EFFECTS OF GELATIN ORIGIN, BOVINE-HIDE AND TUNA-SKIN, ON THE PROPERTIES OF COMPOUND GELATIN-CHITOSAN FILMS

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ABSTRACT

With the purpose to improve the physico-chemical performance of plain gelatin and chitosan films, compound gelatin-chitosan films were prepared. The effect of the gelatin origin (commercial bovine-hide gelatin and laboratory-made tuna-skin gelatin) on the physico-chemical properties of films was studied. The dynamic viscoelastic properties (elastic modulus *G'*, viscous modulus, *G''* and phase angle) of the film forming solutions upon cooling and subsequent heating revealed that the interactions between gelatin and chitosan were stronger in the blends made with tuna-skin gelatin than in the blends made with bovine-hide gelatin. As a result, the fish gelatin-chitosan films were more water resistant (~18% water solubility for tuna *vs* 30% for bovine) and more deformable (~68%

breaking deformation for tuna *vs* 11% for bovine) than the bovine gelatin-chitosan films. The breaking strength of gelatin-chitosan films, whatever the gelatin origin, was higher than that of plain gelatin films. Bovine gelatin-chitosan films showed a significant lower water vapor permeability (WVP) than the corresponding plain films, whereas tuna gelatin-chitosan ones were only significantly less permeable than plain chitosan film. In spite of gelatin-chitosan interactions, all the chitosan-containing films exhibited antimicrobial activity against *S. aureus*, a relevant food poisoning. Mixing gelatin and chitosan may be a means to improve the physico-chemical performance of gelatin and chitosan plain films, especially when using fish gelatin, without altering the antimicrobial properties.

Key words: bovine-hide gelatin, chitosan, fish gelatin, physico-chemical properties, *S. aureus*, edible films

INTRODUCTION

As a consequence of the problems associated with disposal of packaging plastics, there is a growing interest concerning the development of biodegradable materials. These newer materials can be obtained from several sources, which include proteins (collagen/gelatin, soya, whey, wheat, etc), polysaccharides (chitosan, starch, cellulose) and lipids (wax, fatty acids) (Gennadios, Hanna & Kurth, 1997; Tharanathan, 2003). Although the entire substitution of petrochemical polymers with bioplastics may not be possible due to the worse physicochemical properties of the latter, it is necessary to research on the improvement of such properties, as well as on the new applications, in order that bioplastics corner the market and, therefore, the traditional synthetic plastics are partially substituted.

Gelatin is a protein with a broad range of functional properties and applications, including film-forming ability. Bovine and porcine wastes are the most frequent sources to obtain gelatin of good quality. However other sources of gelatin are becoming increasingly relevant, such as fish bones and skins (Gomez-Guillen, Turnay, Fernandez-Diaz, Ulmo, Lizarbe & Montero, 2002). Whatever the species of origin, gelatin films fail in terms of mechanical properties and water resistance, which may limit its field of application. Several strategies have been used to improve the physical performance of gelatin films. These include chemical or enzymatic treatments (Cao, Fu & He, 2007; Chiou et al., 2008; de Carvalho & Grosso, 2004; Spanneberg, Osswald, Kolesov, Anton, Radusch & Glomb, 2010; Zhang et al., 2010)) and mixing with other polymers, as composite films may be designed to take advantages of pure components (Garcia, Pinotti, Martino & Zaritzky, 2004). For example mixing with apolar

components such fatty acids or oils reduces water vapour transmission rate (Jongjareonrak, Benjakul, Visessanguan & Tanaka, 2006; Limpisophon, Tanaka & Osako, 2010; Perez-Mateos, Montero & Gomez-Guillen, 2009). Furthermore polymers can establish new bonds that may enhance the properties of the resulting materials (Denavi, Perez-Mateos, Anon, Montero, Mauri & Gomez-Guillen, 2009; Sionkowska, Wisniewski, Skopinska, Kennedy & Wess, 2004). Gelatin has been blended with casein (Chambi & Grosso, 2006), pectin (Liu, Liu, Fishman & Hicks, 2007), chitosan (Arvanitoyannis, Nakayama & Aiba, 1998; Kolodziejska & Piotrowska, 2007; Kolodziejska, Piotrowska, Bulge & Tylingo, 2006), starch (Arvanitoyannis et al., 1998) and soy protein (Denavi et al., 2009), achieving in general an improvement of its physical performance.

Chitosan (poly b-(1,4)N-acetyl-D-glucosamine) polymer is industrially produced by chemical deacetylation of the chitin found in arthropod exoskeletons. Chitosan is largely utilized not only due to its film forming capability but also because of its antimicrobial properties (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades & Roller, 2001; Jung, Youn, Lee, No, Ha & Prinyawiwatkul, 2010). For this reason, the use of chitosan as an edible coating or film to extend the shelf-life of foods and inhibit pathogens is of growing interest (Aider, 2010; Gomez-Estaca, Montero, Gimenez & Gomez-Guillen, 2007; Lopez-Caballero, Gomez-Guillen, Perez-Mateos & Montero, 2005). Specifically, chitosan has been found to be active against *S. aureus* (Fernandes et al., 2008). Some *S. aureus* strains are able to produce staphylococcal enterotoxins and are the causative agents of staphylococcal food poisonings (Le Loir, Baron & Gautier, 2003). The antimicrobial action of chitosan is supposed to be derived from the positive charge that amino groups present at

89 acidic pH (below 6.5), which lead to cellular membrane depolarization and
90 microbial death. However the main drawback of chitosan under this condition is
91 its intrinsic water solubility, which limits the utilisation as self-standing packaging
92 material. Attempts to increase the water resistance of chitosan have been made
93 including crosslinking with glutaraldehyde, glyoxal or epichlorohydrin (Suto & Ui,
94 1996; Tual, Espuche, Escoubes & Domard, 2000; Zheng, Du, Yu & Xiao, 2000).
95 However, in a subsequent work (Tang, Du & Fan, 2003) it was confirmed that
96 the antimicrobial capacity of chitosan films diminishes with an increase in the
97 cross-linking. Mixing of chitosan with other biopolymers to obtain more insoluble
98 matrices has been proved as an effective means to improve the water
99 resistance of chitosan maintaining its antimicrobial properties (Fernández-Saiz
100 et al., 2008). Chitosan has also been blended with other biopolymers such as
101 methylcellulose and starch resulting in an improvement of its physico-chemical
102 properties (Garcia et al., 2004; Garcia, Pinotti & Zaritzky, 2006).

103 According to Taravel & Domard (1995), the interactions between gelatin and
104 chitosan are produced by both electrostatic and hydrogen bonding, with the
105 blends taking on new physical properties and thus becoming suited to potential
106 new applications (Sionkowska et al., 2004). Accordingly, combining these
107 biopolymers seems to be a promising way to enhance the physical properties of
108 the resulting materials (Huang, Onyeri, Siewe, Moshfeghian & Madihally, 2005;
109 Mao, Zhao, Yin & Yao, 2003). There are some reports dealing with the physico-
110 chemical properties of compound fish gelatin-chitosan films as well as its
111 improvement by adding different crosslinkers (Arvanitoyannis et al., 1998;
112 Kolodziejska et al., 2007; Kolodziejska et al., 2006). However there is no
113 previous report on the effect of the gelatin origin on the film forming ability and

the physico-chemical properties of the gelatin-chitosan admixtures. The objective of the present work has thus been to study the physico-chemical properties of compound gelatin-chitosan films as function of gelatin origin: a commercial bovine-hide gelatin and a laboratory-obtained tuna-skin gelatin. The antimicrobial activity of the resulting films over *S. aureus* was also evaluated.

MATERIALS AND METHODS

Materials

Tuna-skin gelatin was extracted according to a patented method (Gómez-Guillén & Montero, 2001). A commercial type A bovine-hide gelatin (Bloom 200/220) was purchased from Sancho de Borja S.L. (Zaragoza, Spain). Chitosan from shrimp shells (85 % deacetylated; 141,000 Da) was purchased from Guinama (Valencia, Spain). Glycerol and sorbitol were from Panreac (Barcelona, Spain).

For the microbial analyses, brain heart infusion (BHI) broth was purchased from Oxoid (Basingstoke, UK) and bacteriological agar were from Scharlau (Barcelona, Spain). *Staphylococcus aureus* (CECT 240) was obtained from the Spanish Type Culture Collection (Valencia, Spain).

Preparation of the film-forming solutions and films

Seven different formulations were prepared: The batches were: B (bovine-hide gelatin), T (tuna-skin gelatin), Ch (chitosan), B-Ch 0.75% (bovine-hide gelatin plus 0.75% chitosan), T-Ch 0.75% (tuna-skin gelatin plus 0.75% chitosan), B-Ch 1.5% (bovine-hide gelatin plus 1.5% chitosan), T-Ch 1.5% (tuna-skin gelatin

138 plus 1.5% chitosan). The gelatin film-forming solutions (solutions B and T) were
139 prepared at a concentration of 4 g gelatin/100 ml of distilled water. The chitosan
140 film-forming solution (solution Ch) was prepared by dissolving chitosan in a
141 proportion of 3 g/100 mL in 0.15 M acetic acid. The gelatin-chitosan film-forming
142 solutions (solutions B-Ch 0.75%, B-Ch 1.5%, T-Ch 0.75%, T-Ch 1.5%) were
143 prepared by mixing a 4% bovine-hide or tuna-skin gelatin solution with a 1.5%
144 or 3% solution of chitosan in 0.15 M acetic acid, in a proportion of 1:1 (v/v), to
145 obtain a film-forming solution containing 2% gelatin and 0.75% or 1.5%
146 chitosan, respectively. A mixture of glycerol and sorbitol was added to the film-
147 forming solutions as previously described (Thomazine, Carvalho & Sobral,
148 2005) at a concentration of 0.15 g each per gram of the total polymeric agent
149 (gelatin and/or chitosan). Plasticizing molecules of this kind reduce inter-chain
150 interactions, improving film flexibility and, consequently, film handling.

151 The film-forming solutions, which had a pH of 4.6 ± 0.3 in all cases, were
152 warmed and stirred at 45 ºC to obtain a good blend. The films were made by
153 casting an amount of 40 mL onto plexiglass plates (12 x 12 cm) and drying at
154 45 ºC in a forced-air oven for 15 h to yield a uniform thickness of 100 μm ± 11 in
155 all cases except the 0.75% chitosan formulations, which was 80 μm ± 7 thick.
156 Prior to the determinations, the films were conditioned at 22 ºC over a saturated
157 solution of NaBr (58% RH) in desiccators for 3 d.

158

159 **Physical characterization of the film-forming solutions**

160 Dynamic viscoelastic analysis of the film-forming solutions was carried out on a
161 Bohlin CSR-10 rheometer/rotary viscometer (Bohlin Instruments Ltd.,

Gloucestershire, UK) using a cone-plate geometry (cone angle = 4º, gap = 0.15 mm). Cooling and heating from 40 to 6 ºC and back to 40 ºC took place at a scan rate of 1 ºC/min, a frequency of 1 Hz, and a target strain of 0.2 mm. The elastic modulus (G'; Pa), viscous modulus (G''; Pa) and phase angle (º) were determined as functions of temperature. Two determinations were performed for each sample, with an experimental error of less than 6% in all cases.

The film-forming solutions were poured into glasses 2.3 cm in diameter and 3.6 cm in height and left to mature in a refrigerator at 2 ºC for 16-18 h. Gel strength at 9±1 ºC was determined on an Instron model 4501 Universal Testing Machine (Instron Co., Canton, MA, USA) with a 100 N load cell, a crosshead speed of 1 mm/s, and a flat-faced cylindrical plunger 1.27 cm in diameter. The maximum force (g) reading was taken when the plunger had penetrated 4 mm into the gelatin gels.

Physico-chemical characterization of the films

Mechanical properties

A puncture test was performed to determine the maximum strength and deformation of the films at the breaking point. The films were placed in a cell 5.6 cm in diameter and perforated to the breaking point using an Instron model 4501 Universal Testing Machine (Instron Co., Canton, MA, USA) with a round-ended stainless-steel plunger 3 mm in diameter at a crosshead speed of 1 mm/s and a 100 N load-cell. Breaking strength was expressed in N and breaking deformation in percent as previously described (Sobral, Menegalli, Hubinger & Roques, 2001). All determinations were the means of at least five measurements.

186 *Thermal properties*

187 Calorimetric analysis was performed using a model TA-Q1000 differential
188 scanning calorimeter (TA Instruments, New Castle, DE, USA) previously
189 calibrated by running high purity indium (melting point 156.4 ºC; enthalpy of
190 melting 28.44 W/g). Sample amounts on the order of 10 mg ± 0.002 weighed
191 out using a Sartorious model ME235S electronic balance (Goettingen,
192 Germany) were tightly encapsulated in aluminum pans and scanned under dry
193 nitrogen purge (50 mL/min). Freshly conditioned films were rapidly cooled to 0
194 ºC and scanned between 0 and 90 ºC, at a rate of 10 ºC/min. Glass transition
195 temperatures, Tg (ºC), were determined on first heating scans consistently with
196 the other physical determinations carried out on the same original (after
197 conditioning at 58% RH) material. Tg was estimated as the midpoint of the line
198 drawn between the temperature at the intersection of the initial tangent with the
199 tangent through the inflection point of the trace and the temperature of the
200 intersection of the tangent through the inflection point with the final tangent. Tg
201 data were the mean values of at least three replications per film sample.

202

203 *Water solubility*

204 Film portions measuring 4 cm^2 were placed in aluminum capsules with 15 mL of
205 distilled water and shaken gently at 22 ºC for 15 h. The solution was then
206 filtered through Whatman no. 1 filter paper to recover the remaining undissolved
207 film, which was desiccated at 105 ºC for 24 h. Film water solubility was
208 calculated using the equation $FS\ (\%) = ((W_o-W_f)/W_o)\cdot 100$, where W_o was the
209 initial weight of the film expressed as dry matter and W_f was the weight of the

undissolved desiccated film residue. All determinations were carried out in triplicate.

Water vapour permeability

Water vapour permeability (WVP) was determined by a gravimetric method. Films were attached over the openings of cells (permeation area = 15.9 cm^2) containing silica gel, and the cells were placed in desiccators with distilled water at 22 ºC. The cells were weighed daily for 7 d. Water vapour permeability was calculated using the equation $WVP = w \cdot x \cdot t^{-1} \cdot A^{-1} \cdot \Delta P^{-1}$, where w was weight gain (g), x film thickness (mm), t elapsed time (h) for the weight gain, and ΔP the partial vapour pressure difference between the dry atmosphere and pure water (2642 Pa at 22 ºC). Results have been expressed as $g \cdot mm \cdot h^{-1} \cdot cm^{-2} \cdot Pa^{-1}$. All determinations were carried out in duplicate.

Microbial analysis

For microbial analysis, prior to film casting, the pH of all the film-forming solutions was adjusted to 6. This pH was considered suitable to enable any potential *in vitro* antibacterial effects of the film-forming solutions and/or films to be attributed to the chitosan rather than to the pH. The antimicrobial activity of both the film-forming solutions and the films was determined against *S. aureus*. The bacteria were stored at -80 ºC in BHI broth with 25 % glycerol until use. Strain was grown in BHI (Oxoid) broth at 37 ºC overnight to a final bacterial concentration of 10^7-10^8 cfu/mL. Spread plates of brain heart agar were prepared. Sterile filter paper disks immersed in the film-forming solutions or

234 pieces of the films themselves were placed on the plate surfaces and incubated
235 at 37 ºC for 24 h. The appearance of a clear area below or around the film or
236 filter paper disks was deemed to be positive for antimicrobial activity.

237

238 **Statistical analysis**

239 Statistical tests were performed using the SPSS® computer program (SPSS
240 Statistical Software, Inc., Chicago, IL, USA). One-way analysis of variance was
241 carried out. Differences between pairs of means were compared using a Tukey
242 test. The level of significance was set at $p \leq 0.05$.

243

244

245 **RESULTS AND DISCUSSION**

246 **Film-forming solutions**

247 The changes in the viscoelastic properties of the film-forming solutions during
248 cooling and subsequent heating have been plotted in Figure 1. Solution B had a
249 higher elastic modulus (G') value and had thermal transition (gelling and
250 melting) points at higher temperatures than solution T. These findings are
251 indicative both of bovine-hide gelatin's greater capacity to refold into triple-helix
252 chains as it cools and its higher thermostability. These findings were fully
253 expected and wholly consistent with the different origins of the gelatins, as
254 reported previously (Joly-Duhamel, Hellio, Ajdari & Djabourov, 2002; Joly-
255 Duhamel, Hellio & Djabourov, 2002). Bovine-hide gelatin has a higher imino
256 acid (Pro+Hyp) content than tuna-skin gelatin (210 residues in bovine gelatin
257 *vs.* 185 residues in tuna gelatin) (Gomez-Estaca, Montero, Fernandez-Martin &

258 Gomez-Guillen, 2009), and this is well known to be related to enhanced
259 physical properties and higher thermostability of the resulting gelatin gels
260 (Ledward, 1986; Norland, 1990).

261 Adding chitosan modified the viscoelastic properties of both gelatins. The
262 maximum G' values were considerably lower than the values for solutions B and
263 T, and the thermal transition points also occurred at lower temperatures. This is
264 indicative of a pronounced loss of the gelatin's ability to refold into triple-helix
265 chains in the presence of chitosan. A decrease in percentage renaturation of
266 food grade gelatin by incorporation of chitosan has been previously reported
267 (Arvanitoyannis et al., 1998). Adding chitosan at the higher concentration led to
268 a substantial increase in the viscous modulus (G'') during cooling. Furthermore,
269 phase angle values at both concentrations were higher than the values for the
270 corresponding gelatin solutions at temperatures below 10 ºC. Both these effects
271 also indicate that chitosan interferes with protein network formation as a result
272 of gelatin-chitosan interactions. These effects were considerably more marked
273 for the tuna-skin gelatin, especially at the higher chitosan concentration, as
274 indicated by the higher increase in G'' and the not so steep slope of the phase
275 angle during cooling and subsequent heating. Thus, the chitosan interacted
276 more with the tuna-skin gelatin than with the bovine-hide gelatin. Interactions
277 between chitosan and collagen have previously been described (Sionkowska et
278 al., 2004) and were also reflected by an increase in the viscosity values. In the
279 present experiment the collagen had previously been denatured to obtain
280 soluble gelatin, presumably heightening this interaction. The interactions
281 between gelatin and chitosan are produced by both electrostatic and hydrogen
282 bonding (Taravel & Domard, 1995). The former are a consequence of the

283 different charges of gelatin, an anionic biopolymer, and the cationic chitosan.
284 The latter occur extensively between the –COOH, -NH_2, and –OH groups on the
285 amino acids in the gelatin and the –OH and –NH_2 groups on the chitosan, and
286 are particularly notable at low temperatures. Regarding the contribution of
287 electrostatic interactions, it should be noted that both gelatins are type A, i.e.,
288 they had been subjected to an acidic pre-treatment, therefore both are
289 presumed to have similar isoelectric points, being net positively charged at pH
290 6. The most likely explanation for such a different interaction could be the
291 differing imino acid content (Pro+Hyp), which was considerably lower in the
292 tuna-skin gelatin. The abundance of pyrolidine iminoacid content (Pro, and
293 especially Hyp), is well known to be directly implicated in the gelling mechanism
294 promoting the formation of junction zones of triple collagen-like helices
295 (Ledward, 1986). In this connection, the lower iminoacid content in the tuna
296 gelatin would lead to a less extensive self-aggregation of the gelatin chains,
297 which might have contributed to an increment of the gelatin-chitosan
298 interactions.

299 The gel strength determinations (Figure 2) carried out on the cold-matured film-
300 forming solutions revealed a decrease in the mechanical properties of the
301 gelatin gels when chitosan was added, which is ascribed not only to a dilution
302 effect of the gelatin in the film-forming solution but also to the gelatin-chitosan
303 interactions. The effect was more pronounced at the higher concentration of
304 added chitosan, indicating that chitosan not only interfered with nucleation point
305 formation but also with triple-helix chain growth during cold maturation. No
306 differences were found between both types of gelatin.

307

Films

Warm-water fish gelatins have been reported to have quite similar properties to mammalian gelatins (Gilsenan & Ross-Murphy, 2000), and the tuna-skin gelatin films showed Tg values that were very slightly lower (within experimental error) than the bovine-hide gelatin films (Figure 3a, curves B and T; Table 1), in agreement with previous reports (Gomez-Estaca et al., 2009). Films made from various chitosans (different degrees of deacetylation, OH and amino groups, crystallinity, etc.) in the presence of various amounts of different hydrophilic plasticizers have been reported to exhibit very high (>>100ºC) glass transition temperatures (Suyatma, Tighzert & Copinet, 2005). The chitosan films tested here, however, had a rather low glass transition temperature, even lower than the values for the two gelatins (Table 1); as the ratio between the polymer and the glycerol plus sorbitol equiproportional mixture remained constant in all cases, the considerable higher water content in Ch (~22%) was presumably a main responsible. The value seemed to be consistent with a phase diagram previously published (Lazaridou & Biliaderis, 2002) for a system comprising 70 % chitosan and 30 % sorbitol at different moisture contents.

As discussed above, chitosan has been reported to interact molecularly with collagen by forming a wide range of blends where new hydrogen bonding networks appear, the triple helical structure of the collagen being replacing as the chitosan level increases (Sionkowska et al., 2004). In other work, the formation of gelatin-chitosan polyelectrolyte complexes has been shown to give rise to a decrease of the crystallinity of the system (Yin, Yao, Cheng & Ma, 1999). Other factors affecting the properties of gelatin-chitosan films are the evaporation temperature and the presence of plasticizers (water and polyols)

(Arvanitoyannis et al., 1998). Furthermore, the interactions between gelatin and chitosan are supposed to be dependent on the physical and chemical properties of the gelatin, which vary considerably with gelatin origin. As a general rule, mammalian gelatins afford better physical properties and thermostability than fish gelatins, and there are, moreover, appreciable differences among gelatins from different fish species (Gomez-Guillen et al., 2002). The main factors determining the physical properties of a gelatin and hence gelatin quality are the amino acid composition and the molecular weight distribution (Gomez-Guillen et al., 2002). Mammal's gelatins are typically richer in imino acids whereas the average molecular weight highly depends on the extraction procedures. Both these factors will presumably affect the interactions between the gelatin and chitosan. Thus, the physico-chemical properties, and ultimately the potential applications of the resulting materials will all be closely related to the properties of the polymer admixture employed.

In the present work, the glass transition temperatures of compound films were higher than those for the single components, indicating some level of interaction/association. At each of the two chitosan levels, the Tg values for both series of compound gelatin-chitosan blends were quite close and at the lower Ch concentration were nearly 6 ºC higher than for the gelatins alone (Table 1). Augmenting the chitosan component resulted in a further increase of around 3 ºC in the Tg. Higher water contents may produce higher plasticization effects in the blended films by an increase in the macromolecules mobility. However, it appeared clearly overcame by the increase matrix density effect caused by the strong associations between gelatins and chitosan, with the final result of glass transition processes occurring at higher temperatures in the

358 blends than in the single films. Initially, the polyols mixture is highly
359 hygroscophic so that water may plasticize both the low-molecular-weight
360 mixture and the high-molecular-weight components. The water-alone
361 contribution to the plasticization of the different polymeric blends seemed hardly
362 difficult to being separated from the whole plasticizer system. Additionally the
363 humidity values for the B-Ch and T-Ch films were quite similar; small
364 differences in water content could be basically modulated by the glycerol plus
365 sorbitol plus water entire system, so that small variations in water are expected
366 not to play an essentially differential role in the plasticization process and Tg
367 values of the complex films. Therefore, the glass transition behaviour of the B-
368 and T-type compounded films with Ch reasonably showed a paralleled
369 evolution.

370 All these findings were consistent with the thermal scan data obtained for the
371 corresponding gels by rheometry, as shown in Figure 1 (bottom, curves a and
372 b). At both Ch levels hysteresis of the sol-gel-sol transitions increased in the
373 film-forming solutions of both gelatin-chitosan blends relative to those in the
374 corresponding gelatins. Adding the larger amount of chitosan increased the
375 complexity of the gel-sol and sol-gel profiles of both systems, particularly T-
376 Ch1.5, which could be interpreted as a certain level of phase separation in the
377 systems. Gelatin-rich phases (more frequent in the T systems than in the B
378 systems) have higher gel-sol and sol-gel transition temperatures and,
379 conversely, chitosan-rich phases have lower gel-sol and sol-gel transition
380 temperatures. Although this gelling behaviour is not necessarily transferred to
381 the respective films, DSC did not reveal any phase separation in the Ch1.5
382 systems.

383 Table 1 lists the glass transition temperature, mechanical properties (breaking
384 strength and breaking deformation), solubility, and water vapour permeability of
385 the different films. Breaking strength and water solubility values were similar for
386 both gelatin films (B and T), a result attributable to the presence of very high-
387 molecular-weight aggregates (Gomez-Estaca et al., 2009). In comparison, the
388 chitosan film (Ch) was stronger than either of the gelatin films (B and T),
389 although it was also more soluble in water. Perhaps the most interesting feature
390 was that the breaking deformation value for film T was ~10 times higher than
391 the values for films B and Ch. Deformation differences among gelatin films have
392 been previously attributed to different imino acid contents, giving bovine-hide
393 gelatin a higher degree of molecular rigidity (Gomez-Estaca et al., 2009). The
394 water vapour permeability values showed all the films to be quite permeable,
395 entirely consistent with films prepared from biopolymers plasticized with polyols.
396 This has been reported to be also a result of increases in the free volume
397 between polymer chains caused by a reduction in intermolecular attractive
398 forces, making the polymer network less dense and thus more permeable (Cuq,
399 Gontard, Cuq & Guilbert, 1997). However, some differences among the
400 formulations were found. Thus, the Ch film was more permeable to water
401 vapour than the B and T films. Gelatin origin was also a factor, T being less
402 permeable than B, again attributable to both the amino acid profile and the
403 molecular weight distribution (Avena-Bustillos et al., 2006; Gomez-Estaca et al.,
404 2009). Comparisons among different authors are somewhat difficult because of
405 the different film manufacture and measurement procedures. There is a study in
406 which the WVP of compound fish gelatine-chitosan films as well as that of the
407 pure components were studied (Kolodziejska et al., 2007). In this work neither

408 the gelatine and the chitosan pure films nor the compound ones were
409 significantly different. In other study (Arvanitoyannis et al., 1998) mammal
410 gelatine and chitosan were blended at a ratio 1:1, plasticized with different
411 amounts and type of plasticizers and dried at different temperatures, resulting in
412 WVPs ranging from 1.1 to $6.3x10^{-11}$ g m/m^2 s Pa, which are three orders of
413 magnitude lower than ours. In this case the single components were not
414 analyzed.

415 On the whole, the physico-chemical properties of the compound gelatin-
416 chitosan films were more appropriated for practical purposes than the properties
417 of the films made from a single biopolymer only (films B, T, and Ch). As a
418 general rule, the compound films had breaking strengths similar to the values
419 for the Ch films ($p \leq 0.05$), the strongest films, and significantly higher ($p \leq 0.05$)
420 than the values for the gelatin films. Breaking deformation either stayed the
421 same (B-Ch blends) or decreased (T-Ch blends) significantly ($p \leq 0.05$)
422 compared with the respective gelatin films. In spite of the decrease of breaking
423 deformation in T-Ch films compared to T ones, these mixtures showed a higher
424 breaking deformation than B, Ch and B-Ch films. Solubility of the compound
425 films was significantly lower ($p \leq 0.05$) compared with that of chitosan films
426 whatever the gelatin employed. Furthermore, in tuna-skin gelatin-chitosan
427 mixtures the solubility was significantly ($p \leq 0.05$) lower than that of the tuna-skin
428 gelatin. Water vapour permeability of the compound films decreased
429 significantly ($p \leq 0.05$) compared with the single biopolymer component films Ch
430 and B, and was similar to the values for T film, the one with the lower WVP
431 among plain films, in all cases. All the alterations in the physico-chemical
432 properties of the films can be attributed to the gelatin-chitosan interactions

433 previously reported by different researchers (Sionkowska et al., 2004; Taravel
434 et al., 1995; Yin et al., 1999) and confirmed in this study by the results for the
435 viscoelastic properties and gel strength of the film-forming solutions. The
436 interactions were observed to be stronger in the case of the tuna-skin gelatin,
437 hence the alterations in the physical and chemical properties upon blending with
438 chitosan were most discernible for the T-Ch formulations, especially regarding
439 the film breaking deformation and water solubility.

440 It was also evaluated the possible loss of antibacterial activity of chitosan upon
441 mixing with the bovine or the tuna gelatins. For this purpose, *S. aureus* was
442 selected as a model of gram-positive microorganism that is also a relevant food
443 poisoning microorganism. As expected, neither the B and T gelatin film-forming
444 solutions nor the B and T gelatin films employed as controls exhibited any
445 antibacterial effect (Figure 4). On the other hand, all the chitosan-containing
446 formulations displayed distinctly discernible antimicrobial effects against *S.*
447 *aureus*. For purposes of economy, only the photographs for the T films have
448 been included here. So, the good inhibitory effects of the chitosan against *S.*
449 *aureus* were maintained in spite of the gelatin-chitosan interactions observed.
450 Other authors (Fernandez-Saiz, Lagaron, Hemandez-Munoz & Ocio, 2008) also
451 found a gliadin-chitosan blend to effectively reduce the growth of *S. aureus*.

452 In conclusion, mixing gelatin and chitosan may be a means to improve the
453 physico-chemical performance of gelatin and chitosan plain films, especially
454 when using fish gelatin, without altering the antimicrobial properties.

455

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613

614 **Figure captions**

615

616 Figure 1. Dynamic viscoelastic properties of the film-forming solutions. B
617 (bovine-hide gelatin), T (tuna-skin gelatin), Ch (chitosan), B-Ch 0.75% (bovine-
618 hide gelatin plus 0.75% chitosan), T-Ch 0.75% (tuna-skin gelatin plus 0.75%
619 chitosan), B-Ch 1.5% (bovine-hide gelatin plus 1.5% chitosan), T-Ch 1.5%
620 (tuna-skin gelatin plus 1.5% chitosan).

621 Figure 2. Gel strength of the film-forming solutions after cold maturation at 2 ºC.
622 Batch designations as in Figure 1.

623 Figure 3. Glass transition behaviour of one (a) and two (b) component films. (a)
624 One-component films: Bovine-hide gelatin, curve B; tuna-skin gelatin, curve T;
625 chitosan, curve Ch. (b) Two-component films: curves B-Ch 0.75%, B-Ch 1.5%,
626 T-Ch 0.75%, T-Ch 1.5%.

627 Figure 4. Antimicrobial activity of the tuna-skin gelatin (T) and the tuna-skin
628 gelatin plus 1.5% chitosan (T-Ch 1.5%) film-forming solutions (FFS) and films
629 against *S. aureus*. Arrows indicate the inhibition areas.

630

Table 1. Physico-chemical properties (glass transition temperature, breaking strength and breaking deformation by puncture test, solubility, and water vapour permeability) of the film batches [B (bovine-hide gelatin), T (tuna-skin gelatin), Ch (chitosan), B-Ch 0.75% (bovine-hide gelatin plus 0.75 % chitosan), T-Ch 0.75% (tuna-skin gelatin plus 0.75 % chitosan), B-Ch 1.5% (bovine-hide gelatin plus 1.5 % chitosan), T-Ch 1.5% (tuna-skin gelatin plus 1.5 % chitosan)].

	B	T	Ch	B-Ch 0.75%	B-Ch 1.5%	T-Ch 0.75%	T-Ch 1.5%
Tg (ºC)	41.6 ± 0.6^{a}	41.0 ± 0.6^{a}	34.6 ± 0.7^{b}	46.9 ± 0.8^{c}	49.7 ± 0.9^{d}	47.4 ± 0.9^{c}	49.6 ± 09^{d}
Breaking strength (N)	10.7 ± 2.2^{a}	8.5 ± 1.6^{a}	23.0 ± 2.9^{b}	18.4 ± 3.6^{b}	18.8 ± 3.3^{b}	13.2 ± 2.2^{a}	20.0 ± 3.5^{b}
Breaking deformation (%)	14.1 ± 5.0^{a}	154 ± 36^{b}	19.2 ± 4.7^{a}	11.0 ± 3.1^{a}	19.9 ± 4.3^{a}	68 ± 6^{c}	40 ± 9^{a}
Solubility (%)	34.3 ± 0.6^{ab}	39.9 ± 1.3^{b}	82 ± 11^{c}	29.4 ± 2.0^{ab}	33.1 ± 1.8^{ab}	17.5 ± 1.6^{a}	19.0 ± 4.4^{a}
WVP (10^{-8}·g·mm·h^{-1}·cm^{-2}·Pa^{-1})	2.20 ± 0.11^{a}	1.65 ± 0.4^{b}	2.43 ± 0.26^{c}	1.91 ± 0.06^{b}	1.44 ± 0.23^{b}	1.75 ± 0.37^{b}	1.98 ± 0.13^{ab}

Different letters (a, b, c) in the same row indicate significant differences ($p \leq 0.05$).
Tg = Glass transition temperature
WVP = Water vapour permeability

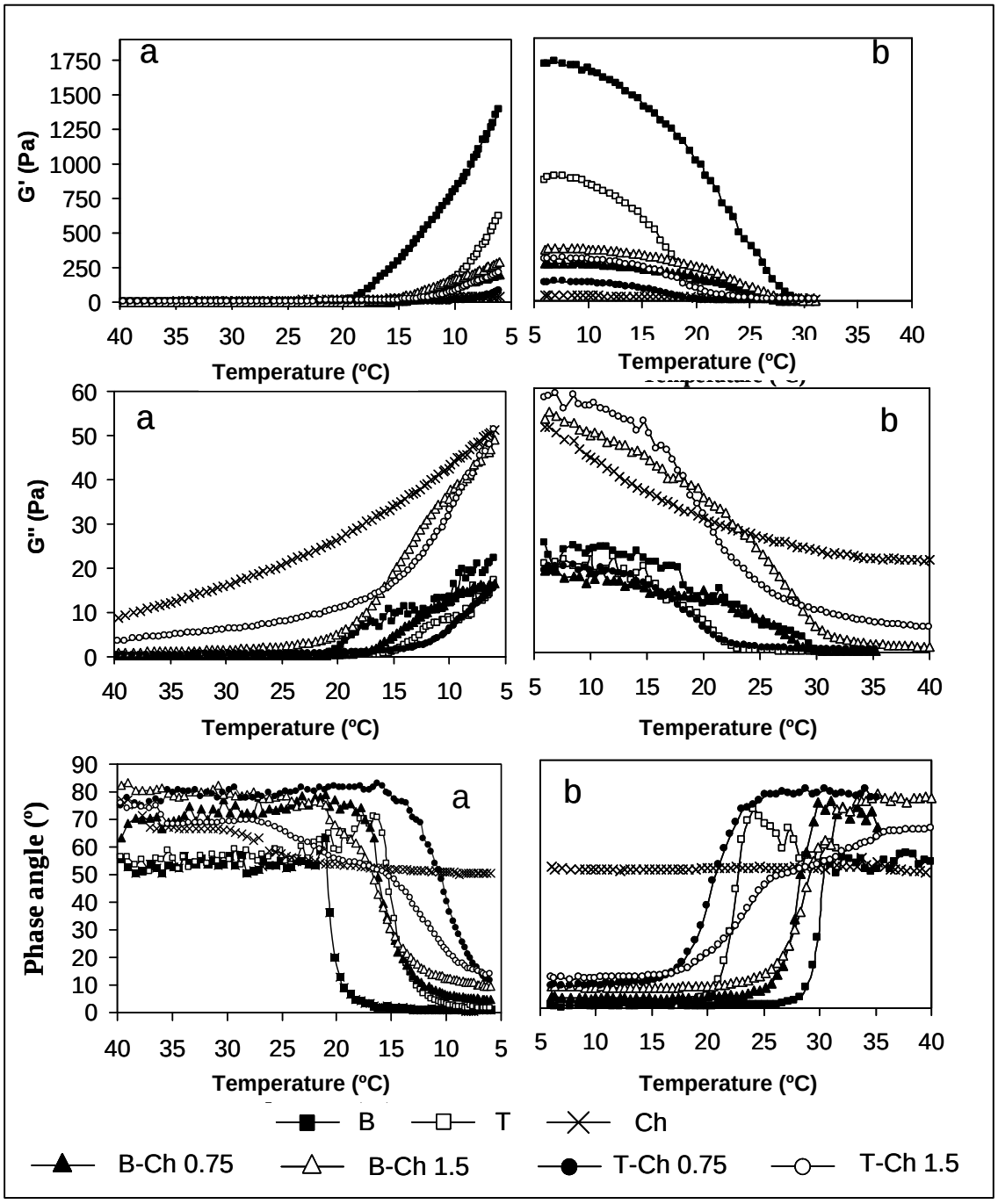
a
b
G' (Pa)
1750
1500
1250
1000
750
500
250
0
40 35 30 25 20 15 10 5
5 10 15 20 25 30 35 40
Temperature (ºC)
a
b
G'' (Pa)
60
50
40
30
20
10
0
40 35 30 25 20 15 10 5
5 10 15 20 25 30 35 40
Temperature (ºC)
a
b
Phase angle (º)
90
80
70
60
50
40
30
20
10
0
40 35 30 25 20 15 10 5
5 10 15 20 25 30 35 40
Temperature (ºC)
B
T
Ch
B-Ch 0.75
B-Ch 1.5
T-Ch 0.75
T-Ch 1.5

Figure 1

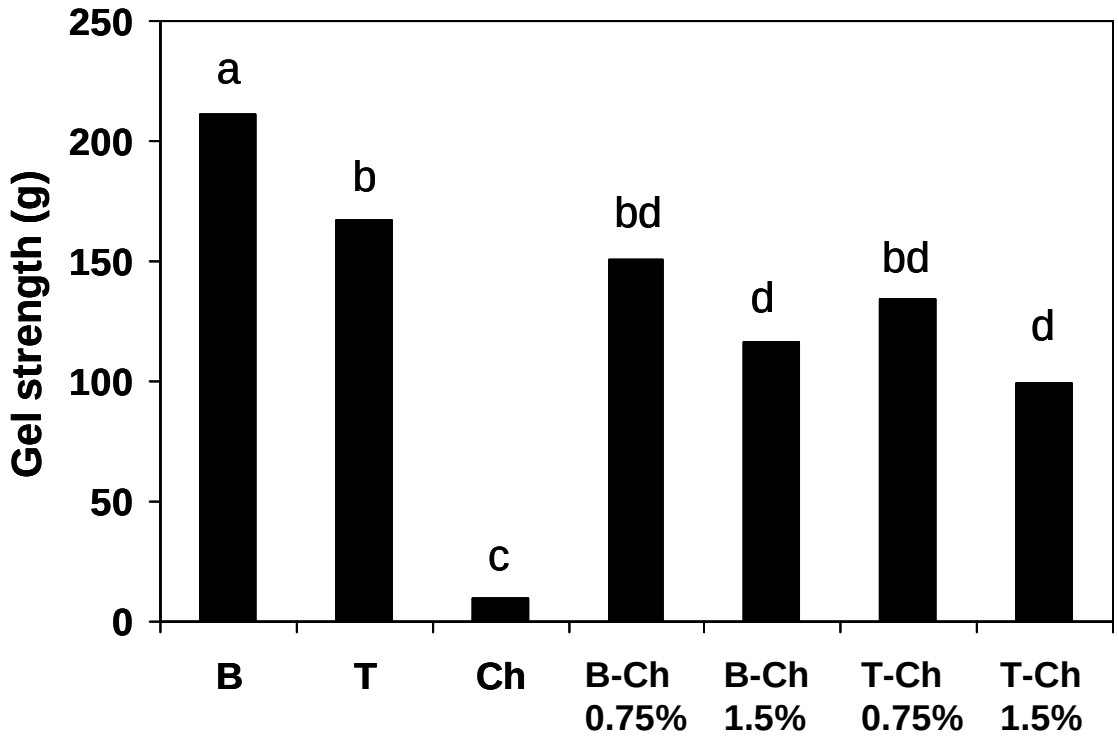
Gel strength (g)
250
200
150
100
50
0
a
b
c
bd
d
bd
d
B
T
Ch
B-Ch 0.75%
B-Ch 1.5%
T-Ch 0.75%
T-Ch 1.5%

Figure 2

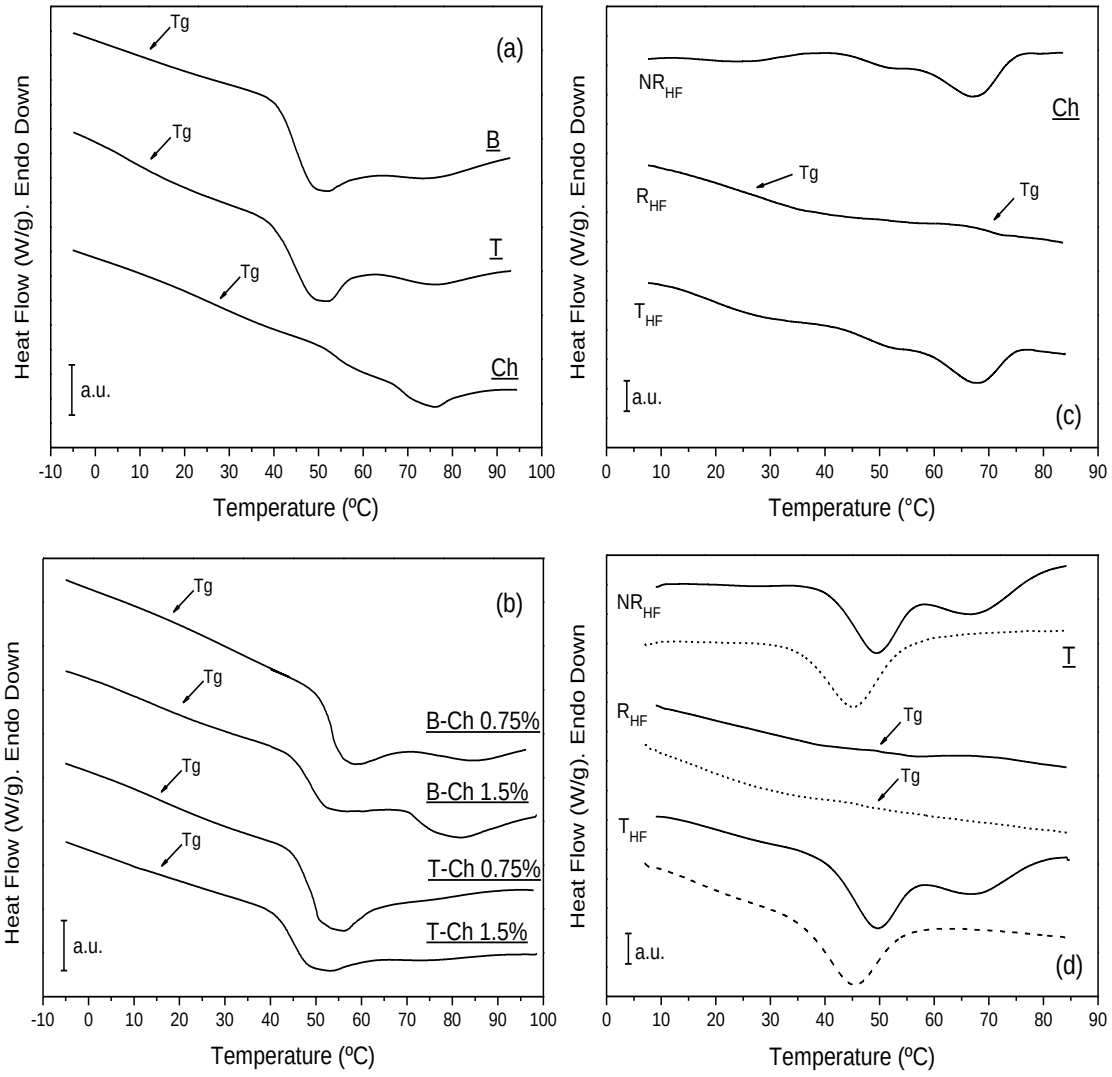
(a)
Tg
B
T
Ch
a.u.
Heat Flow (W/g). Endo Down
Temperature (ºC)
(b)
B-Ch 0.75%
B-Ch 1.5%
T-Ch 0.75%
T-Ch 1.5%
(c)
NR_HF
R_HF
T_HF
Ch
(d)
T

Figure 3

e 4