCG (RCG-WT) and bleached RCG (RCG-BT) displayed similar turbidity profiles which showed that turbidity values increased slightly with pH and reached its maximum at pH 5.0. Poppe [48] reported that the gelatin solution has maximum turbidity at its isoelectric point. From this result, it can be suggested that the isoelectric points of RCG-MT and RCG-UST is estimated to be 6.0 and of RCG-WT and RCG-BT is 5.0. Differences in isoelectric points and turbidity may be caused by clarification processes adopted during gelatin extraction as mentioned by Muyonga, Cole and Duodu [49].

ATR-FTIR

In order to understand the nature of changes that may occur as a result of different pretreatments applied on skins, FTIR spectroscopic analysis was applied

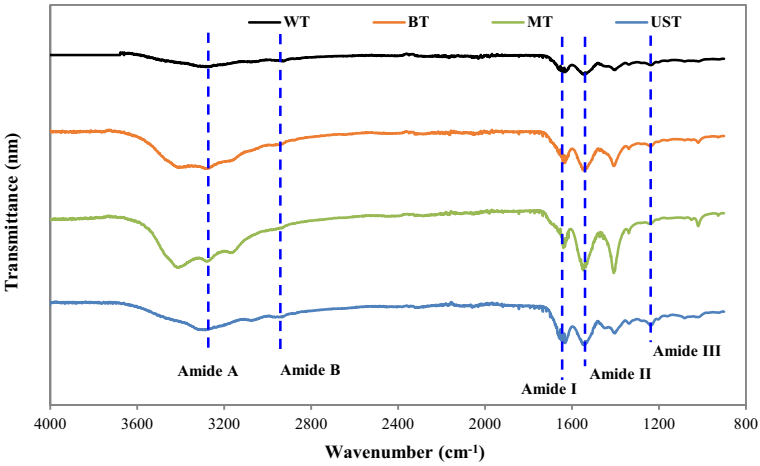

Fig. 3 ATR-FTIR spectra of *R. cemiculus* skin gelatins. WT: without treatment, BT: bleaching treatment, MT: microwave treatment and UST: ultrasound treatment

and results are depicted in Fig. 3. The regions allocated to the bonds are amide I (1617–1624 cm-1), amide II (1540–1545 cm^{-1}), amide III (1229–1233 cm^{-1}), amide A (3207–3296 cm^{-1}) and amide B (2933–2943 cm^{-1}). This profile was in accordance with the one mentioned by Kong and Yu [50]. Amide I vibration was primarily representing C=O stretching and hydrogen bonding coupled with COO^{-}, while Amide II vibration was related to stretching vibrations of C–N groups and bending vibrations of N–H groups [51]. As for Amide III vibration, it reflects both N–H groups deformation and C–N stretching vibrations. Jackson [52] stated that N–H groups deformation resulted from amide linkages and absorptions related to glycine backbone CH groups vibration. Nur Hanani, Beatty, Roos, Morris and Kerry [53] and Tongnuanchan, Benjakul and Prodpran [54] indicated that Amide A illustrates the NH-stretching combined with hydrogen bonding, while Amide B represents asymmetric stretching vibration of $N–H_3^+$ and C–H.

Typical FTIR spectra of all RCG-WT, RCG-UST and RCG-BT were obtained, and two major peak regions were noted at (3600–2800 cm^{-1}) and (1900–900 cm^{-1}). Meanwhile, the obtained spectrum for RCG-MT revealed that all characteristic bonds were slightly shifted. This shift may be attributed to the hydrogen bonding in the polypeptide chain as well as the protein structural re-arrangements resulting from microwave treatment [55]. This result was in accordance with the one found by Binsi, Nayak, Sarkar, Joshy, Ninan and Ravishankar [29], who mentioned that a notable extent of intermolecular cross-links were contained in microwave-extracted gelatin which are resulting from microwave radiation exposure.

Electrophoretic analysis

The SDS-PAGE analysis was carried out to identify gelatin chains. Figure 4 shows the protein patterns of gelatins obtained from different pretreatments. The

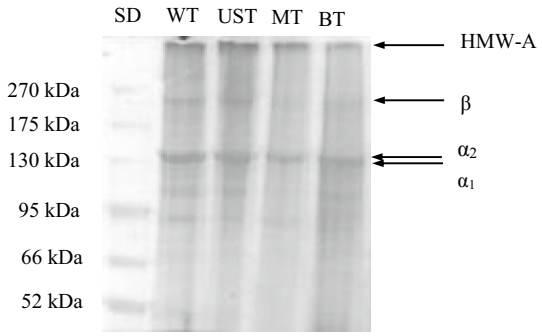

Fig. 4 SDS-PAGE profiles of *R. cemiculus* skin gelatins. SD: molecular weight standard, WT: without treatment, BT: bleaching treatment, MT: microwave treatment and UST: ultrasound treatment. HMW-A indicates high molecular weight aggregates

resulting pattern indicated the presence of all gelatin characteristic chains, namely β and $\alpha_1/\alpha_2$with respective MW of 200 and 120–130 kDa, as previously reported by Gómez-Guillén, Pérez-Mateos, Gómez-Estaca, López-Caballero, Giménez and Montero [56] and Tümerkan, Cansu, Boran, Mac Regenstein and Özoğul [57]. In addition, a similar molecular weight distribution was detected for all extracted gelatins except the one pretreated with microwave. In fact, exposure to microwave radiation during gelatin extraction induced a slight decrease in α_1, α_2 and β chain bands intensity. The gelatin backbone is essentially formed by alpha and beta chains, involved in its triple helix network. It has been reported that the lower the mean molecular weight (MW) of a gelatin chains, the lower the gel strength and viscosity of its solution, where the alpha-chains are the main contributor of gel strength [58]. These results suggested that RCG-MT gelatin may exhibit the lowest gelling, rheological and textural properties. In this context, Mad-Ali, Benjakul, Prodpran and Maqsood [59] demonstrated that the gel properties from goat skin gelatins were influenced by the pretreatment type, and a higher content of α-chains showed the better gelling properties. On the other hand, low molecular weight (MW) peptides were observed in RCG-BT and RCG-MT gelatin patterns, which could be attributed to the excessive collagen hydrolysis during gelatin extraction using these methods which affected their functional and gelling characteristics. The obtained results are in line with several works reported that protein patterns of gelatins are influenced by the extraction conditions Hazirah, Isa and Sarbon [60] and Tümerkan, Cansu, Boran, Mac Regenstein and Özoğul [57].

X-ray diffraction

The X-ray diffraction analysis (XRD) of gelatins obtained from different pretreatments was carried out to examine the crystalline structure and the molecular conformation modifications caused by the applied pretreatment. The XRD patterns of different gelatin samples are represented in Fig. 5. The obtained patterns of gelatins extracted conventionally and after ultrasound treatment showed a broad peak located at about 20° (2θ) reflecting the helical structure of these gelatins. This amorphous peak indicated the space between amino acid residues forming the helix [61] and suggested that the ultrasound treatment does not alter the gelatin structure. The

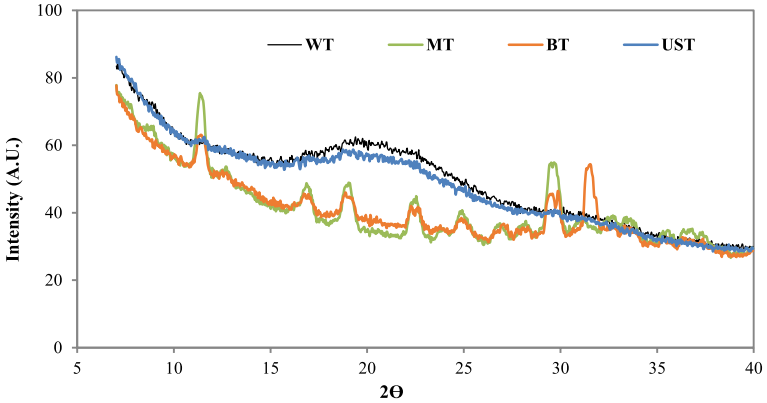

Fig. 5 X-ray diffraction pattern of *R. cemiculus* skin gelatins. WT: without treatment, BT: bleaching treatment, MT: microwave treatment and UST: ultrasound treatment

diffractograms of RCG-MT and RCG-BT displayed several sharp peaks. The two gelatin characteristic peaks located at 11 and 23° were slightly displaced compared to those detected for RCG-WT and RCG-UST suggesting that microwave/bleaching treatments could be responsible for some conformation changes in the molecular structure. In fact, Aewsiri, Benjakul and Visessanguan [62] mentioned that free radicals could be derived from hydrogen peroxide decomposition, and consequently, they may cause the oxidation of gelatin, resulting in protein structure changes. The same findings were approved by LI, MU, Zhang, ZHOU and Lin [63] who mentioned that microwave irradiation does incite additional destructions of gelatin structure.

The other appeared sharp peaks could be related to the inorganic impurities such as salts in the gelatin powder as mentioned by Kchaou, Benbettaieb, Jridi, Nasri and Debeaufort [64] and proved that the microwave and bleaching treatments affected the overall crystallinity of the obtained gelatins. Additionally, Pirestani, Nasirpour, Keramat, Desobry and Jasniewski [65] stated that the functional and structural properties of proteins can be affected by the applied treatment resulting in proteins denaturation through the destruction of certain forces responsible for the stabilization of native conformations, such as electrostatic, hydrogen, disulfide and hydrophobic bonds.

Microstructure of RCGs

The microstructural properties are among the essential aspects of the whole quality of dehydrated products. The pretreatment methods considerably affect the microstructure as mentioned by Krokida and Maroulis [66]. The surface microstructural feature of differently treated gelatins was observed using scanning electron microscopy in comparison with conventionally extracted gelatin. Figure 6 illustrates the micrographs of different RCG samples and shows the topology of

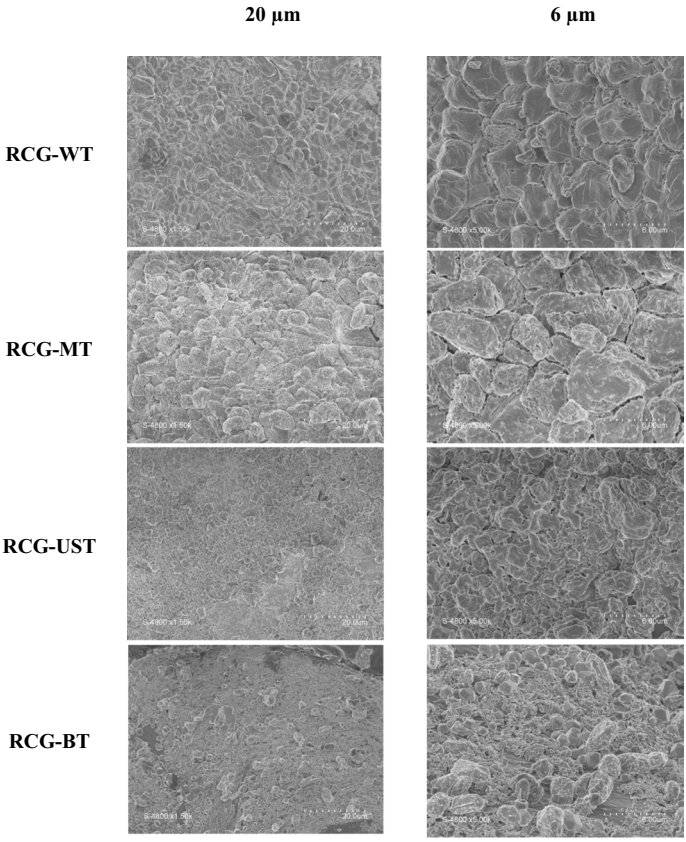

Fig. 6 SEM micrographs of cross-section of *R. cemiculus* skin gelatins. WT: without treatment, BT: bleaching treatment, MT: microwave treatment and UST: ultrasound treatment

the investigated polymer as a function of the applied treatment. A noticeable difference in microstructure was detected when varying the pretreatment. For both RCG-WT and RCG-UST, an almost similar smooth surface morphology was perceived with large fused particles for RCG-WT and fine fused particles for RCG-UST. This was possibly due to cavitation and mechanical oscillations of ultrasounds which decreased sulfhydryl group content and increased protein surface hydrophobicity leading to the reduction of the binding energy among macromolecular proteins and protein conglomerates [20, 67]. Nevertheless, a rough surface was observed in RCG-MT and RCG-BT composed by cracked large-size particles and particles of different sizes emerging on the surface, respectively. The differences in the microstructure of different samples expressed by the significant variation in particles size can be explained by the fact that hydrogen peroxide-induced protein oxidation and led to structural changes [68] as well as the appearance of aggregations [69], expressed on the surface as emerging particles.

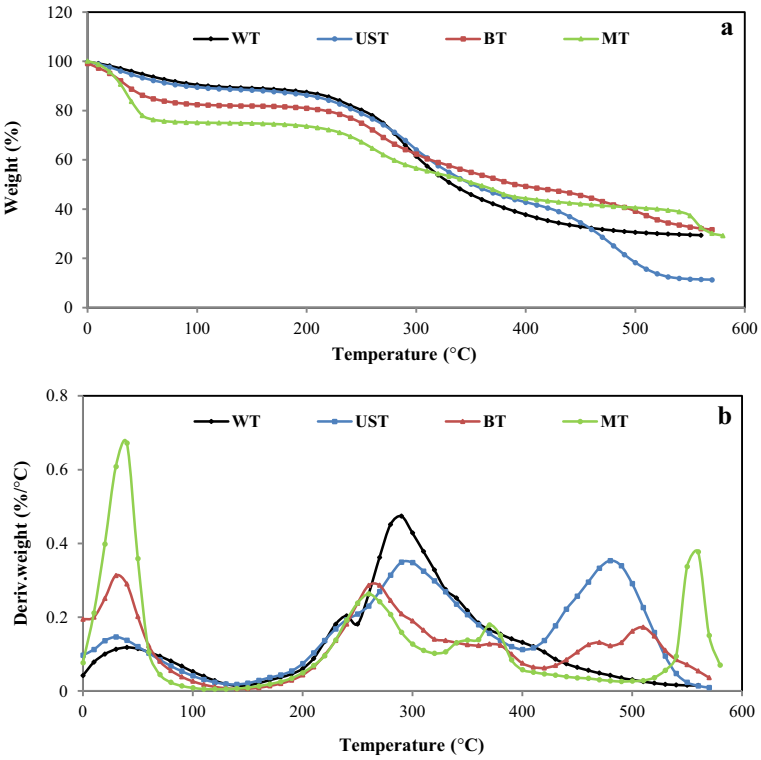

Fig. 7 TGA thermograms of weight loss (**a**) and its derivatives (**b**) of *R. cemiculus* skin gelatin powder with different treatments. WT: without treatment, BT: bleaching treatment, MT: microwave treatment and UST: ultrasound treatment

Thermal analysis

TGA is the conventional technique used to follow materials decomposition and to study their thermal stability. For that purpose, TGA thermograms of differently treated gelatins with accompanying first derivative were determined and are shown in Fig. 7a and b, respectively. As shown in Fig. 7b, all samples showed an initial weight loss detected from 25 to 150 °C which may be due to free water (moisture percent) elimination. The TGA curve of RCG-WT discloses that the major weight loss was ranged between 200 and 400 °C which is related to the gelatin degradation associated with peptide bonds rupture and protein helical structure breakage as mentioned by Benbettaïeb, Karbowiak, Brachais and Debeaufort [70] and Barreto, Pires and Soldi [71]. The TGA curves of both RCG-UST and RCG-BT showed a first major weight loss in a range of 200–400 °C, similarly to RCG-WT, and a second major weight loss extended from 400 to 550 °C which is due to extensive thermal degradation process.

A similar pattern of TGA curve of gelatin has also been stated earlier by [72]. The pattern of the TGA curve of RCG-MT displayed three major weight loss

Table 2 Color parameters of *R. cemiculus* skin gelatin without and with treatment

	Color parameter			
	L*	a*	b*	ΔE
RCG-WT	85.07 ± 0.01^{b}	-0.61 ± 0.01^{d}	10.15 ± 0.02^{b}	–
RCG-UST	80.77 ± 0.06^{d}	-0.37 ± 0.01^{c}	11.3 ± 0.01^{a}	4.46 ± 0.1^{a}
RCG-BT	87.42 ± 0.11^{a}	1.12 ± 0.01^{b}	6.83 ± 0.08^{d}	4.10 ± 0.1^{b}
RCG-MT	81.82 ± 0.06^{c}	1.28 ± 0.01^{a}	9.84 ± 0.01^{c}	3.34 ± 0.1^{c}

[a,b,c,d]Letters in the same column within different pretreatments indicate significant differences ($p < 0.05$)

RCG-WT: Untreated-skin gelatin; RCG-MT: Gelatin from the microwave-pretreated skin; RCG-UST: Gelatin from the ultrasound-pretreated skin; RCG-BT: Gelatin from the bleaching-pretreated skin; a*(redness/greenness); b*(yellowness/blueness); L* (lightness/brightness); Δ*E*: Color difference

phases ranged in 200–300 °C, 350–400 °C and 500–600 °C, with a maximum degradation observed at 560 °C. Therefore, based on this data it could be perceived that among the applied pretreatments on RCG, ultrasound and bleaching guarantee a more thermally stable polymer than microwave.

Color

The color of gelatins extracted under different conditions was measured, and three parameters were recorded, namely L, a and b values (Table 2). As detailed by Oszmiański and Wojdyło [73], a and b represent the chromaticity coordinates. A positive value of a designates the red direction, while a negative one indicates the green direction. For b coordinate, a positive value designates yellow direction and a negative value indicates blue direction. As for L, it indicates lightness, ranging from 0 for black to 100 for white. As shown in treated gelatins and in comparison with control, L, a and b coordinates after treatments were significantly changed. L values increased after bleaching treatment and decreased after ultrasound and microwave treatment. Thus, soaking *R. cemiculus* in H_2O_2 solution might improve the color of gelatin by increasing L. Compared to conventionally extracted gelatin, an increase in a and b values was obtained in gelatin when prior-ultrasonication was applied to reach the highest b value. Such changes proved the yellowness of the obtained powder. This may be due to the generation of free amino group in the resulting gelatin. As a result, those free amino groups might be included in non-enzymatic browning reaction with carbonyl groups present in RCG skin as described by Sinthusamran, Benjakul and Kishimura [74]. Therefore, prior-ultrasonication affected the color of gelatin extracted from skin of *R. cemiculus*. Among all gelatin samples, RCG-MT displayed the lowest total difference in the color value (3.34). These findings showed that the type of the applied pretreatment could differently affect the color of the extracted gelatin.

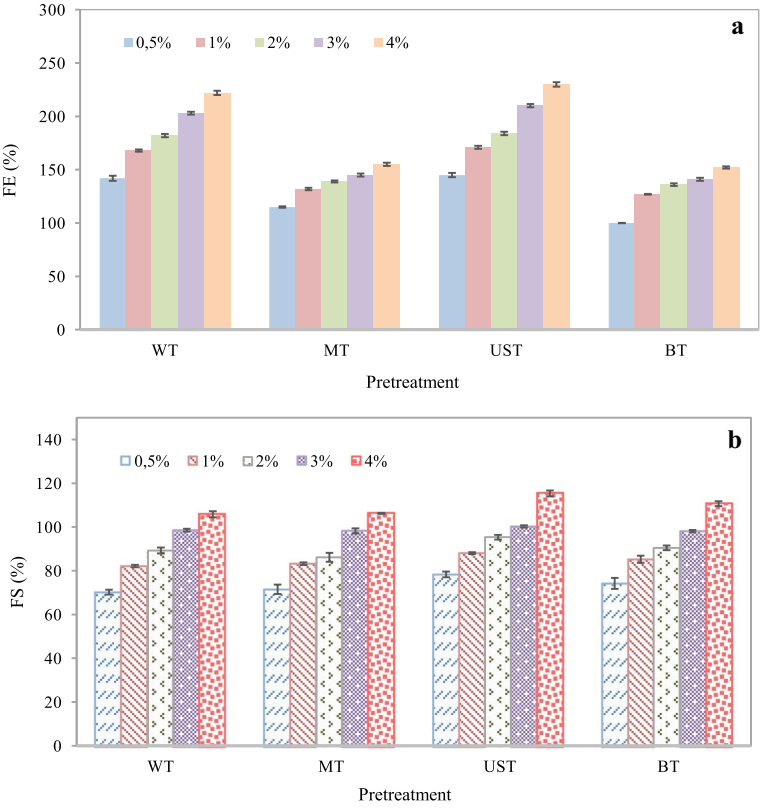

Fig. 8 Foaming properties of gelatins from *R. cemiculus* skin gelatin without and with treatment in terms of foam expansion (**a**) and foam stability (**b**)

Techno-functional properties

Foam capacity and stability

Thanks to its foaming properties, gelatin is used to manufacture various foods such as marshmallows. Both foam expansion (FE) and foam stability (FS) at 60 min after whipping were established to evaluate RCG foam capacity and foam stability for all pretreated gelatins.

FE and FS of differently pretreated gelatins at various concentrations (0.5, 1, 2, 3 and 4%) are depicted in Fig. 8. Foam expansion and stability values of RCG irrespective of the applied pretreatment increased with increasing polymer concentration. These results are in line with those found by Jridi et al. [75] who reported an increase in FE and FS for cuttlefish gelatin. Zayas [76] reported that denser and more stable foams were obtained with higher concentration of proteins due to an increase in the thickness of interfacial films. In overall, RCG-UST at

4% displayed the highest FE and FS, suggesting that ultrasound treatment could lead to the improvement of the foaming properties of RCG.

Proteins foaming ability is associated with their transportation, reorganization and adsorption at the air–water interface. To be capable of adsorbing, proteins should contain hydrophobic groups which turn out to be more exposed during protein unfolding, hence enabling foam formation and stabilization [77]. Since RCG-UST presented the highest FE value, it might be the rapidly adsorbing gelatin among all gelatins capable of being maintained at the air–liquid interface during bubbling according to Damodaran [78] and Van der Ven et al. [79] who mentioned that proteins, which quickly adsorb at the newly created air–liquid interface during bubbling and endure unfolding and molecular rearrangement at the interface, revealed better foaming ability than proteins that adsorb gradually.

Emulsifying properties

Proteins emulsifying properties are considered as emulsion activity, which revealed the ability of the protein to aid creation and stabilization of newly produced

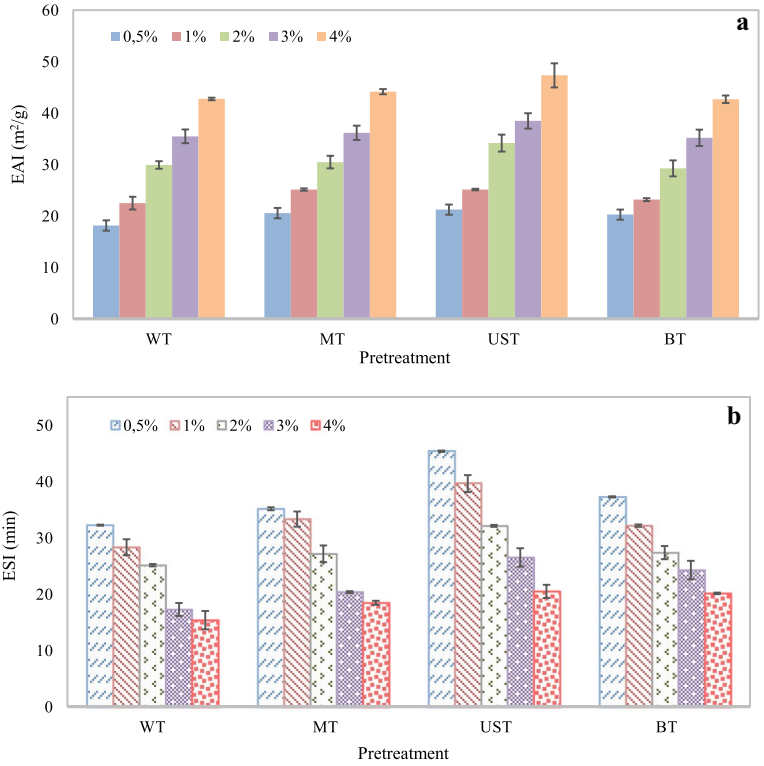

Fig. 9 Emulsifying properties of gelatins from *R. cemiculus* skin gelatin without and with treatment in terms of emulsion activity index (**a**) and emulsion stability index (**b**)

emulsions and the capacity of the proteins to impart strength to emulsion for resistance to stress [80].

Emulsion activity index (EAI) and emulsion stability index (ESI) of differently treated gelatins at different concentrations (0.5–4%) are presented in Fig. 9. The EAI of all extracted gelatins was noted to increase with the increase in polymer concentration as found by Abdelmalek, Gómez-Estaca, Sila, Martinez-Alvarez, Gómez-Guillén, Chaabouni-Ellouz, Ayadi and Bougatef [81] for gelatin extracted from *Loligo vulgaris* skin. This finding was in line with Yamauchi, Shimizu and Kamiya [82] who reported that proteins at high concentrations are capable to better adsorb at interface. Additionally, results proved that at a fixed gelatin concentration, the EAI was the highest for RCG-UST and the lowest for RCG-BT. This possibly resulted from the difference in the intrinsic properties and conformation of proteins between all the extracted gelatins [78]. Additionally, high solubility of the protein in the dispersing phase was found to increase the emulsifying efficiency, since protein molecules rapidly migrated to the surface of the fat droplets [83]. As for RCG-BT EAI low value, it might be explained by the appearance of protein aggregations which might be rigid, preventing then their rapid unfolding at the interface and the effective formation of a film around an oil droplet as explained by Singh and Benjakul [23]. Contrary, the emulsion stability decreased as gelatin concentration increased. The highest ESI (45.36 min) was detected at a concentration of 0.5% for RCG-UST reflecting that the stronger film with higher regularity around oil droplets was obtained after an ultrasound treatment.

Texture profile analysis (TPA)

TPA parameters in terms of hardness, cohesiveness, springiness and chewiness of different gelatin gels are illustrated in Table 3. Hardness, which is referring to the strength of the gel structure when compressed, was determined to precise the force needed to reach certain deformation. Cohesiveness reflects the degree of difficulty in breaking down the internal structure of the gel. Springiness, which known as elasticity, is the rate of regaining the original state. As for chewiness, it defines the energy required to masticate a solid food to a state ready for swallowing. TPA results demonstrated that cohesiveness and springiness were not influenced by the

Table 3 Effect of pretreatment methods on texture parameters of gelatins extracted from *R. cemiculus* skin

	Hardness (N)	Cohesiveness	Springiness (mm)	Chewiness (N × mm)
RCG-WT	13.55 ± 2.05^{a}	0.45 ± 0.08^{a}	11.09 ± 0.15^{a}	86.60 ± 1.40^{a}
RCG-MT	1.49 ± 0.01^{d}	0.42 ± 0.02^{a}	11.34 ± 0.08^{a}	7.07 ± 0.01^{d}
RCG-UST	9.31 ± 0.50^{b}	0.43 ± 0.04^{a}	10.94 ± 0.54^{a}	47.92 ± 3.82^{b}
RCG-BT	3.00 ± 0.42^{c}	0.40 ± 0.01^{a}	10.11 ± 0.86^{a}	13.24 ± 3.09^{c}

^{a,b,c,d}Letters in the same column within different pretreatment indicate significant differences ($p < 0.05$)

RCG-WT: Untreated-skin gelatin; RCG-MT: Gelatin from the microwave-pretreated skin; RCG-UST: Gelatin from the ultrasound-pretreated skin; RCG-BT: Gelatin from the bleaching-pretreated skin

skin pretreatment which may be due to hydrogen bonds and reversibly cross -linked protein network that stabilized the gelatin gel [84]. Meanwhile, RCG-WT exhibited higher hardness and chewiness values compared to the remaining gelatins, which indicates that microwave, ultrasound and bleaching pretreatments significantly ($p < 0.05$) decreased the gel hardness. The order of gel strength was found as: RCG-WT > RCG-UST > RCG-BT > RCG-MT. Indeed, the obtained low gel strength values, when skin was pretreated, could be ascribed to the presence of low molecular mass peptides that could enhance ionic interactions and thus hinder the formation of a robust network. Additionally, the significant change of gel strength values between all gelatins could be due to the differences in intrinsic characteristics, especially the molecular mass distribution of gelatins as demonstrated in Fig. 4. Hence, gelatin with low molecular mass polymers displayed lower gel strength.

Conclusion

Among the various pretreatments used to extract gelatin from *R. cemiculus* skin, ultrasound and bleaching showed higher yield, compared to conventional extraction method. However, gelatin extracted using microwave radiation was found to exhibit structural re-arrangement compared to conventionally extracted gelatin, suggesting that microwave radiation may affect the intermolecular cross-links between protein chains. In addition, results of the present study demonstrated that the extracted gelatin properties were influenced by the applied pretreatment, as shown by the microstructure, TGA and functional properties analysis. Thus, *R. cemiculus* skin gelatin can be interestingly used in different industrial applications depending on the pretreatment applied.

Acknowledgements This research work was conducted in the framework of PHC-Utique Program, (partenariat Hubert Curien « Utique » du Ministère de l'Europe et des Affaires Etrangères français et du Ministère de l'Enseignement et de la Recherche Scientifique tunisien) financed by CMCU (Comité mixte de coopération universitaire), grant N°: 19G0815.

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