1 Development of active biocomposites using a shrimp cooking effluent

2

3 A. Alemán*, F. González, M. Arancibia, M. E. López-Caballero, P. Montero and M. C.

4 Gómez-Guillén.

5 Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC)**

6 C/ José Antonio Nováis, 28040 – Madrid (Spain)

7

8 * Corresponding author. Tel: +34-31-5492300; fax: +34-91-5493627

9 E-mail address: ailen@ictan.csic.es

10 ** This center has implemented and maintains a Quality Management System which

11 fulfils the requirements of ISO standard 9001:2015.

ABSTRACT

A functional microemulsified concentrate was obtained from an industrial shrimp cooking effluent by using a centrifugal separator, resulting in a new effluent with only 5% of dry matter. The shrimp concentrate (C) showed valuable properties for use as a bioactive ingredient, being rich in lipids (~16%) and proteins (~11% w/w), and exhibiting antioxidant (radical scavenging capacity and reducing power) and antimicrobial properties. Chitosan–gelatin (ChG) biocomposites with and without the shrimp concentrate were prepared and characterized. The incorporation of C provided film forming dispersions (S-ChGC) and dried films (F-ChGC) with increased antioxidant and antimicrobial activity. The film microstructure changed with the incorporation of the concentrate, which caused an evident plasticizing effect and reduced the tensile strength. The resulting biocomposites containing the shrimp cooking concentrate showed great potential for use in food applications (as food preservation or food design), medical devices or for cosmetic purposes, as wound dressing or film matrix for application on mucous membranes and epithelia with different functionalities.

Key words: shrimp waste; effluent valorization; chitosan-gelatin biocomposite; film; antioxidant; antimicrobial.

1. INTRODUCTION

There is increasing demand for ready-to-eat fish products, and consequently a large amount of industrial waste is continually generated. The largest volume of commercial cooked shrimp belongs to the genus *Penaeus* and especially to the species *P. notialis* and *L. vanamei*, followed by *P. monodon*. The process of cooking shrimp produces a large volume of effluents, which represents an environmental hazard (Cros et al., 2006). According to the legislation, these effluents containing a high proportion of organic matter must be treated before being discarded in the environment (Vandanjou et al., 2002). Then, previous withdrawal of organic matter may have a double interest. On the one hand, the recovered material could be reused and conditioned for animal or even human food applications, providing added value. On the other hand, the recovery implies a remarkable reduction in the cost of effluent water purification, because the total solids content prior to purification is much lower (Bourseau et al., 2014). As reported in a previous work, shrimp cooking effluent could be a valuable resource, providing a nutritional value of 26.2 g/L of protein, 9.5 g/L of fat and 10.8 g/L of ash (Pérez-Santín et al., 2013). In addition, highly unsaturated lipids, bioactive peptides, minerals (Na, K, Ca, Mg, Cu, Fe, Zn and Mn) and carotenoids (mainly free astaxanthin – cis and trans isomers - and derived esters) with antioxidant capacity were also reported (Pérez-Santín et al., 2013).

The use of waste from the food industry to obtain polymeric materials has increased in recent years. The polymer compound could be used to prepare valuable biocomposites for many uses, such as food packaging, biomedical devices, and for cosmetic purposes. In this connection, the blending of chitosan and gelatin to formulate films has been extensively studied because of the great biocompatibility and non-toxic, biodegradable

nature of the resulting materials (Qiao et al 2017; Bonilla et al., 2017; Hosseini et al., 2013). Chitosan has gained further interest because of its antimicrobial capacity, being able to inhibit the growth of bacteria, yeast and molds. Recently, this polymer has been obtained by less aggressive and less polluting methods (Arancibia et al., 2015, Muxica et al 2017). Gelatin has a broad range of functional properties that make it very versatile, especially in the case of gelatins from marine sources, which have shown excellent film-forming properties, yielding transparent, nearly colorless, highly extensible films (Gómez-Guillén et al., 2011). Reformulation with different polymers and biocompatible compounds is essential when seeking new properties in the resulting biocomposites (Nadi & Kandil 2018). To this respect, the addition of antioxidants and/or antimicrobial compounds can improve the functional properties and versatility of the films, which could be used to prolong the shelf life and safety of coated food products. In this sense, a shrimp (*L. vanammei* and *P. notialis*) cooking effluent concentrate has been previously used for enhance the functionality of chitosan-gelatin composites when applied to fish sausages. The film forming dispersion enriched with the shrimp concentrate extended the shelf life of the fish sausage up to 15 days, while the application of this blend in the form of preformed film provided greater microbiological control (Alemán et al., 2016). The addition of ingredients may alter structural and mechanical properties of the biocomposites, and interactions with the biopolymer film matrix may affect the release of active components and functionality (Giménez et al., 2013, Muxika et al 2017).

Thus, the objective of this work was to evaluate the influence of the addition of a shrimp (*L. vanammei* and *P. notialis*) cooking effluent concentrate on the rheological behavior of chitosan–gelatin film forming dispersions, as well as on the physico-chemical, structural and mechanical properties of the respective preformed chitosan–

gelatin films. Furthermore, the antioxidant and antimicrobial properties of the chitosan–gelatin film forming dispersions and films containing the shrimp concentrate were also evaluated.

2. MATERIALS AND METHODS

2.1. Shrimp concentrate preparation

Shrimp (*L. vanammei* and *P. notialis*) cooking effluent, supplied by Gambastar, S.L. (Burgos, Spain), was recovered within one working day, immediately before being sent to the water treatment plant, and was frozen at –20 °C until use. The frozen cooking effluent was transported to ICTAN. After thawing, the cooking effluent was filtered through gauze and passed through a centrifugal separator (GEA Westfalia Separator, OKA 2-06-566, Düsseldorf, Germany) at 9186xg and constant gravimetric flow rate (50 l/h), in order to simulate a feasible, one-step, non-contaminating industrial procedure. The solid matter resulted in a pasty-looking concentrate (C) that was frozen at –80 °C until analysis.

2.1.1. Proximate analysis of shrimp concentrate (C)

The protein content was determined by a LECO FP-2000 nitrogen/protein analyzer (LECO Corp., St. Joseph, MI, USA using a nitrogen-to-protein conversion factor of 6.25. Moisture and ash contents were evaluated following AOAC (2000). Fat content was determined according to Bligh and Dyer et al. (1959). All determinations were performed at least in triplicate.

2.2. Film-forming dispersions and films preparation

Chitosan (intrinsic viscosity-molecular weight of 830 kDa, 83% deacetylation degree) was obtained by mild processing from chitinous material (cuticles, parapods and pleopods of *L. vannamei*) following the procedure described by Arancibia *et al.* (2015). Chitosan was dissolved in 0.15 M lactic acid under stirring overnight. Gelatin from warm-water fish (Lapi Gelatin, Florence, Italy) was dissolved in hot distilled water (45 °C), and the shrimp concentrate (C) was dispersed in distilled water in the presence of Tween 80. Glycerol (0.2% w/v) was added as plasticizer to the film forming dispersions. All final dispersions were homogenized with a T25 basic Ultra-Turrax (IKA-Werke GmbH & Co. KG, D-79219 Staufen, Germany) at 17500–21500 rpm for 5 min and sonicated (Q700, 132 Qsonica, CT, USA) at 70% amplitude. The formulation of each film-forming dispersion was: S-ChG (chitosan 1%, gelatin 1% and glycerol 0.2%, w/v) and S-ChGC (chitosan 1%, gelatin 1%, shrimp concentrate 1%, glycerol 0.2% w/v and Tween 80 0.02%, w/v). The film-forming dispersions were cast into Petri dishes (9 cm diameter) and dried in an oven (FD 240, Binder, Tuttlingen, Germany) at 45 °C for 21 h. The resulting dried films (F-ChG and F-ChGC, respectively) were conditioned at 58% RH (with saturated KBr solution) and 22 °C for 4 days prior to analysis.

2.3. Viscoelastic properties of film-forming dispersions

A dynamic viscoelastic study of the film-forming dispersions (S-ChG and S-ChGC) was carried out on a Bohlin CVO-100 rheometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry (cone angle 4°, gap 0.15 mm). A dynamic frequency sweep from 0.1 to 10 Hz took place at auto stress, temperature of 10 °C, and target strain of 0.005%. The elastic modulus (G' ; Pa) and viscous modulus (G'' ; Pa) were

plotted as functions of the frequency ramp. To characterize the frequency dependence of G' over the limited frequency range, the following power law was used:

$$G' = G_0' \omega^{n'}$$

Where G_0' is the energy stored and recovered per cycle of sinusoidal shear deformation at an angular frequency of 1 Hz, ω is the angular frequency and n' is the power law exponent; which should exhibit an ideal elastic behavior near zero in gels. At least two determinations were performed for each sample. The experimental error was less than 6% in all cases.

2.4. Film determinations

2.4.1. Thickness, Moisture content and Water solubility

The thickness was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 6–8 random locations in 15 films for each treatment.

The moisture content was determined according to AOAC 2000.

Regarding water solubility, film circumferences 40 mm in diameter were placed in plastic containers with 50 mL distilled water and placed at 22 °C for 24 h. The solution was then filtered through Whatman No. 1 filter paper to recover the remaining undissolved film, which was desiccated at 105 °C for 24 h. Film solubility (FS, %) was calculated using the expression $[(W_o - W_f)/W_o] \times 100$, where W_o was the initial weight of the film expressed as dry matter and W_f was the weight of the undissolved desiccated film residue. All tests were carried out at least in triplicate.

2.4.2. Water vapor permeability

The water vapor permeability (WVP) was determined at least in triplicate at room temperature, following the gravimetric method described by Pérez-Mateos *et al.* (2009) using a desiccator with distilled water.

2.4.3. Color

The color parameters lightness (L^*), redness (a^*) and yellowness (b^*) were measured in the L^* , a^* , b^* mode of the CIE scale using a Konica Minolta 3500-D colorimeter (Osaka, Japan). Results are the average of at least 10 replicates.

2.4.4. Mechanical properties

The breaking strength (BS) and deformation (D) were determined by the puncture test. The tensile strength (TS), elongation at break (EB) and Young's modulus (YM) were determined by the tensile test. Puncture and tensile tests were run at least in triplicate using a TA.XT plus TA-XT2 texture analyzer (Texture Technologies Corp., Scarsdale, NY, USA) as described by Alemán *et al.* (2011).

2.4.5. Microstructure

Low temperature scanning electron microscopy (LowT-SEM) (Oxford CT1500 Cryosample Preparation Unit, Oxford Instruments, England) was used to examine representative microstructural cross-sections of shrimp concentrate (C) and films. Samples were mounted with an optical coherence tomography compound, mechanically fixed onto the specimen holder and cryo-fractured after mounting, as described by Gómez-Guillén *et al.* (2007).

2.4.6. Differential scanning calorimetry (DSC)

Thermal analysis of films was performed using a thermal analyzer model TA-Q1000 (TA Instruments, New Castle, DE, USA) previously calibrated by running high purity indium (melting point, 156.4 °C; melting enthalpy, 28.44 J/g). Samples (around 10 mg) were tightly encapsulated in aluminium hermetic pans. Film samples were scanned under dry nitrogen purge (50 mL/min) between -20°C and 350 °C at a heating rate of 10 °C/min. Representative DSC traces have been displayed from -10°C to 350°C. The shrimp concentrate (C) was scanned under identical conditions between -20°C and 225 °C to avoid possible burning. Glass transition temperature (T_g , °C), peak temperature (T_{max} , °C) and enthalpies of conformational changes (ΔH) were measured at least in triplicate. Before the analysis, the films were dried under silica for one week, and the shrimp concentrate (C) was freeze-dried.

2.4.7. Fourier transform infrared spectroscopy (FTIR) analysis

Infrared spectra between 4000 and 650 cm^{-1} of films were recorded using a PerkinElmer Spectrum 400 Infrared Spectrometer (PerkinElmer Inc., Waltham, MA, USA) equipped with an ATR prism crystal accessory. The spectral resolution was 4 cm^{-1} and 32 scans were averaged for each spectrum. Measurements were performed at room temperature directly on the films (previously conditioned for 5 days in desiccators containing silica gel), which were placed on the surface of the ATR crystal and pressed with a flat-tip plunger until spectra with suitable peaks were obtained. The shrimp concentrate (C) was previously freeze-dried and analysed in the same way. All experiments were performed at least in triplicate and represented as average spectra. Background subtraction was done using the Spectrum software version 6.3.2 (PerkinElmer Inc.).

2.5. Antioxidant activities and Folin–Ciocalteu-reactive substances content

Shrimp concentrate (C) and films (F-ChG and F-ChGC) were suspended in distilled water (w/v) and shaken vigorously until homogeneous dispersions were obtained. All dispersions, including the original film-forming dispersions, were filtered through Whatman No. 1. An aliquot of 20 μ L of sample was used for analysis.

2.5.1. ABTS assay

The ABTS radical [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging capacity was determined as described by Alemán *et al.* (2011). Results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of dry sample based on a standard curve of vitamin C. All determinations were performed at least in triplicate.

2.5.2. FRAP assay

The ferric reducing power (FRAP) assay was carried out as previously described by Alemán *et al.* (2011). Results were expressed as μ moles Fe^{2+} equivalents/g of dry sample based on a standard curve of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. All determinations were performed at least in triplicate.

2.5.3. Photochemiluminescence assay

Superoxide (O_2^-) free radical scavenging capacity was determined by photochemiluminescence (PCL) assay as previously described by Pérez-Santín *et al.* (2013). The results were expressed as mg Vitamin C equivalent/g of dry sample. All samples were measured at least in triplicate.

2.5.4. Folin–Ciocalteu-reactive substances content

The Folin–Ciocalteu-reactive substances content was determined as previously described by Arancibia *et al.* (2014). Results were expressed as mg gallic acid (GA) equivalent/g of dry sample, based on a standard curve of gallic acid. All determinations were performed at least in triplicate.

2.6. Antimicrobial activity

The antimicrobial activity of shrimp concentrate (C), films (F-ChGC and F-ChGC) and film-forming dispersions (S-ChG and S-ChGC), was determined by the diffusion method in agar using sterile filter paper discs (5 mm diameter, Whatman No. 1). Paper discs soaked with 40 µl of the dispersions (shrimp concentrate (C) and filmogenic dispersions (S-ChG and S-ChGC)), as well as disks with a diameter of 15 mm obtained directly from the films, were laid on the surface of agar previously inoculated with 100 µL of the selected microorganisms (10^5 – 10^6 CFU/mL). Paper discs soaked (40 µL) with lactic acid solution (0.15 M) were used for control purposes. The activity was tested against 17 microbial microorganisms, selected for their importance in health or because they are responsible for food spoilage. Strains were obtained from the Spanish Type Culture Collection (CECT). After incubation (24 h), the diameter of the inhibition zone (considered as the antimicrobial activity) was measured with Corel Photo-Paint X3. Results were expressed as mm of inhibition growth (magnification ratio 1:10). Each determination was performed in duplicate.

2.7. Statistical analysis

Statistical tests were performed using the SPSS computer programmer (SPSS Statistical Software Inc., Chicago, IL, USA) for one-way analysis of variance. The difference of

means between pairs was resolved by means of confidence intervals using a Tukey-b test at a level of significance of $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Compositional properties of shrimp concentrate

Shrimp cooking effluent with 37% of dry matter was obtained as a by-product from a crustacean processing factory operating under industrial conditions. A centrifugal separator was used to concentrate the solid matter (shrimp concentrate), in order to simulate a feasible, one-step, non-contaminating industrial procedure. The yield of the recovery process was 86%, giving rise to a new effluent with 5% of dry matter. The resulting cleaned effluent, consisting of $95.0 \pm 0.01\%$ of water, $2.35 \pm 0.02\%$ of protein, $2.08 \pm 0.02\%$ of lipid and $0.13 \pm 0.012\%$ of ash, could provide a considerable reduction of depollution costs for the industry.

The recovered shrimp concentrate (C), with 32% of dry matter, had an intense orange color, shiny appearance and creamy consistency, indicating that a stable emulsion was formed as a result of the strong centrifugation step. The concentrate presented a high lipid component ($16.4 \pm 2.5\%$). The lipid content of shrimp is normally higher in shrimp waste than in the shrimp edible part, owing to the visceral fat content located in the head (Mandeville *et al.*, 1992). The protein and ash contents in C were $11.4 \pm 0.07\%$ and $1.43 \pm 0.14\%$, respectively. The estimated amount of carbohydrate content, calculated by subtracting the sum of percentages of moisture, protein, fat and ash from 100%, was 2.83%. Pérez-Santín *et al.* (2013) reported similar results (lipid 13.5%, protein 11.6%, ash 0.92%) for an active shrimp concentrate obtained under similar conditions, indicating the feasibility of this simple separating system to obtain a valuable product from industrial shellfish cooking wastewater. The shrimp concentrate (C) was explored

as a natural active ingredient to be incorporated in chitosan–gelatin blends to provide them with enhanced antioxidant and antimicrobial properties.

3.2. Viscoelastic properties of film-forming dispersions

The viscoelastic properties of the composite dispersions (S-ChG and S-ChGC) are presented in Figure 1. The mechanical spectra revealed that the elastic modulus (G') and viscous modulus (G'') were considerably higher for S-ChG than for S-ChGC, indicating that the incorporation of C caused strong interference in the complex polymeric matrix formed by gelatin and chitosan. Both S-ChG and, especially, S-ChGC showed predominantly viscous behavior ($G'' > G'$) throughout the whole frequency range. These results indicate that both dispersions were not able to form a gel, acting predominantly as colloidal dispersions, probably because gelatin did not reach the critical gelling concentration. It should be noted that the pH of the original biopolymer solutions was 6.2, and some studies have reported that chitosan–gelatin solutions at pH 6 show increased electrostatic repulsion forces because chitosan is positively charged at this pH (Kołodziejska et al., 2006). This condition might hinder polymer interactions, thus increasing the ability of C to cause interference between chitosan and gelatin.

The mechanical spectra of both S-ChG and S-ChGC were successfully fitted to the power law model, with $R^2 > 0.99$ for G' and G'' in all cases. The S-ChGC dispersion showed much stronger oscillation frequency dependence than S-ChG, as deduced from the higher n' values, which were 0.60 and 1.24 for S-ChG and S-ChGC, respectively. Thus, the incorporation of the shrimp concentrate in the chitosan–gelatin mixture caused notable interference, leading to less cohesive and more unstable polymer dispersion.

3.3. Film properties

Both composite dispersions showed good film-forming capacity, leading to transparent and visually homogeneous films, which were easy to unmold and handle.

3.3.1 Morphological aspects

The thickness of F-ChG was $\sim 34\ \mu\text{m}$. The incorporation of C increased the film thickness to $\sim 54\ \mu\text{m}$. The microstructures of the shrimp concentrate (C) and the cross-sections of F-ChG and F-ChGC films are shown in Figure 2. The concentrate was predominantly formed by uniformly distributed homogeneous lipid droplets, indicating that the centrifugal separation process led to a fine microemulsion, although some protein aggregates remained clearly visible. The cross-section images showed noticeable differences in the microstructure of F-ChG and F-ChGC films. The uniform structure reported by Liu *et al.* (2012) for a chitosan–gelatin film was similar to the structure observed in the present F-ChG film. The addition of C provoked a strong disruption of the continuous dense film structure, in agreement with the rheological properties of the corresponding film-forming dispersions. The lipid droplets lost their overall spherical appearance, but remained homogeneous in size and were uniformly embedded throughout the biopolymer network, leading to a less compact thicker film. The distortion of fat droplets in the film might be associated with evaporation of water during film formation (Aranberri et al., 2004). Anyhow, the regular structure of F-ChGC indicates that C was properly mixed with gelatin and chitosan at the microstructural level.

3.3.2. Differential scanning calorimetry

The DSC behaviour of F-ChG and F-ChGC films is presented in Figure 3. Both traces presented a single glass transition temperature (T_g) at 53.9 °C ($\Delta C_p=0.20$ J/(g·°C)) and 52.1 °C ($\Delta C_p=0.04$ J/(g·°C)) for F-ChG and F-ChGC, respectively, which was assigned to the amorphous portion of the complex biopolymeric matrix formed by the interaction between gelatin and chitosan. Both gelatin and chitosan have been reported to produce highly miscible blend films with similar T_g as in the present work (Bonilla et al., 2017; Hosseini et al., 2013). The DSC trace of the shrimp concentrate (C) showed a transition at $T_g=28.7$ °C ($\Delta C_p=0.16$ J/(g·°C)). The slight reduction of both T_g and heat capacity change of F-ChGC as compared to F-ChG, indicated some ability of C to penetrate between the polymer chains and therefore weaken the interaction between gelatin and chitosan. This effect led to a slight decrease in thermal stability without disturbing the high miscibility of both biopolymers. Contrary to other reports working on gelatin-chitosan films, no evident crystal melting was observed at around 55 °C (Gómez-Estaca et al., 2011; Pérez-Córdoba et al., 2017). This difference could be due to the additional plasticizing effect of lactic acid used for dissolving chitosan, in combination with glycerol.

The film without the shrimp concentrate (F-ChG) showed two pronounced endothermic peaks at $T_{max}=103$ °C (41.1 J/g) and $T_{max}=198$ °C (108 J/g), which could be associated to the disruption of ordered or aggregated structures (crystalline phase) of gelatin and chitosan, respectively. To this respect, melting transitions at 102 °C or 117 °C have been reported to occur in pure bovine gelatin films (Liu et al., 2016) or in tilapia skin gelatin films (Tongnuadnchan et al., 2016), respectively. This transition is attributed to the thermal rupture of hydrogen bonds of the triple helix and the rearrangement into the random coil configuration. On the other hand, an endothermic event at 208 °C, associated to chitosan crystallization, was observed in a previous study with single

chitosan films prepared also with lactic acid (Arancibia et al., 2015). Both endothermic transitions in F-ChG were replaced by one slight single melting event at 130 °C (7.20 J/g) in the F-ChGC film by the addition of the concentrate. The DSC trace of C presented a main endothermic peak at 124 °C (7.72 J/g), probably related to protein thermal breakage. No previous sign of melting associated to protein denaturation was evidenced in C, probably because proteins were mostly aggregated in the cooking effluent. These findings denoted that the addition of the shrimp protein concentrate produced a noticeable plasticizing effect, causing the remaining partially crystalline structure in F-ChG films to disappear. Furthermore, the wide exothermic event representing thermal polymer decomposition in F-ChG was split into two peaks and shifted from 316 °C to 229 °C and 212 in F-ChGC, indicating considerable reduction in film thermal stability by the addition of the shrimp concentrate.

3.3.3. ATR-FTIR spectroscopy

The FTIR spectra of F-ChG and F-ChGC are shown in Figure 4. Both F-ChG and F-ChGC exhibited a broad band between 3100 and 3600 cm^{-1} , which corresponds to stretching vibrations of –OH and –NH groups, due to the presence of intermolecular hydrogen bonds associated with the chitosan pyranose ring and the gelatin amide A. The higher peak intensity in the F-ChGC IR spectrum might be the result of the contribution of the C protein component, which as shown in Figure 4 (inset), presented very high IR absorption at this wavelength range. Similarly, bands at 1633 cm^{-1} , 1538 cm^{-1} and 1230 cm^{-1} , assigned, respectively, to amide I (C=O stretching vibrations), amide II (N-H bending and C-N stretching vibrations) and amide III, were considerably larger in the film containing the shrimp concentrate. Specifically, C sample presented relative maximum IR peak absorptions at 1627 cm^{-1} , 1545 cm^{-1} and 1238 cm^{-1} ,

376 corresponding to the protein amide I, II and III stretching vibrations. The noticeable
377 lipid content in C was reflected mainly by two **characteristic sharp** peaks at 2924 cm^{-1}
378 and 2853 cm^{-1} , attributed to the asymmetric and symmetric stretching vibrations of the
379 aliphatic CH_2 group of fatty acids. The slight shoulder at 1747 cm^{-1} in C sample,
380 assignable to the stretching vibration of the $\text{C}=\text{O}$ group, revealed the presence
381 triglycerides taking part of the lipid component. The predominant contribution of fatty
382 acids to the typical FTIR spectrum of edible oils is characterised by the presence of
383 three main peaks at the wavenumbers of 1746 cm^{-1} , 2924 cm^{-1} and 2854 cm^{-1} (Guillén
384 and Cabo, 2000). The IR absorbance in F-ChG and F-ChGC films peaking at 2926 cm^{-1}
385 in the former and at 2936 cm^{-1} in the latter was mainly assigned to the stretching
386 vibrations of the CH_2 groups of both chitosan and protein amide B of gelatin. The lipid
387 content of C did not increase the absorbance intensity associated to the aliphatic CH_2
388 group stretching vibration in the F-ChGC film, but it caused a noticeable frequency
389 upshift. The small band at 1723 cm^{-1} , appearing similarly in IR spectra of both F-ChG
390 and F-ChGC films, was mostly attributed to the ester groups of lactic acid existing as
391 side chains, forming hydrogen bonds with amino or hydroxyl groups of chitosan (Zhang
392 & Cui, 2012). The great similarity in the band associated with the presence of lactic
393 acid, together with the absence of any detectable frequency shift in amide I (1633 cm^{-1})
394 and amide II (1538 cm^{-1}) bands in both studied films, indicated that there was little
395 hydrogen bonding between C and the complex chitosan–gelatin matrix. Strong peaks at
396 ≈ 1123 , 1082 and $\approx 1034\text{ cm}^{-1}$ appearing in F-ChG film were mostly attributed to the
397 predominantly saccharide structure of chitosan, as well as to the presence of glycerol.
398 As shown in the inset of Figure 4, the shrimp concentrate showed very low IR
399 absorption at this wavenumber range, in accordance to the absence of chitosan and
400 glycerol in its chemical composition. The reduced peak intensities at 1082 and ≈ 1034

cm⁻¹ in the F-ChGC spectrum would then agree with the structural non-specific interference caused by C in the polymeric chitosan–gelatin network.

3.3.4. Mechanical properties

As revealed in Table 1, the addition of the shrimp concentrate affected slightly the mechanical properties of the composite film. The breaking strength and deformation (determined by the puncture test), as well as the elongation at break and Young's modulus (determined by the tensile test), were not significantly different in the F-ChG and F-ChGC films. However, the tensile strength was noticeably higher ($P \leq 0.05$) for F-ChG than for F-ChGC, suggesting that the uniformly distributed lipid droplets of C led to a less rigid film. These results were in accordance with the rheological, microstructural, as well as FTIR and DSC observations, where C was found to interfere with the polymeric chitosan–gelatin blend, causing matrix discontinuity with no apparent chemical interaction. F-ChG showed higher tensile strength and lower elongation at break values than films prepared only with chitosan (Silva-Weiss *et al.*, 2013). It is well known that the presence of proteins in chitosan films could affect their mechanical properties. However, when our films were compared with the values reported for films made from a mixture of chitosan and fish skin gelatin (Gómez-Estaca *et al.*, 2011), breaking strength and breaking deformation were higher for both F-ChG and F-ChGC films.

3.3.5. Water barrier properties

The incorporation of C in the composite film slightly decreased (1.2-fold) the moisture content (Table 2). Arancibia *et al.* (2014) also found that the addition of a protein concentrate from shrimp waste caused a decrease of moisture in the resulting chitosan

films, but in that case, the decrease was more pronounced (two-fold decrease). The high lipid content of C (~16%) decreased the hygroscopicity of F-ChGC, causing a slight moisture reduction, which, however, did not substantially modify the overall mechanical behavior. The moisture values of the F-ChG and F-ChGC films were similar to those obtained by Park and Zhao (2004) for chitosan-based films and by Arancibia *et al.* (2014) for chitosan films with a protein concentrate.

Although the addition of C modified the structure of the chitosan–gelatin film, it hardly altered its water barrier properties. No significant differences ($P \leq 0.05$) in water solubility were found in chitosan–gelatin films with or without the shrimp concentrate (Table 1). On the contrary, the solubility of a chitosan film was decreased by the addition of a protein concentrate from shrimp waste (Arancibia *et al.*, 2014). In this regard, Gómez-Estaca *et al.* (2010) found that, when clove essential oil was added to a gelatin film, the water solubility of the resulting films increased significantly, but when this essential oil was added to a gelatin–chitosan matrix the solubility of the film was maintained. This was explained as being due to specific interactions between gelatin and chitosan, hindering their interaction with water molecules, regardless of the presence of other compounds. The solubility of our films was similar to previous results obtained for gelatin films from squid (Giménez *et al.*, 2009) or cod, (Pérez-Mateo *et al.*, 2009) but it was considerably higher than that of other films obtained from chitosan and gelatin (Gómez-Estaca *et al.*, 2011).

The water vapor permeability of edible films could be improved by the addition of lipid compounds. This may occur because a hydrophilic film can be laminated with a lipid bilayer, by forming a composite film in which the hydrophilic and hydrophobic components are dispersed in a solvent and then dried, or by emulsifying the lipid within the hydrophilic phase (McHugh & Krochta 1994). However, the addition of C, with a

high lipid content (~16%), did not allow a decrease in WVP values of the resulting film (Table 1), because these C lipids were already emulsified with the C protein in a very stable form, as observed by SEM (Figure 2), hindering any chemical interaction with the chitosan–gelatin matrix. In contrast, Arancibia *et al.* (2014) found that the addition of a shrimp protein concentrate significantly increased the WVP values of the resulting chitosan films. As explained above, the mixture of chitosan and gelatin would allow a relatively high water-resistant structure. The WVP values of the F-ChG and F-ChGC films were similar to those reported for tuna gelatin–chitosan films (Gómez-Estaca *et al.*, 2014).

3.3.6. Color properties

Optical properties of edible films affect the appearance of the coated product, which is an important quality factor. Table 1 shows the color parameters, L^* (lightness), a^* (reddish/greenish) and b^* (yellowish/bluish), of F-ChG and F-ChGC. The L^* parameter was similar for the two films, indicating that the presence of C in the film did not affect lightness. However, as expected, the film with C (F-ChGC) showed higher values of a^* and b^* parameters than the control film (F-ChG), which may be attributed mainly to the presence of astaxanthin in the shrimp concentrate, which is mainly responsible for the typical orange crustacean coloration (Ramya Devi *et al.*, 2012).

3.4. Antioxidant activity

Ferric reducing power (FRAP), free radical scavenging capacity (PCL and ABTS) and Folin–Ciocalteu-reactive substance content of the shrimp concentrate (C), the film forming dispersions (S-ChG and S-ChGC) and films (F-ChG and F-ChGC) are shown in Table 2. The shrimp concentrate showed a notable antioxidant activity when it was

measured by FRAP and PCL methods. ABTS radical scavenging capacity, however, was hardly detected, largely attributable to the low water solubility of C. The antioxidant activity of C may be due, largely, to the presence of lipophilic compounds, such as carotenoids, including carotenoprotein and lipoproteins (Liñán *et al.*, 2002). The positive correlation between carotenoid content in shrimp waste, especially astaxanthin, and its antioxidant activity has been previously documented (Liñán *et al.*, 2002; Nuñez-Gastelum *et al.*, 2011). The presence of active small peptides in shrimp concentrate, attributed to endogenous enzymatic hydrolysis and thermal degradation of proteins, may also contribute to its high antioxidant activity (Pérez-Santín *et al.*, 2013). In this regard, hydrolysates with antioxidant activity from various crustaceans have been reported (Manni *et al.*, 2010). Other antioxidant compounds might be principally phenolic compounds and reducing sugars. In this connection, C showed a relatively high content of Folin–Ciocalteu-reactive substances. Although the Folin–Ciocalteu assay is a widely used method for determining total phenol contents, other substances such as sugars and proteins can react with the Folin–Ciocalteu reagent and should be taken into account (Prior *et al.*, 2005). Phenolic compounds with antioxidant capacity have been found previously in shrimp shell wastes (Seymour *et al.*, 1996).

In view of the antioxidant properties of the shrimp concentrate, it was used to enrich the film prepared from chitosan and gelatin. The film without shrimp concentrate (F-ChG) contained Folin–Ciocalteu-reactive substances and exhibited antioxidant activity measured by all the assays, FRAP, PLC and ABTS. The antioxidative properties may be attributable to the peptide fraction of gelatin (Alemán *et al.*, 2011; Giménez *et al.*, 2009) and to the residual free amino group of chitosan, which can react with free radicals, forming a stable macromolecular radical and ammonium group (Xing *et al.*, 2005). The incorporation of C into the film (F-ChGC) significantly increased (3–3.5-fold increase)

the Folin–Ciocalteu-reactive substances content, the reducing power and the superoxide radical scavenging capacity measured by PCL. The ABTS radical scavenging capacity, however, was similar for the two films. These results are in agreement with the antioxidant activity of C. The shrimp concentrate showed similar antioxidant capacity in the chitosan–gelatin films to that of the corresponding filmogenic dispersions. In contrast, an alcalase gelatin hydrolysate showed lower antioxidant capacity in the gelatin film matrix than in the corresponding filmogenic solution (Giménez *et al.*, 2009). The increased antioxidant activity of F-ChGC and S-ChGC could be useful for the preservation of several products, especially certain types of food in which oxidation processes are a limiting factor determining shelf life.

3.5. Antimicrobial activity

Given the antioxidant properties exhibited by F-ChGC, this film and the corresponding film-forming dispersion (S-ChGC) were selected to evaluate their antimicrobial activity. The active compounds diffused from the film-forming solution to the agar (Table 3), leading to inhibition zones higher ($p \leq 0.05$) than those observed with the resulting films, in which activity was evident but was limited to the area in contact with the agar (data not shown). This different effect could be due both to the lower water content in the film in comparison with the film-forming solution (which could impede diffusion) and to the solid state of the film, which might favor the sorption of free active groups, limiting antimicrobial activity to a specific area. Coma *et al.* (2002) stated that chitosan in film form is unable to diffuse through adjacent agar media, probably because of reduced availability of NH_3^+ groups and consequent limitation of antibacterial properties (Elsabee & Abdou, 2013). It should be noted that lactic acid, used to dissolve chitosan, did not show antimicrobial activity (data not shown).

The S-ChGC dispersion inhibited the growth of potentially pathogenic microorganisms such as *L. monocytogenes* and *S. choleraesuis*, as well as microorganisms involved in fish spoilage such as *S. putrefaciens* and *Pseudomonas* sp ($p \leq 0.05$). However, they did not affect the growth of *L. acidophilus* (a potentially probiotic microorganism). On the other hand, molds did not show sensitivity to these formulations. In this regard, Fang et al. (1994) indicated that chitosan concentrations up to 5 mg/mL (pH 5.4) did not effectively inhibit growth of *A. niger*. The concentration of chitosan in this study was 10 mg/mL (pH 6.2). The formulation tested (chitosan–gelatin–shrimp concentrate) was effective against Gram-positive and Gram-negative bacteria, although higher inhibition zones corresponded to Gram-positives (e.g. *B. thermosphacta*, *L. monocytogenes* and *S. aureus*). In this connection, chitosan has been reported to be more effective against Gram-positive than Gram-negative bacteria (Gildberg & Stenberg, 2001). Among the mechanisms of action proposed, chitosan may form a polymer membrane on the cell surface, preventing nutrients from entering the cell. In the case of chitosan with low molecular weight, they can enter the cell by pervasion, disturbing the physiological activities of bacteria and causing them to flocculate (Gómez-Guillén *et al.*, 2011).

The shrimp concentrate alone showed antimicrobial activity when tested against the above-mentioned microorganisms (Table 3). The addition of this concentrate to the chitosan–gelatin solution did not modify its activity. Moreover, sometimes the activity of the complex dispersion increased. Chitosan has good antimicrobial activity if the pH is lower than the isoelectric point. If chitosan and protein are positively charged, the interactions between them are restricted (Jeon *et al.*, 2001). Consequently, chitosan could interact with the negative residual charge on the surface of bacterial cells, disrupting the membrane integrity of the microorganism and thus improving the antimicrobial effect. A similar effect could occur with the protein concentrate. Although

the pH of the dispersions was near neutral, the presence of protonated groups was observed by FTIR (Fig. 3). The antimicrobial activity of F-ChGC or its respective film-forming dispersion (S-ChGC) might therefore improve the shelf life and safety of the covered product.

4. Conclusions

A shrimp cooking effluent microemulsified concentrate, rich in lipid and protein, with antioxidant and antimicrobial properties, was recovered by means of a centrifugal separator. The shrimp concentrate rendered homogeneous and highly plasticized films when mixed with a gelatin-chitosan blend, with intense orange coloration and low transparency. Both the film forming dispersion and the film containing the shrimp concentrate exhibited antioxidant and antimicrobial capacities, being good candidates for development of active materials for use in food, medical or cosmetic industry. Regarding the possibilities of food applications, the film could be used for improving preservation or for food design purposes, for example as a "paste" substitute either in strips, sheets, or in the form of refillable pouches as *Gyozas* or tortellini. Furthermore, the recovery of this concentrate would contribute to environmental care and the sustainability of marine resources.

Acknowledgements

This research was financed by: (i) the Spanish Ministry of Economy and Competitiveness through project AGL2011-27607, AGL2014-52825-R and project AGL2017-84161-C2 and (ii) the Spanish National Research Council (CSIC) through

574 project 201370E036. M. Arancibia was funded by a SENESCYT Scholarship provided
575 by the Ecuadorian government.

576

5. References

- Alemán, A., Giménez, B., Montero, P., & Gómez-Guillén, M. C. (2011b). Antioxidant activity of several marine skin gelatins. *LWT - Food Science and Technology*, 44, 407–413.
- Alemán, A., González, F., Arancibia, M. Y., López-Caballero, M. E., Montero, P., Gómez-Guillén, M. C. (2016). Comparative study between film and coating packaging based on shrimp concentrate obtained from marine industrial waste for fish sausage preservation. *Food Control*, 70, 325–332.
- AOAC. (2000). Official methods of analysis. Gaithersburg, MD, USA: Association of Official Analytical Chemists.
- Aranberri, I., Binks, B. P., Clint, J. H., Fletcher, P. D. (2004). Evaporation rates of water from concentrated oil-in-water emulsions. *Langmuir*, 16, 2069–74.
- Arancibia, M. Y., Alemán, A., Calvo, M., López-Caballero, M. E., Montero, P., & Gómez-Guillén, M. C. (2014). Antimicrobial and antioxidant chitosan solution enriched with active shrimp (*Litopenaeus vannamei*) waste materials. *Food Hydrocolloids*, 35, 710–717.
- Arancibia, M. Y., Alemán, A., Calvo, M., López-Caballero, M. E., Gómez-Guillén, M. C. & Montero, P., 2015. Development of active films of chitosan isolated by mild extraction with added protein concentrate from shrimp waste. *Food Hydrocolloids* 43, 91-99.
- Arancibia, M. Y., Lopez-Caballero, M. E., Gómez-Guillén, M. C., Fernández-García, M., Fernández-Martín, F., Montero, P. (2015). Antimicrobial and rheological properties

600 of chitosan as affected by extracting conditions and humidity exposure. *LWT - Food*
601 *Science and Technology*, 60, 802–810.

602 Bligh, E. G. & Dyer, W. J. (1959). A rapid method for total lipid extraction and
603 purification. *Canadian Journal of Biochemistry and Physiology*, 37, 911-917.

604 Bonilla, J., Bittante, A.M.Q.B., Sobral, P.J.A. (2017). Thermal analysis of gelatin–
605 chitosan edible film mixed with plant ethanolic extracts. *Journal of Thermal Analysis*
606 *and Calorimetry*, 130 (2), 1221-1227.

607 Bourseau P, Massé A, Cros S, Vandanjon L and Jaouen P, 2014. Recovery of aroma
608 compounds from seafood cooking juices by membrane processes. *J Food Eng* 128,157–
609 166.

610 Coma, V., Martial-Gros, A., Garreau, S., Copinet, A., Salin, F., & Deschamps, A.
611 (2002). Edible antimicrobial films based on chitosan matrix. *Journal of Food Science*,
612 67, 1162–1169.

613 Cros, S., Lignot, B., Jaouen, P., Bourseau, P. (2006). Technical and economical
614 evaluation of an integrated membrane process capable both to produce an aroma
615 concentrate and to reject clean water from shrimp cooking juices. *Journal of Food*
616 *Engineering*, 77, 697–707.

617 Elsabee, M. Z., & Abdou, E. S. (2013). Chitosan based edible films and coatings: a
618 review. *Material Science and Engineering. Materials for Biological Applications*, 33,
619 1819-1841.

620 Fang, S.W., Li, C. F., & Shih, D. Y. C. (1994). Antifungal activity of chitosan and its
 621 preservative effect on low-sugar candied kumquat. *Journal of Food Protection*, 57,
 622 136–140.

623 Gildberg, A., & Stenberg, E. (2001). A new process for advanced utilization of shrimp
 624 waste. *Process Biochemistry*, 36, 809–812.

625 Giménez, B., Gómez-Estaca, J., Alemán, A., Gómez-Guillén, M. C, Montero, P. (2009).
 626 Improvement of the antioxidant properties of squid skin gelatin films by the addition of
 627 hydrolysates from squid gelatin. *Food Hydrocolloids*, 23, 1322-1327.

628 Giménez, B., Gómez-Guillén, M. C., López-Caballero, M. E., Gómez-Estaca, J., &
 629 Montero, P. (2013). Role of sepiolite in the release of active compounds from gelatin-
 630 egg white films. *Food Hydrocolloids*, 27, 475–486.

631 Gómez-Estaca, J., Gómez-Guillén, M. C., Fernández-Martín, F., & Montero, P. (2011).
 632 Effects of gelatin origin, bovine-hide and tuna-skin, on the properties of compound
 633 gelatin–chitosan films. *Food Hydrocolloids*, 25, 1461–1469.

634 Gómez-Estaca, J., López de Lacey, A., López- Caballero, M. E., Gómez-Guillén, M. C.,
 635 & Montero, P. (2010). Biodegradable gelatin-chitosan films incorporated with essential
 636 oils as antimicrobial agents for fish preservation. *Food Microbiology*, 27, 889–896.

637 Gómez-Guillén, M. C., Giménez, B., López-Caballero, M. E., & Montero, M. P. (2011).
 638 Functional and bioactive properties of collagen and gelatin from alternative sources: A
 639 review. *Food Hydrocolloids*, 25, 1813–1827.

640 Gómez-Guillén, M. C., Ihl, M., Bifani, V., Silva, A., & Montero, P. (2007). Edible films
 641 made from tuna-fish gelatin with antioxidant extracts of two different murta ecotypes
 642 leaves (*Ugni molinae* Turcz). *Food Hydrocolloids*, 21, 1133–1143.

643 Guillén, M.D. & Cabo, N. (2000). Some of the most significant changes in the Fourier
 644 transform infrared spectra of edible oils under oxidative conditions. *Journal of the*
 645 *Science of Food and Agriculture*, 80, 2028-2036.

646 Hosseini, S.F., Rezaei, M., Zandi, M., & Ghavi, F.F. (2013). Preparation and functional
 647 properties of fish gelatin-chitosan blend edible films. *Food Chemistry*, 136 (3-4), 1490-
 648 1495.

649 Jeon, Y. J., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of
 650 chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers*, 44, 71–76.

651 Liñán-Cabello, M. A., Paniagua-Michel, J., & Hopkins, P. M. (2002). Bioactive roles of
 652 carotenoids and retinoids in crustaceans. *Aquaculture Nutrition*, 8, 299–309.

653 Liu, Z., Ge, X., Lu, Y., Dong, S., Zhao, Y., & Zeng, M. (2012). Effects of chitosan
 654 molecular weight and degree of deacetylation on the properties of gelatine-based films.
 655 *Food Hydrocolloids*, 26, 311–317.

656 Liu, F., Majeed, H., Antoniou, J., Li, Y., Ma, Y., Yokoyama, W., Ma, J., & Zhong, F.
 657 (2016). Tailoring physical properties of transglutaminase-modified gelatin films by
 658 varying drying temperature. *Food Hydrocolloids*, 58, 20-28.

659 Mandeville, S., Yaylayan, V., & Simpson, B. K. (1992). Proximate analysis, isolation
 660 and identification of amino acids and sugars from raw and cooked commercial shrimp
 661 waste. *Food Biotechnology*, 6, 51–64.

662 Manni, L., Ghorbel-Bellaaj, O., Jellouli, K., Younes, I., & Nasri, M. (2010). Extraction
 663 and Characterization of Chitin, Chitosan, and Protein Hydrolysates Prepared from
 664 Shrimp Waste by Treatment with Crude Protease from *Bacillus cereus* SV1. *Applied*
 665 *Biochemistry and Biotechnology*, 162, 345–357.

666 McHugh, T. H. & Krochta, J. M. (1994). Water vapor permeability properties of edible
 667 whey protein–lipid emulsion films. *Journal of the American Oil Chemists Society*, 71,
 668 307–312.

669 Muxika, A., Etxabide, A., Uranga, J., Guerrero, P. & de la Caba, K. 2017. Chitosan as a
 670 bioactive polymer: Processing, properties and applications. *International Journal of*
 671 *Biological Macromolecules* 105, 1358–1368

672 Nady, N. & Kandil, S.H. 2018. Nobel blend for producing porous chitosan-based films
 673 suitable for biomedical applications. *Mebranes*, 8, 2-18. doi: 10.3390

674 Núñez-Gastélum, J. A., Sánchez-Machado, D. I, López-Cervantes, J., Paseiro-Losada,
 675 P., Sendón, J., Sanches-Silva, A. T., Costa, H. S., Aurrekoetxea, G. P., Angulo, I., &
 676 Soto-Valdez, H. (2011). Physical and chemical properties of pigmented oil obtained
 677 from shrimp heads. *Grasas y Aceites*, 62 doi:10.3989/gya.102710.

678 Park, S. & Zhao, Y. (2004). Incorporation of a High Concentration of Mineral or
 679 Vitamin into Chitosan-Based Films. *Journal of Agricultural and Food Chemistry*, 52,
 680 1933–1939.

681 Pérez-Córdoba, L.J., Norton, I.T., Batchelor, H.K., Gkatzionis, K., Spyropoulos, F., &
 682 Sobral, P.J.A. (2017). Physico-chemical, antimicrobial and antioxidant properties of

683 gelatin-chitosan based films loaded with nanoemulsions encapsulating active
 684 compounds. *Food Hydrocolloids*, in Press.

685 Pérez-Mateos, M., Montero, P., & Gómez-Guillén, M. C. (2009). Formulation and
 686 stability of biodegradable films made from cod gelatin and sunflower oil blends. *Food*
 687 *Hydrocolloids*, 23, 53-61.

688 Pérez-Santín, E., Calvo, M.M, López-Caballero, M. E., Montero, P., & Gómez-Guillén,
 689 M. C. (2013). Compositional properties and bioactive potential of waste material from
 690 shrimp cooking juice. *LWT - Food Science and Technology*, 54, 87– 94.

691 Prior, R., Wu, X., & Schich, K. (2005). Standardized Methods for the Determination of
 692 Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *Journal of*
 693 *Agricultural and Food Chemistry*, 53, 4290–4302.

694 Qiao, C., Ma, X., Zhang, J. And Yao, J. 2017. Molecular interactions in gelatin/
 695 chitosan composite films. *Food Chemistry* 235, 45-50.

696 Ramyadevi, D., Subathira, A., & Saravanan, S. (2012). Potential Recovery of Protein
 697 from Shrimp Waste in Aqueous two Phase System. *Research Journal of Chemical*
 698 *Sciences* 2, 47–52.

699 Seymour, T. A., Li, S. J., & Morrissey, M. T. (1996). Characterization of a natural
 700 antioxidant from shrimp shell waste. *Journal of Agricultural and Food Chemistry*, 44,
 701 682–685.

702 Silva-Weiss, A., Bifani, V., Ihl, M., Sobral, P. J. A., & Gómez-Guillén, M. C. (2013).
 703 Structural properties of films and rheology of film-forming solutions based on chitosan

704 and chitosan-starch blend enriched with murta leaf extract. *Food Hydrocolloids*, 31,
705 458–466.

706 Tongnuanchan, P., Benjakul, S., Prodpran, T., Pisuchpen, S., Osako, K. (2016).
707 Mechanical, thermal and heat sealing properties of fish skin gelatin film containing
708 palm oil and basil essential oil with different surfactants. *Food Hydrocolloids*, 56, 93-
709 107.

710 Vandanjon, L., Cros, S., Jaouen, B., Quéméneur, F., Bourseau, P. (2002). Recovery by
711 nanofiltration and reverse osmosis of marine flavours from seafood cooking waters.
712 *Desalination*, 144, 379–385.

713 Xing, R., S. Liu, Guo, Z., Yu, H., Wang, P., Li, C., Li, Z., & Li, P. (2005). Relevance of
714 molecular weight of chitosan and its derivatives and their antioxidant activities in vitro.
715 *Bioorganic & Medicinal Chemistry*, 13, 1573–1577.

716 Zhang, Z., & Cui, H. (2012). Biodegradability and biocompatibility study of poly
717 (chitosan-lactic acid) scaffolds. *Molecules*, 17, 3243–3258.

718 Zheng, L. Y., & Zhu, J. F. (2003). Study on antimicrobial activity of chitosan with
719 different molecular weights. *Carbohydrate Polymer*, 54, 527–530.

720

721

722

Figure 1. Frequency sweep test in terms of Elastic modulus (G') and Viscous modulus (G'') of chitosan-gelatin films without (F-ChG) and with the shrimp concentrate (F-ChGC).

741
742
743
744
745
746
747

748
749
750
751
752
753
754
755
756

Figure 2. Low Temperature-SEM micrographs of the shrimp concentrate (C) and cross-section of chitosan-gelatin films without (F-ChG) and with the shrimp concentrate (F-ChGC).

755
756

757
759 Figure 3. DSC traces of the freeze-dried shrimp concentrate (C) and chitosan-gelatin
760 films without (F-ChG) and with the shrimp concentrate (F-ChGC).
761
762
763
764
765
766
767
768

769
770
771

772
773
777
778
779
780
778

Figure 4. ATR-FTIR spectra of the freeze-dried shrimp concentrate (C) (inset) and the chitosan-gelatin films without (F-ChG, dotted line) and with the shrimp concentrate (F-ChGC, solid line). Wavenumber values plotted above main peaks correspond to sample F-ChGC.

Table 1. Physicochemical properties of chitosan-gelatin films without (F-ChG) and with the shrimp concentrate (F-ChGC).

	F-ChG	F-ChGC
Tensile strength (MPa)	41.2 ± 0.45 ^b	26.4 ± 1.5 ^a
Elongation at break (%)	8.53 ± 0.45 ^a	8.82 ± 0.80 ^a
Breaking strength (N)	36.5 ± 2.55 ^a	38.2 ± 2.2 ^a
Deformation (%)	105 ± 6.2 ^a	106 ± 6.6 ^a
Young's modulus (MPa)	37.2 ± 5.3 ^a	31.9 ± 3.3 ^a
Moisture (%)	23.2 ± 0.29 ^b	19.7 ± 1.1 ^a
Film solubility (%)	93.5 ± 4.2 ^a	90.6 ± 2.8 ^a
WVP* ($\times 10^{-8} \text{ g m}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}$)	2.06 ± 0.27 ^a	2.16 ± 0.13 ^a
Lightness (L^*)	33.5 ± 0.20 ^a	33.5 ± 0.10 ^a
Redness (a^*)	-0.76 ± 0.01 ^a	5.14 ± 0.19 ^b
Yellowness (b^*)	-0.16 ± 0.03 ^a	6.52 ± 0.19 ^b
Transparency	30.4 ± 0.04 ^b	18.8 ± 0.46 ^a

Results are the mean ± standard deviation. n=3. One-way ANOVA: Different letters indicate significant differences among the different films ($P \leq 0.05$).

* WVP: Water vapour permeability

Table 2. Antioxidant activity and Folin reactive substances of shrimp concentrate (C), chitosan-gelatin film forming dispersions (S-ChG, S-ChGC) and films (F-ChG, F-ChGC).

Sample	ABTS (mgVitCeq/g)	FRAP (μ molFe/g)	PCL (mgVitCeq/g)	Folin reactive substances (mg/g)
C	0.86 ± 0.01^a	45.8 ± 3.3^c	13.5 ± 0.65^c	25.0 ± 1.4^c
S-ChG	2.22 ± 0.18^b	4.50 ± 0.94^a	1.06 ± 0.36^a	1.32 ± 0.13^a
S-ChGC	2.17 ± 0.21^b	13.3 ± 1.4^b	3.41 ± 0.44^b	3.75 ± 0.43^b
F-ChG	2.05 ± 0.06^b	4.71 ± 0.16^a	0.72 ± 0.27^a	1.29 ± 0.15^a
F-ChGC	2.18 ± 0.11^b	13.6 ± 1.1^b	2.51 ± 0.47^b	3.67 ± 0.39^b

Results are the mean $\pm$ standard deviation, n=3. One-way ANOVA: Different letters indicate significant differences among the samples ($P \leq 0.05$).

Table 3. Antimicrobial activities (cm of inhibition growth) of shrimp concentrate (C) and chitosan-gelatin film-forming dispersions (S-ChG and S-ChGC).

Microorganism	C	S-ChG	S-ChGC
<i>Aeromonas hydrophila</i>	0.66 ± 0.03 ^a	0.65 ± 0.01 ^a	0.66 ± 0.01 ^a
<i>Aspergillus niger</i>	-	-	-
<i>Bacillus cereus</i>	-	0.60 ± 0.03	-
<i>Bacillus coagulans</i>	0.68 ± 0.02 ^b	-	0.75 ± 0.13 ^a
<i>Brochothrix thermosphacta</i>	0.72 ± 0.05 ^b	0.69 ± 0.04 ^b	0.91 ± 0.09 ^a
<i>Clostridium perfringens</i>	-	0.74 ± 0.05 ^a	0.68 ± 0.01 ^a
<i>Enterococcus faecium</i>	-	0.68 ± 0.02 ^a	0.66 ± 0.00 ^a
<i>Escherichia coli</i>	0.64 ± 0.02 ^a	0.61 ± 0.00 ^a	-
<i>Lactobacillus acidophilus</i>	-	-	-
<i>Listeria monocytogenes</i>	0.58 ± 0.01 ^c	0.66 ± 0.04 ^b	0.75 ± 0.08 ^a
<i>Photobacterium phosphoreum</i>	0.76 ± 0.01 ^a	0.61 ± 0.02 ^b	0.57 ± 0.00 ^b
<i>Pseudomonas fluorescens</i>	0.57 ± 0.00 ^a	-	0.53 ± 0.03 ^a
<i>Salmonella choleraesuis</i>	0.69 ± 0.02 ^a	0.67 ± 0.04 ^a	0.68 ± 0.02 ^a
<i>Shewanella putrefaciens</i>	0.57 ± 0.05 ^a	0.54 ± 0.05 ^a	0.57 ± 0.00 ^a
<i>Shigella sonnei</i>	0.66 ± 0.04 ^a	0.67 ± 0.03 ^a	0.66 ± 0.00 ^a
<i>Staphylococcus aureus</i>	-	0.68 ± 0.03 ^a	0.70 ± 0.07 ^a
<i>Yersinia enterocolitica</i>	1.23 ± 0.02 ^a	0.69 ± 0.01 ^b	-

Results are the mean ± standard deviation. Different letters (a,b,c) in the same row indicate significant differences between solutions (p≤0.05)