Bioaccessibility and antimicrobial properties of a shrimp demineralization extract blended with chitosan as wrapping material in ready-to-eat raw salmon

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ABSTRACT

A shrimp extract (SME) obtained from the mild-acid demineralization treatment of shrimp shells to produce chitosan was collected. It was mainly composed of fat ($\approx 73\%$), protein ($\approx 19\%$), and ash ($\approx 9\%$) and contained considerable amounts of calcium (≈ 1.9 g/100 g), astaxanthin (≈ 30 mg/100 g) and unsaturated fatty acids ($\approx 27\%$ MUFA, $\approx 39\%$ PUFA). The SME was used in combination with chitosan for wrapping raw salmon to produce a ready-to-eat product enriched in calcium. No significant changes in hardness were found, as compared to the unwrapped salmon. Estimated intakes of bioaccessible calcium increased significantly by 3.6-fold, whereas intake of bioaccessible fat was reduced by 15%. SFA were the main fatty acid group reduced ($\approx 80\%$), whereas MUFA and PUFA were only reduced by $\approx 20\%$ each. Total viable counts, pseudomonads, enterobacteria, and specific fish spoilers were reduced by 2–4 log CFU/g in wrapped sample during the chilled storage period (19 days).

Keywords

Shrimp waste; demineralization; calcium; polyunsaturated fatty acids; Bioaccessibility;
chitosan film.

1. INTRODUCTION

Current consumer trends towards healthy, nutritive, and convenient foods have driven research on functional foods. At the same time, both scientists and industry are continuously doing research on new preservation treatments and technologies in order to improve microbial and oxidative quality and extend shelf life. This is especially true in the case of highly perishable foods such as fresh fish. In this context, edible films and coatings are useful tools not only for improving quality and shelf life but also for incorporating functional ingredients as a part of food design (Blanco-Pascual & Gómez-Estaca, 2016). Chitosan is a natural cationic linear aminopolysaccharide obtained from chitin that is employed in the development of edible films and coatings. It possesses fat-binding properties and antimicrobial and antioxidant activity, and is an excellent carrier for sustained release and delivery of active compounds (Dutta, Tripathi, Mehrotra, & Dutta, 2009; Higuera, López-Carballo, Gavara, & Hernández-Muñoz, 2015; Panith, Wichaphon, Lertsiri, & Niamsiri, 2016), thus being a very interesting matrix for the development of functional foods.

The process of obtaining chitosan from crustacean shells involves sequential demineralization, deproteinization, and deacetylation, in which a considerable amount of waste containing proteins, lipids, calcium, magnesium, and pigments is produced (Gedda, Pandey, Lin, & Wu, 2015; Gildberg & Stenberg, 2001). Attempts have been made to give value to this waste, e.g., obtaining protein concentrates by enzymatic treatment after the demineralization step (Arancibia, Alemán, Calvo, López-Caballero, Montero, & Gómez-Guillén, 2014; Gildberg & Stenberg, 2001), but there is still room for improvement. For example, most of the calcium present in shrimp shells is discarded without its active extraction for further use. In a work by Gedda, Pandey, Lin, and Wu (2015), this calcium was extracted and employed as a green calcium source for the

design of calcium oxide-nanoplates with antimicrobial properties. Other components from shrimp cephalothorax that could be valorized during the process of obtaining chitosan are unsaturated lipids, α -tocopherol, and astaxanthin (Gómez-Estaca, Calvo, Álvarez-Acero, Montero, & Gómez-Guillén, 2017).

Bioaccessibility is defined as the fraction of a compound that is solubilized from a food during gastrointestinal digestion, and is considered as a measure of maximum oral bioavailability. Bearing this in mind, it is obvious that evaluating a functional compound's bioaccessibility is a more accurate measure of the amount of that compound that can reach the systemic circulation than simply determining its concentration in the food. Bioaccessibility determination becomes especially relevant in the case of chitosan-containing foods because it may chelate important nutrients such as minerals or polyunsaturated fatty acids, reducing the nutritional value of the food.

In the present work, the extract obtained from a mild demineralization treatment (using lactic acid) of shrimp heads and cuticles was collected, dried and characterized. A conventional chitosan film was selected to support the demineralization extract, with the aim to be used as a wrapping material for raw salmon pieces with functional potential to increase the intake of bioaccessible calcium, magnesium and fatty acids. At the same time, the effect of SME on the antimicrobial capacity of the chitosan-based film was also evaluated by studying the shelf life of wrapped raw salmon pieces.

2. MATERIALS AND METHODS

2.1. Materials

Frozen shrimp (*Litopenaeus vannamei*), kindly provided by Angulas Aguinaga (Burgos, Spain), was used for obtaining the shrimp mineral extract (SME) from the chitin mild

demineralization process. Fresh salmon was acquired in a local market. Chitosan (deacetylation degree 90.7%; molecular weight 140,000 Da) was supplied by Guinama (Valencia, Spain). Fatty acid standards were from Accustandard, Inc. (Barcelona, Spain). Culture media for microbiological analyses were from Oxoid (Basingstoke, UK) except Tryptone Soy Agar that was from Merck (Darmstadt, Germany). The kit to measure cytotoxicity was from Promega (Madrid, Spain). All other reagents were from Sigma-Aldrich (Madrid, Spain).

2.2. Obtaining of the shrimp demineralization extract (SME)

Frozen shrimp was thawed and peeled. Head and exoskeleton were mixed (1:3 w/v) with lactic acid (75 g/L) and kept in agitation for 36 hours at 21 °C. Then the mixture was filtered through gauze. The resulting liquid phase was recovered, freeze-dried and stored at -20 °C until use.

2.3. Chemical characterization of SME

Moisture and ashes of the SME were determined according to official methods (AOAC, 2000). The fat content was determined as per Bligh and Dyer Bligh and Dyer (1959). The protein content was calculated from the amino acid analysis, which was determined using a Biochrom 20 amino acid analyzer (Pharmacia, Barcelona, Spain), following a procedure described previously Gomez-Estaca, Gimenez, Montero, and Gomez-Guillen (2009).

Calcium, magnesium, sodium, potassium, zinc, iron, copper, manganese, and phosphorus cations were quantified on an atomic absorption spectrophotometer (Perkin Elmer, model 5100 PC, Norwalk, Connecticut, USA) with an air and acetylene oxidizing flame. The sample was prepared by acid digestion in the presence of nitric

acid and hydrogen peroxide with a microwave furnace (Microwave Digestion LabStation, Milestone Inc., Shelton, USA).

Total astaxanthin content was determined as described elsewhere Gómez-Estaca, Calvo, Álvarez-Acero, Montero, and Gómez-Guillén (2017). For fatty acid determination, 10 mg of lipid extract was derivatized into fatty acid methyl esters in triplicate and analyzed in an Agilent 7820A gas chromatograph with FID detector as previously described (Gómez-Estaca, Calvo, Álvarez-Acero, Montero, & Gómez-Guillén, 2017). The internal standard C13:0 was used for quantification, which was added to the sample in the non-methylated state before methylation, whereas identification was accomplished by comparison of the retention times with standards. Results were expressed as relative percentage of the identified fatty acids or as mg fatty acid/g lipid extract.

All determinations were performed at least in triplicate.

2.4. Cytotoxicity assay of SME

Cytotoxicity was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) colorimetric method using the RAW 264.7 cell line and the CellTiter 96 AQueous Non-radioactive cell proliferation assay kit. Cells (10^4 – 10^5 cells/mL) were cultured in DMEM supplemented with 10% (v/v) of fetal bovine serum, penicillin (100 units/mL), and gentamicin (50 µg/mL) in 96-well plates, and incubated at 37 °C in an atmosphere with 95% relative humidity and a CO₂ flow of 5%. SME was firstly suspended in DMSO, subsequently diluted in PBS (1 mg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL and 0.1 µg/mL), and finally filtered to sterilize. Cell viability was assayed after 24 h of incubation. A control sample without SME addition containing a comparable amount of diluted DMSO, and a sample

with 70% ethanol as positive control were also tested. The absorbance of each well was measured at 570 nm using a microplate reader (Bio-Tek, Winooski, VT, USA), and cell viability was expressed as % considering the absorbance of the control sample as 100%.

2.5. Film preparation

Chitosan was dissolved in 0.15 M lactic acid solution (2 g/100 mL) under overnight stirring. Separately, SME was dispersed in distilled water (6 g/100 mL) by stirring for 1 h, resulting in a uniform and stable solution. The film-forming solution (FFS) was prepared by mixing both solutions at 1:1 ratio to a final concentration of 1 g/100 mL of chitosan and 3 g/100 mL of SME. For comparative purposes, a chitosan film-forming solution without SME addition was also prepared at the same concentration (1 g/100 mL). Film-forming solutions (40 g) were cast in Perspex dishes (144 cm²) and dried in an oven (FD 240, Binder, Tuttlingen, Germany) at 45 °C for 21 h. The dried films were conditioned at 58% RH and 22 °C for 3 days prior to use. The resulting films are referred to as Ch (chitosan film) and Ch-SME (chitosan films with added SME). No plasticizers were needed to obtain flexible films (Arancibia, Aleman, Lopez-Caballero, Gomez-Guillen, & Montero, 2015).

2.6. Physico-chemical characterization of the films

Film thickness was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 5 random locations in 10 films.

For film water solubility determination, circular pieces of film 40 cm in diameter were placed in plastic containers with 50 mL of distilled water and gently shaken for 24 h at 22 °C. The remaining undissolved film was recovered by filtration through Whatman No. 1 filter paper, and afterwards desiccated at 105 °C for 24 h. Film solubility (FS, %) was calculated using the equation:

$$FS (\%) = \left(\frac{W_o - W_f}{W_o} \right) * 100 \quad (1)$$

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.

Film color was measured using a Konica Minolta CM-3500d spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan) set to D65 illuminant/10° observer. Film specimens were measured against the surface of a standard white plate and the CIELAB color space was used to obtain the color coordinates L^* [black (0) to white (100)], a^* [green (−) to red (+)], and b^* [blue (−) to yellow (+)]. Results are the average of at least 15 replicates.

For opacity determination, rectangular film pieces were cut and placed directly in a quartz spectrophotometer test cell, using an empty test cell as the reference. The opacity index of the films was calculated by the following equation:

$$O = \frac{Abs_{600}}{x} \quad (2)$$

where Abs_{600} is the value of absorbance at 600 nm and x is the film thickness (mm). Measurements were taken in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan).

The mechanical properties of the films were determined by tensile and puncture tests, in triplicate, using a TA.XT plus TA-XT2 texture analyzer (Texture Technologies Corp., Scarsdale, NY, USA), as described by Blanco-Pascual, Aleman, Gomez-Guillen, and Montero (2014).

Film water vapor permeability was determined according to Sobral, Menegalli, Hubinger, and Roques (2001). Films were attached over the openings of cells (permeation area = 1.54 cm²) containing silica gel, and the cells were placed in desiccators with distilled water at 22 °C. The cells were weighed daily for 7 d. Water vapour permeability was calculated using the following equation:

$$WVP = \frac{w * x}{t * A * \Delta P} \quad (3)$$

where w was weight gain (kg), x film thickness (m), t elapsed time for the weight gain (s), and ΔP the partial vapour pressure difference between the dry atmosphere and pure water (2642 Pa at 22 °C). All tests were carried out in triplicate.

2.7. Practical application as raw salmon wraps

Preparation of salmon samples

Fresh salmon was acquired in a local market. The fish were headed, gutted, filleted, washed, and skinned. Salmon pieces measuring approximately 3×2 cm were cut and wrapped with Ch or Ch-SME films in a muscle:film relation of approximately 20:1 in weight (S-Ch and S-Ch-SME batches, respectively). An unwrapped control batch (S batch) was also prepared for comparative purposes.

Texture analysis

Shear strength of unwrapped and wrapped salmon was evaluated using a Kramer shear cell. A TA.XT2i universal machine (Stable Micro Systems, Surrey, UK) equipped with a 5 Kg static load cell (crosshead speed 100 mm/min) was used. The samples were analyzed 15 h after preparation, in order to allow film swelling. Samples were sheared

with the muscle myotomes parallel to shear cell slots. Results were expressed as N/g, and at least eight replications were performed for each sample.

Bioaccessibility study

Calcium and magnesium bioaccessibility was determined in both films and wrapped salmon samples, using an *in vitro* simulated gastrointestinal digestion static model previously described (Laparra, Velez, Montoro, Barbera, & Farre, 2003), consisting in a gastric phase at pH 2 with pepsin followed by an intestinal one at pH 6.5 with pancreatin and bile salts. The bioaccessible fraction was obtained by filtration through Whatman No. 1 filter paper and concentrated by freeze-drying prior to calcium and magnesium analysis, which was performed as described above. For bioaccessibility calculation, the calcium and magnesium content of undigested samples were also determined. Results were expressed as mg bioaccessible calcium or magnesium/g film or wrapped salmon.

A quantitative and qualitative analysis of lipid bioaccessibility of wrapped salmon samples was also performed. For this purpose, the same *in vitro* simulated gastrointestinal digestion static model as described above was used. The fat contained in the bioaccessible fractions was extracted with 100 mL of chloroform by vigorous shaking, decantation, and evaporation. The bioaccessible fat was determined gravimetrically, and qualitative fatty acid analysis was performed as described above. For the bioaccessibility calculation, the fat content and fatty acid composition of undigested salmon samples were also determined. Estimated intake values of each fatty acid group (g/100 g sample) were determined by taking into consideration both their contents and their bioaccessibilities.

Storage trial

For microbiological analyses, a total amount of 10 g of muscle was collected and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 90 ml of buffered 0.1% peptone water in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain). After 1 min in a Stomacher blender (model Colworth 400, Seward, London, UK), appropriate dilutions were prepared for the following microorganism determinations: (i) total bacterial counts (TBC) on spread plates of Iron Agar 1% NaCl incubated at 15 °C for 3 days; (ii) H₂S-producing organisms, as black colonies, on pour plates of Iron Agar incubated at 15 °C for 3 days; (iii) luminescent bacteria on spread plates of Iron Agar 1% NaCl incubated at 15 °C for 5 days; (iv) pseudomonads on spread plates of Pseudomonas Agar Base with added CFC (Cetrimide, Fucidine, Cephalosporine) supplement for *Pseudomonas* spp. incubated at 25 °C for 48 h; (v) Enterobacteriaceae on double-layered plates of Violet Red Bile Glucose agar incubated at 30 °C for 48 h [after first adding 5 mL of Tryptone Soy Agar and incubating at room temperature for 1 h]; (vi) lactic acid bacteria on double-layered plates of MRS Agar incubated at 30 °C for 72 h. All microbiological counts are expressed as the log of the colony-forming units per gram (log CFU/g) of sample and performed in triplicate.

The total volatile basic nitrogen (TVBN) was determined in triplicate and the results were expressed as mg N/100 g Antonacopoulos and Vyncke (1989).

2.8. Statistical analyses

Statistical tests were performed using the SPSS® computer program, version 15.0 (SPSS Statistical Software, Inc., Chicago, IL, USA). One-way and two-way analyses of variance (ANOVA) were carried out. Differences between pairs of means were assessed on the basis of confidence intervals using the Tukey-b test. The level of significance was $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Characterization of SME

After freeze-drying, a homogenous orange-red colored powder was obtained, the main components of which were fat (73.3 ± 2.5 g/100 g), protein (18.5 ± 0.1 g/100 g), and ash (9.5 ± 0.1 g/100 g). The most abundant fatty acids were oleic (C18:1n9c), palmitic (C16:0), and linoleic (C18:2n6c), which accounted for more than 50% of the total amount of fatty acids identified (Table 1). Considerable amounts of stearic (C18:0), docosahexaenoic (C22:6n3), and eicosapentaenoic (C20:5n3) acids were also found. Saturated fatty acids (SFAs) accounted for $\approx 34\%$ of the total amount of fatty acids identified, whereas MUFAs and PUFAs represented ≈ 27 and $\approx 39\%$, respectively. These results are similar to those reported in previous works in which the fatty acid composition of lipid extracts from shrimp (*L. vannamei*) cephalothoraxes was analyzed (Gómez-Estaca, Calvo, Álvarez-Acero, Montero, & Gómez-Guillén, 2017; Takeungwongtrakul, Benjakul, Santoso, Trilaksani, & Nurilmala, 2015). Owing to the fact that the same species was employed, agreement of results is not surprising. The Σ PUFA/ Σ SFA and Σ PUFA ω -6/ Σ PUFA ω -3 ratios of the SME fat indicate the interest of using it as a food additive to improve the fatty acid profile of foods. Suppressive effects on various diseases have been found by improving these ratios in the diet (Calder, 2012; Simopoulos, 2002). Also contributing to the potential health-related benefits of SME is the considerable amount of astaxanthin present (0.294 ± 0.013 mg/g), possessing antioxidant and anti-inflammatory activities (Gómez-Guillén, Montero, López-Caballero, Baccan, & Gómez-Estaca, 2018).

The most abundant amino acids were Gly, Glu, Asp, and Ala, and significant amounts of essential amino acids were also present, which accounted for approximately 1/3 of

the total (Table 2). Therefore, the incorporation of SME into the formulation of edible films based on carbohydrates would strengthen protein input from a nutritional point of view. The contents of some minerals in SME are also shown in Table 2. Calcium was the most abundant element, although significant amounts of phosphorous, sodium, potassium, and magnesium were also found. According to the literature, calcium carbonate is the main component of the ash fraction of shrimp shells (Rodde, Einbu, & Varum, 2008). Results of the heavy metals analyzed (Zn, Fe, Cu, Mn) revealed similar or lower contents than those found in a previous work on white shrimp (*Litopenaeus vannamei*) shells, in which no health risk for humans was suggested (Banerjee, Chen, & Wu, 1996). In this connection, the cytotoxicity analysis performed revealed no cytotoxic effects at concentrations ≤ 1 mg/mL (Figure S1), this result is not surprising in view to the SME composition. Indeed, astaxanthin has been shown to alleviate cytotoxicity in H9c2 cells through inhibition of mitochondria-mediated apoptosis (Fan, Sun, Fu, Hou, Li, Yang, et al., 2017). To the best of our knowledge, this is the first work in which the cytotoxicity of a shrimp demineralization extract has been evaluated.

3.2. Characterization of edible films

The study of the physicochemical properties of edible films is crucial in order to evaluate their suitability and performance when applied as food wrappings. Some of these properties, such as color, opacity, mechanical properties, water vapor permeability, and solubility, are listed in Table S1. Both film-forming solutions yielded flexible, uniform and transparent films to naked eye that were easy to handle (Figure S2). The average thickness of Ch and Ch-SME films were 52 ± 4 μm and 75 ± 9 μm , respectively. The film with SME incorporated had an orange-red color that was evidenced by the objective measure of color, showing higher redness and yellowness than the control film ($p \leq 0.05$). Also, as a consequence of the addition of SME, the

lightness of the film diminished slightly, and opacity increased ($p \leq 0.05$), presumably owing to a light-scattering effect of the solids incorporated (Gómez-Estaca, Calvo, Sánchez-Faure, Montero, & Gómez-Guillén, 2015).

The mechanical properties of the film were affected differently by the incorporation of SME, that is, puncture force and tensile strength diminished whereas puncture deformation and elongation at break remained stable. Chitosan forms films with a compact, rigid structured network, whereas the main components of SME are lipids and low molecular weight peptides that are likely to be incorporated in the chitosan matrix between adjacent polymer chains, reducing cohesion forces and thus reducing the film's resistance to breaking. Elongation and deformation were probably unchanged because of the plasticizing effect of low molecular weight peptides and lipids (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009). Nevertheless, the mechanical properties were in the same range as those of edible films made from chitosan or proteins such as gelatin, muscle proteins, or wheat gluten (Gomez-Estaca, Montero, Fernandez-Martin, & Gomez-Guillen, 2009; Gómez-Estaca, Montero, & Gómez-Guillén, 2014; Hernandez-Munoz, Kanavouras, Ng, & Gavara, 2003), pointing to their suitability as film coatings or wrappings for food. Regarding WVP, Ch-SME film showed a higher value than Ch one ($p \leq 0.05$). This result may be a result of a looser packing of chitosan chains as a consequence of the presence of SME components, which increased free volume of the polymeric structure and thus enhanced permeability (Balaguer, Fajardo, Gartner, Gomez-Estaca, Gavara, Almenar, et al., 2014). This is consistent with mechanical properties. Comparing results with the literature, the WVP of Ch-SME film is in the same order of magnitude than those obtained for gelatin, chitosan or gliadins (Fajardo, Balaguer, Gomez-Estaca, Gavara, & Hernandez-Munoz, 2014; Gomez-Estaca, Gomez-Guillen, Fernandez-Martin, & Montero, 2011). Another

important factor of an edible film is its water solubility, as many food products present high moisture. Both films showed the characteristic high solubility of chitosan films at acidic pH, although the Ch-SME film showed a significantly lower value ($p \leq 0.05$). The desired solubility of an edible film depends on the intended application. In the present work, the films developed were used to wrap salmon *sashimi*, acting as a multifunctional active coating, so they were designed to be eaten. Consequently, a relative solubility of the film would be desirable in order to facilitate chewing.

3.3. Performance of films as functional salmon wrappers

Both films were homogeneous and transparent enough to allow visualization of the wrapped salmon (Figure S3), in spite of the higher opaqueness of Ch-SME film. Both films tended to swell and soften in contact with the wet surface of the salmon portion, however they did not disintegrate. Since the film was meant to be ingested together with the fish, far from being a drawback this effect was desirable from a sensory point of view. With regard to the rheological properties of the salmon samples, the Kramer test evidenced a slight trend towards hardening of the wrapped samples (4.9 ± 0.9 , 6.2 ± 0.9 , and 6.8 ± 1.2 N/g for S, S-Ch, and S-Ch-SME, respectively), but significant differences were not found ($p \leq 0.05$).

Calcium and magnesium contents and bioaccessibility

Calcium and magnesium contents and bioaccessibility of the salmon samples are shown in Table 3. This table also shows the contents and bioaccessibility of these minerals in the edible films. As expected, the contents of both minerals in the Ch-SME film were significantly higher than in the Ch film ($p \leq 0.05$), owing to the mineral content of SME; however, bioaccessibility was low, probably owing to the chelating effect of chitosan (Uraki, Fujii, Matsuoka, Miura, & Tokura, 1993). Wrapping the salmon with the Ch-

SME film caused a significant increase ($p \leq 0.05$) in the contents of the two elements analyzed, which was especially high in the case of calcium (7-fold). From these results, it can be estimated that 100 g of S-Ch-SME sample would provide ≈ 22 mg of bioaccessible calcium, whereas S and S-Ch would only provide ≈ 6 mg, which is a 3.6-fold increase. In the case of magnesium, narrower but significant ($p \leq 0.05$) differences were obtained, S, S-Ch, and S-Ch-SME samples providing ≈ 26 , ≈ 24.0 and ≈ 30.3 mg of bioaccessible magnesium/100 g, respectively.

Total fat and fatty acids contents and bioaccessibility

The salmon employed in the present study presented a typical fat content of ≈ 20 g/100 g muscle. It is worth noting that wrapping the salmon caused a significant decrease ($p \leq 0.05$) in the fat content, irrespective of the film used (Table 4). Bearing in mind that the salmon-to-film ratio was high (20:1, w:w), this may be due to an impairment of fat extraction owing to the fat-binding properties of chitosan (Ma, Zhang, & Zhong, 2016; Panith, Wichaphon, Lertsiri, & Niamsiri, 2016). Comparing the two wrapped samples, it can be seen that S-Ch-SME showed a higher fat content (Table 4), which can be due to saturation of chitosan-binding sites by interaction with the SME components, as well as to the fat provided by SME. With regard to the bioaccessibility results, wrapping the salmon with the chitosan film (S-Ch batch) caused a 3-fold decrease as compared to the unwrapped salmon. This can be due to the previously mentioned fat-binding ability of chitosan and it suggests the usefulness of chitosan films as a way of reducing the fat bioavailability of fatty foods. Indeed, the total amount of bioaccessible fat was reduced by $\approx 80\%$ (Table 4). Although this kind of application would be more interesting in foods containing saturated fats, it may also be an option for weight-loss diets. In a study by Santos et al. Santos, Espadaler, Mancebo, and Rafecas (2012), guinea pigs were fed with chitosan-supplemented diets and a lower fat absorption was found. In contrast, the

S-Ch-SME batch showed a similar fat bioaccessibility to that of the unwrapped control (S batch), which was most probably also due to a saturation effect of chitosan-binding sites. In this sample, the reduction of total bioaccessible fat was $\approx 15\%$ (Table 4).

The fatty acid profile was significantly modified as a result of wrapping with the films, although the effect was different depending on the type of film (Table 5). On the one hand, the Ch film induced a reduction in the contents of many fatty acids, especially MUFA and also PUFA, resulting in a decrease in the $\Sigma\text{PUFA}/\Sigma\text{SFA}$ ratio. This result agrees with the reduction in total fat content shown in Table 4. Santos and Rafecas found that chitosan selectively binds unsaturated fatty acids, increasing excretion of these fatty acids Santos and Rafecas (2009). On the other hand, the Ch-SME film wrapping significantly ($p \leq 0.05$) increased the contents of SFA and PUFA, which were the most abundant fatty acid groups in SME (Table 1), as compared to unwrapped salmon. Consequently, neither the $\Sigma\text{PUFA}/\Sigma\text{SFA}$ nor the $\Sigma\text{PUFA}\omega-6/\Sigma\text{PUFA}\omega-3$ fatty acid ratios were modified. As already discussed, most of the chitosan-binding sites in the Ch-SME film would be taken up by the SME components, and the increase in SFA and PUFA would be attributable to the non-bound fat contained in SME. With regard to bioaccessibility, both wrapped samples showed lower bioaccessibility values than unwrapped salmon for the three fatty acid groups, especially the one with the Ch film; however, all fatty acids (except C22:4n6) showed bioaccessibility values higher than 70%. Taking into consideration both the fat content and the bioaccessibility of the three fatty acid groups (SFA, MUFA, and PUFA), Table S2 has been constructed to summarize the estimated intake values for each fatty acid group for the different samples. As can be seen, both wrapped samples provided a lower amount of bioaccessible fatty acids irrespective of unsaturation, in agreement with estimated fat intake values presented in Table 4. Nevertheless, SFA was the fatty acid group that was

reduced most with respect to the unwrapped sample (more than 80%), whereas MUFA and PUFA were only reduced by $\approx 20\%$ in the salmon wrapped with the shrimp mineral extract film developed (S-Ch-SME batch).

Storage trial

Results of microbiological analyses are shown in Figure 1. The initial microbial load (total viable bacteria) of the unwrapped salmon (S batch) was $(5.5 \pm 0.2 \log \text{ CFU/g})$. These values were slightly higher than those obtained in previous works on salmon fillets (Gómez-Estaca, López De Lacey, Gómez-Guillén, López-Caballero, & Montero, 2009; Rode & Hovda, 2016). However, this result is not at all surprising, bearing in mind the process of cutting the raw salmon into pieces, which would probably have induced some microbial contamination in spite of the hygienic conditions employed. This is consistent with the *Enterobacteriaceae* counts found at the beginning of storage, which were probably related to contamination by manipulation. The presence of specific fish spoilers such as H_2S -producing microorganisms and luminescent colonies, which are presumptive indicators of *S. putrefaciens* and *P. phosphoreum* respectively (Lopez-Caballero, Sanchez-Fernandez, & Moral, 2001; Lopez-Caballero, Torres, Sanchez-Fernandez, & Moral, 2002), as well as pseudomonads, was also noticeable already on the first day of storage. In contrast, lactic acid bacteria were below the detection limit.

The two edible films developed produced a striking change in the microbial population of the muscle after 1 day of storage (S-Ch and S-Ch-SME batches), reducing practically all the groups to levels below the detection limit (Figure 1). As storage continued, total viable bacteria and pseudomonads in the S batch progressively increased, reaching a maximum of $\approx 9 \log \text{ CFU/g}$ at day 14 of storage for both microorganism groups. H_2S -producing microorganisms reached their maximum earlier (day 7), after a lag period of

3 days, whereas luminescent bacteria increased to a maximum of ≈ 8 log CFU/g at day 5 and then remained stable until the end of storage. Enterobacteria and lactic acid bacteria showed a lower growth; the former increased by only 1 log CFU/g during the whole storage period, while the latter reached a maximum of 3.6 ± 0.1 log CFU/g at day 14, after a lag period that lasted for 7 days. The antimicrobial effect exerted by the SME-added edible film (S-Ch-SME batch) at day 1 was extended by up to 3 days (total viable counts, luminescent bacteria, pseudomonads), 5 days (enterobacteria) or even the whole storage period of 19 days (H_2S -producing microorganisms), showing counts below the detection limit. In contrast, the microbial growth inhibition exerted by the chitosan films (S-Ch batch) was mostly limited to the first day of storage, indicating that not only chitosan but also SME exerted an antimicrobial effect. In spite of this, microbial counts were lower than those of the S batch during the whole storage period for all microorganisms tested except lactic acid bacteria. This was also observed in the S-Ch-SME batch, in which microbial counts were generally even lower than those of the S-Ch batch in all microorganism groups.

Initial values of around 9 mg TVBN/100 g muscle were found, indicating the freshness of the fish (Figure S4). As expected, TVBN accumulated progressively as storage continued, attaining a maximum value of ≈ 24 mg/100 g, which is lower than the 30 mg/100 g value that is considered the limit of fish freshness. A slight increase in TVBN was detected in the S-Ch-SME batch with the application of the film; however, its concentration in this batch remained practically constant during storage. Similarly, TVBN values in the S-Ch batch stayed relatively stable during storage. Despite the microbial growth observed in both wrapped batches, these groups did not reach a sufficient concentration to produce an increase in volatile compounds. Arancibia et al. reported that a chitosan coating, alone or incorporating an extract with a protein-lipid

concentrate from shrimp waste, reduced TVBN in prawns during 14 days of chilled storage Arancibia, López-Caballero, Gómez-Guillén, and Montero (2015).

4. CONCLUSION

A type of waste commonly produced during a mild demineralization process for obtaining chitosan was recovered and characterized. It showed no cytotoxicity and has potential as a functional food ingredient, mainly thanks to its calcium content and fatty acid composition. A functional edible film was prepared by blending this shrimp demineralization extract (SME) with chitosan, showing a good performance as wrapping material for raw salmon pieces. The Ch-SME film in the wrapped fish improved the calcium and magnesium contents and reduced the estimated saturated fatty acids intake. At the same time, the presence of the SME boosted the antimicrobial capacity of the chitosan based film and extended salmon shelf life.

5. ACKNOWLEDGMENTS

The authors wish to thank the Spanish Ministry of Economy and Competitiveness for financial support through projects AGL2011-27607, AGL2014-52825-R and 201370E036, as well as for J. Gómez-Estaca's "Juan de la Cierva" fellowship.

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Figure captions

Figure 1. Microbial counts in unwrapped (S batch), chitosan edible film-wrapped (S-Ch batch), and shrimp mineral extract-chitosan film-wrapped (S-Ch-SME batch) salmon.

Table captions

Table 1. Fatty acid composition of the shrimp mineral extract (SME) expressed as % of the total amount of fatty acids identified.

Table 2. Amino acid composition and contents of some minerals in the shrimp mineral extract (SME).

Table 3. Calcium and magnesium contents of the films (Ch and Ch-SME) and the salmon samples (S, S-Ch, and S-Ch-SME), and their bioaccessibilities.

Table 4. Fat content, fat bioaccessibility and estimated intake of fat of salmon samples. S: unwrapped salmon; S-Ch: chitosan film-wrapped salmon; S-Ch-SME: shrimp mineral extract-chitosan film-wrapped salmon.

Table 5. Fatty acid profile of salmon samples and their bioaccessibilities. S: unwrapped salmon; S-Ch: chitosan film-wrapped salmon; S-Ch-SME: shrimp mineral extract-chitosan film-wrapped salmon.

Supplementary material

Figure S1. Cell viability expressed as the % of viable cells with respect to an untreated control after 24 h exposition to different concentrations of SME, as compared to a positive control treated with ethanol.

Figure S2. Image of the films developed. Ch: chitosan film; Ch-SME: shrimp mineral extract-chitosan film.

Figure S3: Images of the salmon samples. S: unwrapped salmon; S-Ch: chitosan film-wrapped salmon; S-Ch-SME: shrimp mineral extract-chitosan film-wrapped salmon.

Figure S4. TVBN in the unwrapped (S batch), chitosan edible film-wrapped (S-Ch batch), and shrimp mineral extract-chitosan film-wrapped (S-Ch-SME batch) salmon.

Table S1. Some physicochemical properties of the films: optical properties (lightness, L^* ; redness, a^* ; yellowness, b^* ; opacity), water solubility, water vapor permeability, and mechanical properties (puncture force, puncture deformation, tensile strength, and elongation at break).

Table S2. Estimated intake values for each fatty acid group for the different samples, and their reductions (%) with respect to unwrapped salmon. S: unwrapped salmon; S-Ch: chitosan film-wrapped salmon; S-Ch-SME: shrimp mineral extract-chitosan film-wrapped salmon.

Figure 1. Microbial counts in unwrapped (S batch), chitosan edible film-wrapped (S-Ch batch), and shrimp mineral extract-chitosan film-wrapped (S-Ch-SME batch) salmon.

Table 1. Fatty acid composition of the SME expressed as % of the total amount of fatty acids identified.

Fatty acid	%
C14:0	1.16 ± 0.01
C15:0	1.24 ± 0.01
C16:0	19.56 ± 0.05
C17:0	1.81 ± 0.01
C18:0	8.90 ± 0.02
C20:0	0.51 ± 0.01
C21:0	0.20 ± 0.04
C22:0	0.40 ± 0.01
C24:0	0.27 ± 0.04
Σ SFA ¹	34.05 ± 0.2
C16:1n7	2.08 ± 0.01
C18:1n9c	19.98 ± 0.03
C18:1n7c	3.55 ± 0.02
C20:1n9	1.16 ± 0.00
C24:1n9	0.54 ± 0.03
Σ MUFA ²	27.32 ± 0.09
C18:2n6c	14.08 ± 0.03
C18:3n3	1.08 ± 0.00
C20:2n6	1.71 ± 0.00
C20:3n3	0.35 ± 0.00
C20:3n6	0.16 ± 0.01
C20:4n6	3.96 ± 0.01
C20:5n3	6.74 ± 0.03
C22:4n6	0.22 ± 0.03
C22:5n3	0.97 ± 0.02
C22:6n3	8.91 ± 0.07
Σ PUFA ³	38.63 ± 0.21
Σ PUFAω-3 ⁴	18.05 ± 0.12
Σ PUFAω-6 ⁵	20.58 ± 0.06
Σ PUFA/Σ SFA ⁶	1.13
Σ PUFAω-6/Σ PUFAω-3 ⁷	1.14

¹Σ SFA: total amount of saturated fatty acids. ²Σ MUFA: total amount of monounsaturated fatty acids. ³Σ PUFA: total amount of polyunsaturated fatty acids. ⁴Σ PUFAω-3: total amount of ω-3 polyunsaturated fatty acids. ⁵Σ PUFAω-6: total amount of ω-6 polyunsaturated fatty acids. ⁶Σ PUFA/Σ SFA: polyunsaturated/saturated fatty acids ratio. ⁷Σ PUFAω-6/PUFAω-3: ω-6/ω-3 polyunsaturated fatty acids ratio.

Table 2. Amino acid composition and contents of some minerals in the shrimp mineral extract (SME).

Amino acid composition (residues/1000 residues)		Mineral content (mg/100 g SME)	
Asp	116	Ca	1913 ± 113
Thr	38	Mg	113 ± 4
Ser	57	Na	454 ± 22
Glu	112	K	294 ± 14
Gly	134	Zn	1.98 ± 0.25
Ala	94	Fe	8.54 ± 0.15
Cys	5	Cu	3.32 ± 0.14
Val	49	Mn	0.33 ± 0.07
Met	21	P	647±28
Ile	32		
Leu	64		
Tyr	31		
Phe	39		
His	20		
Lys	55		
Arg	39		
Pro	55		
OH-Pro	0		
OH-Lys	39		
TEAA*	316		

*TEAA (Total essential amino acids ($\sum$ Ile+Leu+Lys+Met+Phe+Thr+Val+His))

Table 3. Calcium and magnesium contents of the films (Ch and Ch-SME) and the salmon samples (S, S-Ch, and S-Ch-SME), and their bioaccessibilities.

	Ca²⁺ content (mg/100 g film or salmon)	Bioaccessibility (%)	Mg²⁺ content (mg/100 g film or salmon)	Bioaccessibility (%)
Ch film	13 ± 0.2	26.3 ± 5.1	1.9 ± 0.1	0.8 ± 0.2
Ch-SME	1180 ± 40	9.4 ± 0.3	76.9 ± 21.2	11.0 ± 0.8
S	8.5 ± 0.2	79.2 ± 16.5	42.2 ± 0.8	60.9 ± 13.8
S-Ch	8.2 ± 0.3	74.9 ± 1.2	42.4 ± 1.0	56.7 ± 7.8
S-Ch-SME	60.9 ± 0.8	36.4 ± 7.3	47.1 ± 0.8	64.2 ± 1.9

Table 4. Fat content, fat bioaccessibility and estimated intake of fat of salmon samples. S: unwrapped salmon; S-Ch: chitosan film-wrapped salmon; S-Ch-SME: shrimp mineral extract-chitosan film-wrapped salmon.

	Fat content (g/100 g)	Bioaccessibility (%)	Estimated fat intake (g/100 g)
S	19.4±1.0	75.2±4.3	14.6 ±3.1
S-Ch	12.4±0.1	23.2±5.0	2.9±1.0
S-Ch-SME	16.0±0.6	77.4±4.7	12.4±2.8

Table 5. Fatty acid profile (mg fatty acid/g lipid extract) of salmon samples and their bioaccessibilities. S: unwrapped salmon; S-Ch: chitosan film-wrapped salmon; S-Ch-SME: shrimp mineral extract-chitosan film-wrapped salmon.

	S		S-Ch		S-Ch-SME	
	mg/g	Bioaccessibility (%)	mg/g	Bioaccessibility (%)	mg/g	Bioaccessibility (%)
C14:0	16.7±0.2	89.2	15.6±0.8	76.9	16.8±0.2	82.4
C15:0	1.4±0.0	92.8	1.4±0.1	71.4	1.6±0.0	75
C16:0	73.1±0.3	90.8	72.8±2.6	76.2	76.3±0.3	82
C17:0	1.4±0.0	85.7	1.4±0.1	78.5	1.6±0.0	81.2
C18:0	22.5±0.0	91.5	22.2±0.7	77.9	23.4±0.3	82.5
C20:0	2.9±0.0	89.6	2.6±0.2	80.8	3.0±0.1	80
C22:0	1.3±0.0	100	1.3±0.0	76.9	1.4±0.0	85.7
C24:0	0.5±0.0	100	0.5±0.0	80.0	0.5±0.0	100
ΣSFA	119.8±0.5a	90.8±2.1C	117.8±4.5a	76.8±1.9A	124.6±0.9b	82.1±2.8B
C16:1n7	21.6±0.4	90.7	20.0±0.9	78.5	21.7±0.2	82.5
C18:1n7c	26.4±0.3	90.9	25.2±1.2	78.6	26.8±0.2	83.2
C18:1n9c	397.3±2.1	91.2	372.9±17.4	79.9	398.6±4.7	83.9
C20:1n9	23.1±0.3	91.8	21.2±1.1	80.7	23.1±0.4	84.0
C22:1n9	3.7±0.1	91.9	3.4±0.1	82.4	3.8±0.1	84.2
C24:1n9	2.6±0.0	92.3	2.6±0.1	76.9	2.7±0.0	81.5
ΣMUFA	474.7±3.2b	91.2±2.5B	445.3±20.8a	79.8±1.8A	476.7±5.6b	83.8±1.4A
C18:2n6c	148.5±2.1	90.3	139.7±5.4	79.0	149.4±1.9	83.0
C18:3n3	82.2±1.3	90.6	76.4±3.0	79.2	81.9±0.8	83.0
C18:3n6	1.4±0.0	85.7	1.3±0.1	76.9	1.4±0.0	78.6
C18:4n3	4.7±0.0	93.6	3.7±0.3	91.9	4.3±0.2	90.7
C20:2n6	11.2±0.0	91.0	10.2±0.4	80.4	11.3±0.1	84.1
C20:3n3	6.9±0.1	92.7	6.3±0.4	80.9	6.8±0.3	86.8
C20:3n6	2.5±0.0	88.0	2.3±0.1	73.9	2.5±0.0	80.0
C20:4n3	7.5±0.1	92.0	6.9±0.2	79.7	7.5±0.0	84.0
C20:4n6	2.1±0.0	85.7	2.2±0.1	72.7	2.4±0.2	83.3
C20:5n3	20.4±0.2	89.7	19.0±0.0	78.4	21.0±0.8	81.4

C22:4n6	0.6±0.0	50.0	0.6±0.0	50	0.7±0.0	28.3
C22:5n3	10.7±0.1	90.6	10.4±0.0	76.0	10.9±0.3	81.6
C22:6n3	23.7±0.6	87.8	25.7±3.0	66.1	25.6±3.4	76.6
ΣPUFA	288.0±3.9a	84.6±3.1C	279.0±10.0a	72.9±1.9A	300.1±4.6b	77.2±2.2B

Different letters in the same row indicate significant differences ($p \leq 0.05$) among different samples for total fatty acid content (lower case letters) or bioaccessibility (capital letters).

Highlights

1. Waste recovered from obtaining shrimp chitosan (fat 73%, protein 19%, ash 9%)
2. The waste (SME) is a source of calcium and unsaturated fatty acids
3. Development of chitosan-SME edible films suitable for raw salmon wrapping
4. Salmon microbial quality improved
5. Bioaccessible calcium content improved