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## Food Chemistry

### Effect of glucose substitution by low-molecular weight chitosan-derivatives on functional, structural and antioxidant properties of Maillard reaction-crosslinked chitosan-based films

--Manuscript Draft--

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<b>Abstract:</b>	<p>In this study, the effects of different temperatures, incubation times and types of reducing sugars, including glucose and different low molecular weight (Mw) chito-oligosaccharides (COS) with varying acetylation degree (AD), on the extent of Maillard reaction (MR) on chitosan-based films were studied. Interestingly, an improvement of structural and functional properties of all MR-crosslinked films was noted, which is more pronounced by heating at higher temperature and exposure time. These findings were proved through FTIR and X-ray diffraction analyses. In addition, color change and Ultraviolet spectra demonstrate that glucose addition provides the high extent of MR, followed by COS1 and COS2 . These results were confirmed by enhanced water resistance and thermal properties. Moreover, MR-chitosan/COS films showed the highest mechanical properties, whereas, glucose-loaded films were brittle, as demonstrated by scanning electron microscopy micrographs. Furthermore, MR-chitosan/COS1 films exhibited the better antioxidant behavior followed by chitosan/glucose and chitosan/COS2 films, mainly at higher heating-conditions.</p>

## Highlights

- Maillard reaction (MR) was induced in chitosan-glucose and chitosan/**chito-oligosaccharides** films;
- The type of reducing sugar involved in MR had a great impact on its extent;
- MR rate was influenced by temperature and heating time;
- Functional and structural properties of films were modified by heat treatments.
- Antioxidant properties were highly enhanced by the MR products.

24 **Effect of glucose substitution by low-molecular weight chitosan-derivatives on**  
25 **functional, structural and antioxidant properties of Maillard reaction-crosslinked**  
26 **chitosan-based films**

27  
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47 **Abstract**

48 In this study, the effects of different temperatures, incubation times and types of reducing  
49 sugars, including glucose and different low molecular weight (Mw) chito-oligosaccharides  
50 (COS) with varying acetylation degree (AD), on the extent of Maillard reaction (MR) on  
51 chitosan-based films were studied. Interestingly, an improvement of structural and functional  
52 properties of all MR-crosslinked films was noted, which is more pronounced by heating at  
53 higher temperature and exposure time. These findings were proved through Fourier-transform  
54 infrared and X-ray diffraction analyses. In addition, color change and Ultraviolet spectra  
55 demonstrate that glucose addition provides the high extent of MR, followed by COS1 (Mw <  
56 4.4 kDa; AD, 18.20%) and COS2 (Mw < 4.4 kDa; AD, 10.63%). These results were confirmed  
57 by enhanced water resistance and thermal properties. Moreover, MR-chitosan/COS films  
58 showed the highest mechanical properties, whereas, glucose-loaded films were brittle, as  
59 demonstrated by scanning electron microscopy micrographs. Furthermore, MR-chitosan/COS1  
60 films exhibited the better antioxidant behavior followed by chitosan/glucose and  
61 chitosan/COS2 films, mainly at higher heating-conditions. Thereby, MR-crosslinked  
62 chitosan/COS based films were attractive to be applied as functional and active coating-  
63 materials in various fields.

64 **Keywords:**

65 Chitosan; Chito-oligosaccharides; Films; Maillard reaction conditions; Physicochemical  
66 characterization; antioxidant potential.

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## 70 1. Introduction

71 Nowadays, many research has been made to develop natural, biodegradable and non-toxic  
72 bio-based packaging to enhance the shelf-life of fresh or packaged commercialized food as  
73 alternative to synthetic packaging, which are the main cause of environmental pollution  
74 (Galiano et al., 2018; Liu et al., 2019). Indeed, coating material should protect food from all  
75 pathogenic contamination, oxidation and drying and perfectly prevent the **Ultraviolet (UV)**  
76 radiation. For this reason, several polymers such as polysaccharides and proteins were widely  
77 used for biofilm preparation (Affes et al., 2020; Etxabide, Urdanpilleta, Guerrero, & de la Caba,  
78 2015; Gennadios, Handa, Froning, Weller & Hanna, 1998; Hamdi, Nasri, Ben Azaza, Li, &  
79 Nasri, 2020; Kchaou, Benbettaieb, Jridi, Nasri, & Debeaufort, 2019). Thanks to its film-forming  
80 properties, antioxidant and antimicrobial activities, chitosan-based film has received growing  
81 interest to be applied in different fields including food, biotechnology, cosmetics and  
82 biomedical domains (Aranaz et al., 2018; Chang et al., 2019; Hajji, Younes, Affes, Boufi, &  
83 Nasri, 2018; Li, Lin, & Chen, 2014).

84 The physicochemical properties of chitosan films were depended on chitosan  
85 characteristics mainly its molecular weight (Mw), acetylation degree (AD) and  
86 depolymerization degree (Affes et al., 2021; Hamdi et al., 2019; Leceta, Guerrero, & de la Caba,  
87 2013a; Li et al., 2014). All these parameters should be taken into account to choose the better  
88 film formulation that gives good barrier properties against light and water, mechanical  
89 resistance and biological activities. Further, in order to enhance chitosan film characteristics  
90 several chemical, physical and enzymatic modifications methods were envisaged to enlarge  
91 their domain of applications including Maillard reaction (MR), heat treatment and the  
92 combination of two polymers (Affes et al., 2020c; Fernández-de Castro et al., 2016; Gullón et  
93 al., 2016; Hajji et al., 2018; Leceta, Guerrero, Ibarburu, Dueñas, & de la Caba, 2013b). **Given**

94 the good antioxidant and antibacterial activities of MR products (MRP) in food industry, their  
95 use as preservatives is attracting increasing interest (Gullón et al., 2016; Hamdi et al., 2020).

96 Maillard reaction is a non-enzymatic crosslinking complex biochemical process, which  
97 is done by heat treatment of amino acids in the presence of reducing sugars, and that produces  
98 fluorescent, brown final MRP named melanoidins with high biological properties (Affes et al.,  
99 2020c; Etxabide, Urdanpilleta, Gómez-Arriaran, de la Caba, & Guerrero, 2017; Sun et al., 2017;  
100 Li et al., 2014). Three stages in the MR are usually considered. The initial products are called  
101 Schiff bases. They form Amadori products via rearrangement, which undergo further reactions  
102 to form irreversible advanced glycation end products (Kchaou et al., 2018). Having amino  
103 groups makes chitosan a candidate to react with the carbonyl group of reducing sugars, and  
104 allows it to participate in the MR (Gullón et al., 2016; Li et al., 2014). The rate of this chemical  
105 reaction is influenced by many factors, including the type, the concentration and the ratio of  
106 carbonyl/amino groups, pH, temperature, time of exposure and water activity (Hamdi et al.,  
107 2020; Kchaou et al., 2018; Kchaou et al., 2019; Gullón et al., 2016; Xu, Huang, Xu, Liu, &  
108 Xiao, 2019).

109 In a previous study (Affes et al., 2020c), the factor amino-containing compound was  
110 investigated using chitosan and high Mw-chitosan-depolymerization-products (CDP) (1244.7,  
111 482.03 and 163.56 kDa), while maintaining constant the same added-carbonyl-containing  
112 compound, which is glucose at a concentration of 0.5 mg/ml, in all films' formulations. Changes  
113 undergone by crosslinking and MR development, after heat-treatment during 24 h at 90 °C,  
114 demonstrated better functional, thermal and mechanical properties in higher Mw chitosan-based  
115 films. Although many studies, dealing with MR crosslinked-chitosan-based films, have been  
116 achieved and the factors affecting the reaction, including characteristics (Mw and AD of  
117 chitosan) and concentration of amino groups donors, pH and water activity, have been  
118 evaluated, there are no many previous reports on the effect of the others factors, particularly the

119 nature of reducing sugars (Fernández-de Castro et al., 2016; Gullón et al., 2016; Kosaraju et al.,  
120 2010; Li et al., 2014). Thus, in the present work, chitosan was used as constant amino-  
121 containing compound and other parameters affecting MR development were studied.

122 The present research aims to evaluate the effects of different heating conditions, including  
123 temperature and incubation time, and types of reducing sugars (glucose and different low  
124 molecular weight (Mw) chito-oligosaccharides (COS) with varying acetylation degree (AD)),  
125 at a concentration of 5 mg/ml, on the extent of MR on chitosan-based films. To this end,  
126 chitosan, chitosan/glucose and chitosan/COS-based films were thermally treated at 90 and 110  
127 °C and for 6 and 24 h and their functional, structural, thermal, mechanical and antioxidant  
128 properties were compared.

129 To the best of our knowledge, this is the first study dealing with the use of low-Mw COS,  
130 having different acetylation degree, as carbonyl-containing compounds, to develop MR on  
131 chitosan-based films, to optimize films crosslinking conditions, as well as to correlate MR rate  
132 with resulted films physico-chemical and antioxidant properties.

## 133 2. Materials and methods

### 134 2.1. Materials

135 Shrimp chitosan (Ch) (Molecular weight (Mw), 1244.70 kDa; Acetylation degree (AD),  
136  $7.60 \pm 0.54\%$ ) was prepared as described in our previous study (Affes et al., 2019) and  
137 employed as filmogenic biopolymer for films preparation. Two different chito-oligosaccharides  
138 (COS) were prepared by chitosan hydrolysis during 24 h using the chitosanolytic crude extract  
139 from *Portunus segnis* blue crab viscera (pH 4.0, 40 °C and E/S ratio = 100 U/g chitosan) (Affes  
140 et al., 2019) and the chitosanolytic preparation from *Bacillus licheniformis* strain (pH 5.0, 50  
141 °C and E/S ratio = 70 U/g chitosan) (Affes et al., 2020b), respectively. The mixtures  
142 chitosan/chitosanases were incubated at appropriate conditions and the obtained soluble parts



143 were freeze-dried, characterized by using **steric exclusion chromatography (SEC-HPLC)** and  
144 the first derivative **Ultraviolet (UV)** spectrophotometric method and referred as COS1 (Mw, <  
145 4.4 kDa; AD,  $18.20 \pm 1.19\%$ ) and COS2 (Mw, < 4.4 kDa; AD,  $10.63 \pm 0.17\%$ ) using the  
146 digestive and bacterial chitosanases, respectively.

147 COS1, COS2 and D (+) anhydrous-glucose (Glu) ( $C_6H_{12}O_6$ ;  $180 \text{ g mol}^{-1}$ ) were used as  
148 reducing sugars to initiate the Maillard Reaction (MR) in chitosan-based films. Anhydrous  
149 glycerol was purchased from Fluka (98% purity, Fluka Chemical, Germany) and used as  
150 plasticizer for the films. All other reagents were of analytic grade.

## 151 **2.2. Films preparation**

152 Films forming solutions (FFS) were prepared by dissolving chitosan (10 mg/ml) in acetic  
153 acid (1%, v/v). Then, three categories of films were prepared by the addition of three different  
154 reducing sugars to promote MR development; glucose, COS1 and COS2, at a concentration of  
155 5 mg/ml. Glycerol was added as a plasticizer to the solutions at a concentration of 15% (w/w  
156 dry chitosan matter) and the FFS were maintained under stirring for 30 min. Subsequently, a  
157 volume of 34.0 ml of each FFS was cast in Polystyrene Petri dishes (13.5 x 13.5 cm diameter)  
158 and left to dry for 48 h at 25 °C. Dried films were manually peeled off from the surface and  
159 non-heated films were considered as blank and referred as F1 (Ch), F2 (Ch-Glu), F3 (Ch-COS1)  
160 and F4 (Ch-COS2).

161 All prepared films were then conditioned at 25 °C and 50% **relative humidity (RH)** before  
162 analyses, except for **Fourier-transform infrared spectroscopy (FTIR)**, **X-ray diffraction (XRD)**  
163 and **thermogravimetric analyses (TGA)**, films were equilibrated at 0% RH.

## 164 **2.3. Heating treatment of films**

165 Then, to induce MR, the films (with or without reducing sugar) were heated in an oven  
166 at two different temperatures ( $90 \pm 2$  and  $110 \pm 2$  °C) and for different incubation times (6 and

167 24 h). The films heated at 90 °C during 6 and 24 h were named F1-90-6 h, F1-90-24 h, F2-90-  
168 6 h, F2-90-24 h, F3-90-6 h, F3-90-24 h, F4-90-6 h and F4-90-24h, respectively for F1, F2, F3  
169 and F4. While, the films heated at 110 °C were named F1-110-6 h, F1-110-24 h, F2-110-6 h,  
170 F2-110-24 h, F3-110-6 h, F3-110-24 h, F4-110-6 h and F4-110-24h for F1, F2, F3 and F4  
171 respectively.

## 172 2.4. Physicochemical characterization of the films

### 173 2.4.1. Color properties

174 Color development was studied using a CIE colorimeter (CR-5, Konica Minolta, Sensing  
175 Europe B.V). Color of the films was expressed as L\* (lightness/brightness), a\* (greenness/  
176 redness) and b\* (blueness/yellowness). The total color change ( $\Delta E^*$ ) of the films was  
177 determined as follows:

$$178 \quad \Delta E = \sqrt{((L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2)} \quad \text{Eq. (1)}$$

179 where  $L_0^*$ ,  $a_0^*$ ,  $b_0^*$  are the colorimetric parameters of the film F1, as standard, and  $L^*$ ,  $a^*$ ,  $b^*$   
180 are the values of the modified films.

181 The obtained CIE Lab values were then used to calculate the browning index (BI) as  
182 mentionned in equation 2:

$$183 \quad BI = \frac{(100 \times (z - 0.31))}{0.172} \quad \text{with} \quad z = \frac{a^* + 1.75 (L^*)}{5.645 (L^*) + (a^*) - 3.012 (b^*)} \quad \text{Eq. (2)}$$

### 184 2.4.2. UV-visible light spectroscopy

185 The films were cut into rectangles (1.0 x 3.0 cm) and placed in the test cell of an **UV-**  
186 **visible (UV-vis)** spectrophotometer (Shimadzu UV-2401PC). Light transmission of the films  
187 was determined in the wavelength range from 200 to 800 nm. An empty test cell was used as a  
188 reference.

### 189 2.4.3. FTIR-ATR analysis

190 FTIR spectra of the studied films were assessed using a spectrometer (Agilent  
191 Technologies, Cary 630 series) equipped with an attenuated reflection accessory (ATR)  
192 containing a diamond/ZnSe crystal, at 25 °C. Data analysis and treatment was carried out by  
193 using the OMNIC spectra software (Thermo Fisher Scientific).

#### 194 **2.4.4. Water content and solubility**

195 The water content (WC) of the films ( $\text{g}_{\text{moisture}}/100 \text{ g}_{\text{film}}$ ), was determined by measuring  
196 the weight loss of each film sample (100 mg) after drying in an oven at 105 °C until constant  
197 weight was reached according to the following equation:

$$198 \quad \text{WC (\%)} = \frac{(m_i - m_f)}{m_i} \times 100 \quad \text{Eq. (3)}$$

199 where  $m_i$  is the initial film weight (g) and  $m_f$  is the final is the final film dry weight (g). Three  
200 samples of each formulation were analyzed.

201 The solubility of the films in water (WS) was studied according to the Gennadios et al.  
202 (1998) method. WS was calculated as follows:

$$203 \quad \text{WS (\%)} = \frac{[(m_i \times (100 - \text{MC})) - m_f]}{(m_i \times (100 - \text{MC}))} \times 100 \quad \text{Eq. (4)}$$

204 where  $m_i$  is the initial film weight (g),  $m_f$  is the final dry film weight (g) and WC is the water  
205 content of each film sample (%). All tests were carried out in triplicate.

#### 206 **2.4.5. Water contact angle**

207 The contact angle measurements were measured using the sessile drop method on a GBX  
208 equipment (Digidrop, Romans, France), equipped with an image analysis software (Visiodrop).  
209 Six measurements per films were carried out. All the tests were conducted in an environmental  
210 chamber at 25 ( $\pm 2$ ) °C and with a relative humidity of 50 ( $\pm 1$ ) %.

#### 211 **2.4.6. Film thickness**

212 Film thickness was measured using a micrometer (Digimatic IP65, Mitutoyo, France).  
213 Six measurements at different positions were done for each film sample. The mean value was  
214 taken into account for mechanical properties parameters calculation.

#### 215 **2.4.7. Mechanical properties**

216 Tensile strength (TS, MPa) and elongation at break (EAB, %) of the films were  
217 determined using a rheometer apparatus (Physica MCR, Anton Paar, GmbH, France) equipped  
218 with a mechanical properties measuring geometry. Rheoplus software was used for the  
219 estimation of TS and EAB, corresponding respectively to the maximum load and the final  
220 extension at break from the tensile stress vs. strain curves. Six measurements for each films  
221 formulation were carried out at 25 °C.

#### 222 **2.4.8. Thermal stability analysis**

223 The thermal stability of the film was studied, at a temperature range from 30 to 600 °C,  
224 using a thermogravimetric analysis (TGA, Q500 High Resolution, TA Instruments). The  
225 temperature of maximum degradation ( $T_{max}$ , °C), the weight loss ( $\Delta w$ , %) and final residue at  
226 600 °C (%) values were determined using TA Universal Analysis 2000 software (Version 4.5  
227 A, TA instruments).

#### 228 **2.4.9. X-ray diffraction analysis**

229 The crystallinity of the selected films was performed by x-ray diffraction analysis, at  $2\theta$   
230 from 7 to 40 °, using a Philips diffractometer equipped with monochromatic Cu  $K_{\alpha}$  radiation  
231 source.

#### 232 **2.4.10. Films microstructure**

233 The microstructure of the films was assessed by surface and cross-section observations  
234 using a scanning electron microscopy (SEM, Hitachi S4800), at different magnifications.

## 235 **2.5. Antioxidant activity**

236 The capacity of film samples (10 mg) to scavenge 2,2'-Azino-bis (3-  
237 ethylbenzothiazoline-6-sulfonic acid) (ABTS<sup>+</sup>) and 1,1-diphenyl-2-picrylhydrazyl (DPPH)  
238 radicals was studied according to the methods of Re et al. (1999) and Bersuder, Hole, & Smith  
239 (1998), respectively. The absorbances of the final solutions were measured at 734 and 517 nm,  
240 after 10 min and 24 h of incubation in the dark, respectively for DPPH and ABTS scavenging  
241 activity.

242 The ability of films (10 mg) to reduce iron (III) was evaluated according to the method  
243 described by Yidirim, Mavi, & Kara (2001) with slight modification of the incubation time at  
244 50 °C during 3 h. The absorbance of the final solutions was measured at 700 nm.

245 The total antioxidant ability of the films (10 mg) to reduce Mo (VI) to Mo (V) and to  
246 form a green phosphate/Mo (V) complex at acidic pH was studied as reported by Prieto, Pineda,  
247 & Aguilar, (1999). The absorbance was measured at 695 nm. All the experiments were carried  
248 out in triplicate.

## 249 **2.6. Statistical analysis**

250 Statistical analyses were performed with IBM SPSS Statistics v.20. Normal distribution  
251 and equality of variance were tested with the Shapiro-Wilk and Levene's tests respectively.  
252 Statistically significant differences were then calculated using the Kruskal-Wallis test for non-  
253 normal distributed data with the dunn's post hoc test. Differences were considered significant  
254 at *p value* < 0.05. Standard deviation errors were used to compare all experiments.

## 255 **3. Results and discussion**

### 256 **3.1. Effect of Maillard reaction on films optical and spectral properties**

#### 257 **3.1.1. Films color change**

258 To evaluate the effect of MR on chitosan-based films, different reducing sugars including  
259 glucose and chito-oligosaccharides (COS1 and COS2) were added to the FFS. Films were  
260 incubated at 90 and 110 °C for 0, 6 and 24 h to perform MR. During reaction, reducing sugars  
261 reacted with amino acid residues of chitosan to form the final MR products (MRP). The final  
262 stage of MR was evaluated by color change measurement. Films color was measured using  
263 CIELab scale and L\* (darkness/whiteness), a\* (greenness/redness) and b\*  
264 (blueness/yellowness) parameters were used to calculate difference in color ( $\Delta E^*$ ) and  
265 browning index (BI).

266 Results illustrated in **Table 1** showed that L\* values of the control unheated films F1, F3  
267 and F4 are similar ( $p > 0.05$ ). However, it decreased significantly in the film F2 containing  
268 glucose (Glu). Further, after thermal treatment of the films, L\* values decreased significantly  
269 indicating that these films turn darker. For all the formulations, this decrease was more  
270 noticeable after heating at 110 °C, as compared to 90 °C and after incubation time during 24 h  
271 in comparison with those incubated for 6 h. Furthermore, the decrease of L\* values was more  
272 pronounced in the films containing glucose followed by the films containing COS1 and COS2  
273 as compared to the films of chitosan. After heating at 90 °C, there are no significant decrease  
274 in L\* values of chitosan films without reducing sugar, however, negligible change of L\* values  
275 was observed after heating at 110 °C. Darker color of the films indicate the development of  
276 MR, due to the interaction between chitosan and reducing sugars (Glu or COS), allowing to the  
277 production of dark MRP. These brown products produced during the final stage of MR are  
278 melanoidins (Affes et al., 2020c).

279 Interestingly, thermal treatment of all the films decreased their a\* values and increased  
280 their b\* values, as compared to the control unheated films, turning to green and yellow color  
281 region, respectively ( $p < 0.05$ ). These variations were more significantly pronounced as

282 temperature and heating time increased and in the films containing glucose followed by the  
283 films containing COS1 and COS2 as compared to chitosan films.

284 Moreover,  $\Delta E^*$  values, obtained by comparing the color of the films with the film F1,  
285 showed a slight difference between the control films more pronounced in the film F2 ( $3.6 \pm$   
286  $0.90$ ). However, a high significant change of color was obtained in the MR-crosslinked films,  
287 which is correlated with the increase of the heating time and temperature and differed toward  
288 the used reducing sugar. Indeed, this effect was highest at  $110\text{ }^\circ\text{C}$  and after 24 h of treatment  
289 and when glucose was added as reducing sugar ( $18.80 \pm 0.22$ ), followed by COS1 ( $11.30 \pm$   
290  $0.34$ ) and COS2 ( $8.34 \pm 0.36$ ) comparing to chitosan film ( $4.20 \pm 0.34$ ).

291 Besides, the BI, which give an overall evaluation of the progress of the MR according to  
292 the development of colored compounds, was measured and results are illustrated in **Table 1**. In  
293 all the heated films, the BI values were higher as temperature and incubation time were higher.  
294 Further, low BI values were obtained in the treated free-reducing sugar chitosan-based films  
295 ( $2.30 \pm 0.39$  for F1-110-24 h). However, similarly to the trend of  $\Delta E$ , the BI of heated films  
296 conjugated with Glu ( $120.85 \pm 5.27$  for F2-110-24 h) was higher than that of chitosan/COS1  
297 ( $33.79 \pm 0.91$  for F3-110-24 h) and chitosan/COS2 films ( $18.01 \pm 1.87$  for F4-110-24 h).

298 All of these results indicates that MR was more pronounced for chitosan/Glu films  
299 followed by chitosan/COS1 and chitosan/COS2 films, comparing with chitosan film, as  
300 evidenced by the intensity of the change of color to dark-yellowish. This fact could indicate  
301 that severer reactions occurred in the chitosan films conjugated with Glu than in the films  
302 containing COS, allowing to the generation of higher amount of melanoidins. Similarly, Xu et  
303 al. (2019) illustrated that milder color change was obtained for soybean protein isolate (SPI)  
304 films conjugated with COS as compared to Glu, implying that COS was less susceptible to the  
305 MR with SPI than Glu. Moreover, it appeared that color change was higher in the films  
306 containing COS1 than those containing COS2, this fact can be attributed to the difference in

307 depolymerisation degree (DP) allowed by the different method of chitosan depolymerization  
308 despite the higher AD of COS1 (18.20%) than COS2 (10.63%).

309 Furthermore, results demonstrate that the development of MR was more significant when  
310 temperature and heating time increased. In this context, Leceta et al. (2013a) reported that color  
311 parameters changed notably for chitosan-based films after being heat-treated at 105 °C for 24  
312 h, which could mean that the film structure changed. Kchaou et al. (2019) reported that the  
313 color change of heated gelatin-glucose films increased as a function of heating temperature  
314 indicating better extent of MR of these films at higher temperature. Further, Etxabide et al.  
315 (2015) and Kchaou et al. (2018) proved that color change of gelatin-lactose and gelatin-glucose  
316 films, respectively, was more pronounced as heating time increased, turning toward dark yellow  
317 at longer times, due to the production of higher amount of brown melanoidins (Affes et al.,  
318 2020c). Darker films, generated after MR development, have stronger barrier ability in the  
319 visible region and are advantageous to prevent oxidative deterioration by coating sensitive to  
320 light foods (Yang et al., 2015).

### 321 **3.1.2. Ultraviolet-visible (UV-vis) spectroscopy**

322 UV-vis spectroscopy was carried out in the range 200-800 nm in order to analyze the  
323 effect of crosslinking as a function of heating time and temperature and using different reducing  
324 sugars. Spectra, presented in **Fig. 1A, 1B, 1C, 1D**, showed that the control unheated films had  
325 poor barrier properties to light in the UV region, being the preventive effect slightly better for  
326 films conjugated with reducing sugars, particularly with glucose.

327 Further, as can be seen, the absorbance of all the heated films highly increased as function  
328 of temperature and heating time. Interestingly, in all cases, the absorbance was higher when the  
329 films were heated at 110 °C than 90 °C and after 24 h of reaction time than 6 h. Indeed, the  
330 conditions 110 °C - 24 h were more efficient than 110 °C - 6 h followed by 90 °C - 24 h and 90  
331 °C - 6 h to develop high UV absorbance compounds. Furthermore, when chitosan films without



332 reducing sugar were heated a slight increase was obtained, mainly after heating at 110 °C, as  
333 compared to F1. This modification was attributed to the caramelisation reaction due to heating  
334 of chitosan (Li et al., 2014). Moreover, there was a sharp increase in absorbance (A) in the  
335 range of 200 – 500 nm for the heated films F2, F3 and F4, as compared to the control unheated  
336 films. This increase was clearly higher in the films F2 conjugated with Glu followed by the  
337 films containing COS1 and the films conjugated with COS2. These results are in line with the  
338 color change.

339         The concomitant rise of absorption of the heated films containing reducing sugars indicate  
340 the formation of MRP generated by the development of MR, which is a condensation reaction  
341 between nitrogen-containing compounds of chitosan and carbonyl group of reducing sugars (Li  
342 et al., 2014). The interactions between functional amino and carbonyl groups, present in  
343 chitosan and glucose or COS, respectively, are associated to the development of intermediate  
344 colorless compounds (Amadori) absorbing at 294 nm, prior to the generation of brown pigments  
345 (melanoidins), absorbing at 420 nm, as final MRP (Kchaou et al., 2018; Gullón et al., 2016).  
346 Results showed that in all cases, the values of absorbance at 294 nm ( $A_{294\text{ nm}}$ ) were significantly  
347 higher than those obtained at 420 nm ( $A_{420\text{ nm}}$ ). Similarly, Kosaraju, Weerakkody, & Augustin,  
348 (2010) proved that the thermal treatment of chitosan with the addition of glucose found higher  
349 rates of intermediate browning products than final browning products. In addition, they were  
350 greater when temperature and heating time were higher. Moreover, the absorbance at 280 and  
351 420 nm of the heated films conjugated with glucose were higher than those of the heated films  
352 containing COS1 and COS2 comparing to heated chitosan films. This means much more  
353 intermediate compounds of the MR and darker color. These results indicated that temperature,  
354 incubation time and the type of reducing sugar affected crosslinking by MR extension. In the  
355 same context, Gullón et al. (2016) reported that reaction conditions, especially temperature and  
356 reaction time, affect the formation of chitosan/glucose derivatives by MR.

### 357 **3.1.3. Fourier-transform infrared spectroscopy (FTIR)**

358 Chemical changes of chitosan film structure, due to reducing sugars addition and MR  
359 development at different heating times and temperatures, were studied using FTIR  
360 spectroscopy. FTIR spectra presented in Fig. 2A, 2B, 2C, 2D showed that similar characteristic  
361 peaks were obtained for all the films. All films spectra revealed the presence of the stretching  
362 vibrations of hydroxyl group (O-H), CH<sub>2</sub> and CH<sub>3</sub> groups, amide I (C=O in the NH-COCH<sub>3</sub>  
363 group), amide II (N-H) and C-H bending vibration of CH<sub>2</sub> groups obtained at 3370, 2875, 1630,  
364 1550, and 1450 cm<sup>-1</sup>, respectively. These three latter absorption bands showed a shift to a lower  
365 wavenumber as compared to chitosan powder, which were 1650, 1562 and 1420 cm<sup>-1</sup>,  
366 respectively as described in our previous study (Affes et al., 2019). This shift was due to the  
367 relaxation of the chains additionally to the effect of glycerol addition and its interaction with  
368 chitosan (Affes et al., 2020a ; Fernández-de Castro et al., 2016). Furthermore, for the control  
369 films, the intensity of the N-H band obtained at 1550 cm<sup>-1</sup> was higher than the intensity of the  
370 band of the carbonyl groups C=O at 1630 cm<sup>-1</sup>. The higher intensity of the amine groups is a  
371 result of the presence of activated amine groups (-NH<sub>3</sub><sup>+</sup>) generated during the films evaporation  
372 (Fernández-de Castro et al., 2016).

373 Furthermore, small shifts of the amide I and amide II peaks from 1630 and 1550 cm<sup>-1</sup> to  
374 around 1615 and 1536 cm<sup>-1</sup>, respectively, were observed for all MR-crosslinked films. Indeed,  
375 the shift of the absorption peak of the Amide I to 1615 cm<sup>-1</sup> suggests the formation of Schiff  
376 base (C=N) between the reducing sugar termination and the chitosan amino groups (Affes et  
377 al., 2020c ; Kchaou et al., 2018). The shift of the characteristic peak of the Amide II of MR-  
378 crosslinked films was ascribed to the chitosan conformational transformations occurring during  
379 the crosslinking reaction between chitosan and reducing sugars.

380 Nevertheless, after thermal treatment of all the films, the intensity of the different bands  
381 of the spectra decreased as compared to unheated films. This decrease was more pronounced in

382 the films containing glucose, especially F2-110-24 h, and when the films were heated during  
383 higher time (24 h) and at higher temperature (110 °C). These results were in accordance with  
384 WS values variation between control and heated films. As well, the difference in the intensity  
385 between the 1630 and 1550 cm<sup>-1</sup> absorption bands became smaller. Similarly, Gullón et al.  
386 (2016) stated that the intensity of chitosan polymer powder decreased after thermal treatment  
387 at 60 °C during 32 h.

388 All these results proved the crosslinking of the films due to MR development between  
389 carbonyl and amine groups thanks to heating treatment. This reaction was more noticeable when  
390 reducing sugars were added, especially glucose. These results corroborate with the study of  
391 Fernández-de Castro et al. (2016) for heated chitosan and chitosan/COS films. Leceta et al.  
392 (2013b) demonstrated also the change in the chemical structure of chitosan films after heat  
393 treatment by FTIR spectra variation analysis.

### 394 **3.2. Water resistance features of chitosan based films modified by MR**

395 Water sensitivity of the films, which is an important factor in the shelf life of packaging  
396 materials, was studied by measurement of moisture content (WC), water solubility (WS) and  
397 water contact angle (WCA).

398 Results illustrated in **Table 2** showed that WC values of the control unheated films  
399 containing glucose (14.07%), COS1 (13.98%) and COS2 (14.15%) were significantly higher  
400 than WC value of chitosan film (12.28%). This is probably due to the humectant character of  
401 glucose and to the WC of COS1 and COS2. Additionally, the heat treatment of the different  
402 films proved a high decrease of WC values as compared to the control films. This decrease was  
403 higher as the temperature and the heating time were higher ( $p < 0.05$ ). Indeed, heating  
404 conditions of 110 °C for 24 h showed the lowest WC values followed by 110 °C-6 h, 90 °C-24  
405 h and 90 °C-6 h, for all the films. Furthermore, the highest WC decrease was observed in the  
406 heated films containing glucose, especially F2-110-24 h (from 14.07 to 9.60%). The decrease

407 of WC by thermal treatment is due to the interaction between the amino group of chitosan and  
408 the carbonyl group of the different reducing sugars (glucose, COS1 or COS2) through MR.  
409 Similarly, Kchaou et al. (2018) reported that WC values of gelatin films increased with the  
410 addition of glucose and decreased gradually after heating at 90 °C with heating time and glucose  
411 content. Rivero, García, & Pinotti (2012) stated that heating of chitosan film at 100, 160 and  
412 180 °C decreased its moisture content value which is lower at higher temperature.

413 In addition to its importance to assess the extension of MR, it is crucial to control film  
414 solubility to be applied as food coatings, allowing several potential benefits. To this end, films  
415 WS was evaluated. As shown in **Table 2**, the addition of glucose, COS1 and COS2 to control  
416 unheated chitosan film lead to a significant increase of its WS from 12.12% to 16.19, 18.56 and  
417 19.00%, respectively. The higher WS of chitosan/COS films is due to the total water solubility  
418 of the added COS as described in our previous studies (Affes et al., 2019; Affes et al., 2020b).

419 Contrarily, when the films were heat-treated, WS values decreased significantly, as  
420 compared to unheated films, and proportionally to the increase of temperature and heating time  
421 indicating a change in the chemical structure of the films by thermal treatment. The high  
422 decrease of WS values in the films containing reducing sugars, especially in the films  
423 containing glucose, is due to the development of interactions between amino and carbonyl  
424 groups of chitosan and reducing sugars, respectively, induced by temperature, allowing to the  
425 crosslink of the films through MR. The decrease of free amino groups in film matrix lead to  
426 obtain more compact structure less sensitive to water with WS values (Umemura & Kawai,  
427 2007). Likewise, Fernández-de Castro et al. (2016) proved that chitosan/oligosaccharide film  
428 showed higher WS value than chitosan film and that thermal treatment at 105 °C decreased  
429 significantly WS by the development of brown compounds through MR. In addition, Umemura  
430 & Kawai (2007) stated that the solubility in acetic acid of chitosan films conjugated with  
431 glucose and cellobiose decreased after thermal treatment at 50 °C for 15 h. In this context,

432 Etxabide et al. (2015) reported that the WS of gelatin films decreased with the addition of  
433 lactose and that this reduction of WS is more pronounced at higher concentrations of lactose  
434 and after treatment at 105 °C for longer time.

435 Water contact angle (WCA) values are considered as a good indicators of the degree of  
436 hydrophilicity of the film surface, being higher when hydrophilicity is lower. The final state of  
437 a water drop informs about the surface wettability (Leceta et al., 2013a). The surface properties  
438 of the films were evaluated by WCA measurements. Results depicted in **Table 2** showed the  
439 variation of WCA of chitosan films as a function of temperature, heating time and type of  
440 reducing sugars. WCA values slightly decreased after 20 s ( $T_{20s}$ ) of depositing the drop as  
441 compared to 10 s ( $T_{10s}$ ), due to water evaporation. Additionally, control chitosan film (F1)  
442 showed the higher WCA values. However, the addition of glucose (85.09 °), COS1 (65.50 °)  
443 and COS2 (59.20 °) to chitosan films decreased significantly the WCA as compared to F1. The  
444 hydrophilicity of these latter films was probably attributed to their higher WC that make them  
445 more susceptible to absorb water molecules. After thermal treatment, WCA regress  
446 significantly for all the films as compared to the unheated ones indicating that heating leads to  
447 an increase of films hydrophilicity as a result of Maillard crosslinking reactions. This reduction  
448 was more important when temperature and heating time were higher. Further, control and  
449 heated chitosan-based films without reducing sugar addition were considered as hydrophobic  
450 films as they exhibit WCA values higher than 90 °. Similarly, Kchaou et al. (2018) stated that  
451 WCA of gelatin-glucose films decreased after thermal treatment at 90 °C as a function of  
452 heating time. Furthermore, Leceta et al. (2013b) proved that the heat-treatment of chitosan films  
453 containing 15% glycerol for 24 h at 105 °C caused a slight decrease in WCA values as compared  
454 to un-heated films.

### 455 **3.3. Thickness and mechanical properties changes of MR treated chitosan-based films**

456 Results from **Table 2** show that overall films possess similar thickness of approximately  
457 31.0  $\mu\text{m}$  ( $p > 0.05$ ). The mechanical properties, including tensile strength (TS) and elongation  
458 at break (EAB), of the different films were measured and displayed in **Table 2**, except for the  
459 heated films containing glucose because they were too brittle to be tested.

460 Based on the tensile stress vs. strain curves, TS and EAB values of chitosan-based film  
461 **showed slight non-significant decrease** when glucose was added to the control film F2. This  
462 decrease was probably attributed to the high concentration of glucose (5 mg/ml) incorporated  
463 in the film. **Heating of this films generate slightly more tearable and brittle films, with not**  
464 **significant differences.** Similarly, Hamdi et al. (2020) demonstrated that no significant effect  
465 on mechanical properties of control chitosan film was obtained with the addition of glucose, at  
466 a concentration of 0.4 mg/ml, and that heating of chitosan/glucose film increase its mechanical  
467 behavior.

468 In addition, **a non-significant** decrease of TS and EAB was obtained with the addition of  
469 COS1 and COS2 for the control films F3 and F4. Further, mechanical parameters values of the  
470 film F4 were slightly higher than F3. This variation can be explained by the higher AD of COS1  
471 (18.20%) than COS2 (10.63%). Similarly, Hamdi et al. (2019) reported that AD influences  
472 chitosan film-forming mechanical properties and that lower AD chitosan provides better film  
473 mechanical behavior.

474 Moreover, heat treatment of chitosan, chitosan/COS1 and chitosan/COS2 films **slightly**  
475 improve their mechanical properties. The film F3-110-24 h showed **the highest increase** of EAB  
476 as compared to F3 and the film **F1-110-24 h** showed the highest TS value ( $p < 0.05$ ). Further,  
477 this increase of mechanical properties values was correlated with the thermal treatment  
478 conditions in the following order of reactivity: 110 °C-24 h > 110 °C-6 h > 90 °C-24 h > 90 °C-  
479 6h. Thus suggesting that higher temperature and heating time were more efficient to obtain  
480 more flexible and stretchable chitosan and chitosan/COS films.

481 The slight enhancement of mechanical properties of chitosan films by heating treatment,  
482 especially for F1-110-24 h, is in accordance with results obtained by Leceta et al. (2013b) who  
483 demonstrated that heating of chitosan film at 105 °C for 24 h increased its TS and EAB values.  
484 Moreover, these results were in agreement on with those of Hamdi et al. (2020) who stated that  
485 heat treatment at 90 °C for 24 h did not affect significantly the mechanical parameters of  
486 chitosan blank film. The better flexibility and stretchability of heated films as compared to  
487 control films was attributed to the formation of more compact network through MR crosslinking  
488 (Park et al., 1999).

#### 489 **3.4. Thermal properties of MR crosslinked chitosan-based films**

490 The thermal stability was assessed by means of thermogravimetric analysis (TGA), in a  
491 range of temperature between 30 and 600 °C, in order to study the changes occurred by the  
492 effect of different heating conditions on chitosan-based films with and without reducing sugars  
493 addition. The thermal decomposition data in terms of corresponding weight loss ( $\Delta w$ ),  
494 temperature of maximum degradation ( $T_{max}$ ) and final residual mass (R) of the films, measured  
495 from the curve of the derivate weight loss as fonction of temperature as shown in Fig. 1E, 1F,  
496 1G, 1H, are detailed in Table S1.

497 On the TGA derivate weight curves of the control chitosan-based films F1, F2, F3 and F4  
498 three transformation steps corresponding to the main stages of weight loss were distinguished.  
499 The first stage of transformation ( $\Delta w_1$ ) observed below 100 °C is related to the loss of free and  
500 bound water.  $\Delta w_1$  values increased for the chitosan-based films conjugated with reducing  
501 sugars F2, F3 and F4 as compared to F1, which is in line with the increase of WC values of  
502 these films. Whereas,  $\Delta w_1$  values of overall heated films decreased comparing to control  
503 unheated films. This decrease, that was further correlated with the decrease of WC of heated  
504 films, was higher as temperature and heating time were higher.

505 A second narrow weight loss ( $\Delta w_2$ ) appeared at approximately 140 - 230 °C and was  
506 probably attributed to the entrapped water through hydrogen bonds and the elimination reaction  
507 of NH<sub>3</sub> as suggested by Martins, Cerqueira, & Vicente (2012) or due to the evaporation of  
508 glycerol as considered by Leceta et al. (2013b).  $\Delta w_2$  values decreased for all the heated films  
509 and disappeared for chitosan films containing glucose heated at 110 °C (F2-110-6 h and F2-  
510 110-24 h).

511 The third major stage of weight loss ( $\Delta w_3$ ), corresponding to  $\Delta w$  around 44.94 and  
512 57.14%, displayed the degradation or the decomposition of chitosan and COS chains and were  
513 higher for heated films comparing to control films (Martins et al., 2012).

514 Regarding  $T_{max}$  values, no change was noted for the film F2 incorporated with glucose  
515 (300 °C) as compared to F1 (297 °C). However, the addition of COS to control chitosan-based  
516 films decreased their  $T_{max}$  values, that were 277.3 and 267 °C for F3 and F4, respectively. After  
517 thermal treatment,  $T_{max}$  values of all heated films increased as compared to unheated films. This  
518 increase was more pronounced when the films were heated at 110 °C comparing to 90 °C and  
519 during 24 h as compared to 6 h. When comparing to the chitosan heated films, glucose  
520 incorporation increased  $T_{max}$  values of heated films, however,  $T_{max}$  values of chitosan films  
521 conjugated with COS remains lower despite having increased. Similarly, the residual weight at  
522 600 °C (R) was higher for all the heated films as compared to unheated films.

523 These variations of thermal stability behavior were attributed to the different extent of  
524 MR crosslinking, through creation of new bonds in film matrix, between the different films  
525 formulations and the different heating conditions. The increase of thermal stability of heated  
526 chitosan films containing reducing sugars is explained by the development MRP which could  
527 interact with chitosan chains to stabilize the film network leading to more thermally stable films  
528 with enhanced functional properties (Hamdi et al., 2020; Kchaou et al., 2019; Leceta et al.,



2013b). An increase of thermal stability was further stated for heated free-reducing sugars chitosan films by thermal treatment, but in a less extend than for MR.

### 3.5. Effect of MR crosslinking on the structural properties of chitosan-based films

#### 3.5.1. X-ray diffraction (XRD) analysis

XRD analysis is a proven tool to study the changes of the molecular conformation of chitosan-based films acquired by reducing sugar addition and thermal treatment. The XRD patterns of the different films formulations before and after treatment at 90 and 110 °C for 24 h, were illustrated in Fig. 2E, 2F, 2G, 2H.

As shrimp chitosan is a semi-crystalline polysaccharide due to its regular chain it present two peaks at  $2\theta = 10$  and  $20^\circ$  associated with its hydrated conformation (Affes et al., 2019). Similarly, XRD chromatogram of chitosan film showed that it was in a partially crystalline state and has two main characteristic peaks with a slight shift, at  $2\theta$  around 12 and  $20^\circ$ . Comparably XRD chromatograms were obtained by Chang et al. (2019), Leceta et al. (2013) and Rivero et al. (2012) for chitosan-based films. When glucose was added to chitosan film (F2), the same peaks were observed but with slightly lower intensity as compared to F1. Whereas, it is clearly shown that the addition of COS reduced the crystallization of chitosan films F3 and F4, where the peak at  $12^\circ$  disappeared and the intensity of the peak at  $20^\circ$  decreased comparing to F1. This change in the crystallinity open the incorporation of COS1 and COS2 was ascribed to the non-crystalline structure of these two chitosan derivatives (Affes et al., 2019; Affes et al., 2020b).

After thermal treatment of the different films, the crystallinity decreases remarkably as a function of heating temperature as evidenced in the decrease in the intensity of the peak at  $20^\circ$ . Indeed, films heating at 110 °C provides less crystalline structure than 90 °C. Further, the intensity of this peak ( $20^\circ$ ) was lower in the films containing COS followed by glucose containing films, as compared to F1. As well, the peak at  $12^\circ$  disappeared in all the heated

554 films. Similarly, diffractograms of chitosan films cured at different temperatures showed the  
555 disappearance of the peak located at  $2\theta = 15^\circ$  and demonstrated that films heating at higher  
556 temperature,  $160^\circ\text{C}$ , leads to lower crystallinity than  $100^\circ\text{C}$  (Rivero et al., 2012). Furthermore,  
557 Leceta et al. (2013b) stated that chitosan film structure was influenced by the effect of  
558 temperature. These results were in contradiction with those of Fernández-de Castro et al. (2016)  
559 who stated that chitosan and chitosan/COS (1:1) films heat treated and non-treated showed the  
560 same XRD patterns with amorphous behaviour and a single wide diffraction peak at  $20.2^\circ$ .

### 561 **3.5.2. Films microstructure**

562 In order to investigate the microstructural modifications in the elaborated chitosan-based  
563 films through MR, the final films microstructure was qualitatively visualized by surface  
564 electron microscopy (SEM) analysis. Crosslinked films through MR at  $110^\circ\text{C}$  for 24 h were  
565 selected for the SEM analysis, and compared with control unheated films, except for heated  
566 film containing glucose where the film F1-90-6 h was tested because F1-110-24 h was too brittle  
567 and it is difficult to be fixed on SEM support.

568 Characteristic SEM micrographs of surface and cross-sections of films were shown in  
569 **Fig. 3**. The surface of all the films was flat, smooth and homogenous without apparent porosity.  
570 However, the films F2 and F2-90-6 h showed the appearance of some cracks more pronounced  
571 in the heated film F2-90-6 h, due to the addition of glucose (5 mg/ml).

572 Moreover, cross-sectional images of the control films showed compact and homogenous  
573 structure without any evidence of irregularities. Further, crosslinking provides and increases of  
574 films cross-sections homogeneity and order, except of the film F2-90-6 h were some cracks  
575 were visualized. Results were in accordance with mechanical properties values that increased  
576 for heated chitosan and chitosan/COS films and decreased for heated chitosan/glucose films.

577 The more compact structure of the heated chitosan and COS films, as compared to control  
578 films, was attributed to the crosslinking through MR thanks to the action of temperature leading

579 to high interaction between chitosan and COS. Likewise, Fernández-de Castro et al. (2016)  
580 reported that chitosan and chitosan/oligosaccharides based films had homogenous  
581 microstructure with relatively roughness and that no irregularities, caused by crosslinking  
582 through heat treatment at 105 °C overnight, were observed in the heat treated films.

583 However, the less compact structure of heated chitosan/glucose films despite the high  
584 extent of MR in this films, was probably attributed to the very high concentration of glucose.  
585 Hamdi et al (2020) reported that the incorporation of glucose at concentrations from 0.5 to 2%  
586 (w/w chitosan) to chitosan film improves its microstructure.

### 587 **3.6. Antioxidant property of activated films by Maillard reaction**

588 To evaluate the antioxidant activity of blank and MR-crosslinked chitosan-based films  
589 and to correlate antioxidant potential to films structural characteristics, different *in vitro*  
590 antioxidant tests were assayed including DPPH and ABTS<sup>+</sup> radicals scavenging activities,  
591 reducing power test and the total antioxidant activity.

592 As shown in **Fig. 4**, for the four tested mechanisms, chitosan-based film F1 exhibited the  
593 lowest antioxidant behavior. The addition of glucose increased slightly the antioxidant activities  
594 values of the film F2 ( $p < 0.05$ ). Further, when COS were incorporated in the control films, a  
595 significant increase of the antioxidant potential, via the different tests, was noted which is more  
596 pronounced for the film F3 than F4. The higher antioxidant activity of the film F3 than F4 was  
597 attributed to the better antioxidant activity of COS1 (Mw, < 4.4 kDa; AD,  $18.20 \pm 1.19\%$ ) than  
598 COS2 (Mw, < 4.4 kDa;  $10.63 \pm 0.17\%$ ) as shown in our previous studies (Affes et al., 2019;  
599 Affes et al., 2020b). Thus can be a result of the difference in the degree of polymerization, ion  
600 composition, and the presence of high units of D-glucosamine in the structure of COS1 despite  
601 its high AD than COS2 (Anraku et al., 2018; Li, Liu, Xing, Qin, & Li, 2013). Therefore, for the  
602 control films, F3 showed the better antioxidant behavior, followed by F4 and F2 as compared  
603 to F1.

604           Regarding thermally treated films, values were significantly higher in heated films than  
605 in the blank films, even by heating of the film F1, due to the development of active MRP. This  
606 increase more noticeable after heating at 110 °C for 24 h followed by 110 °C- 6h, 90 °C-24 h  
607 and 90 °C-6 h allowing to conclude that heating at higher temperature (110 °C) and incubation  
608 times (24 h) generates better antioxidant films. Further, the heated film F1 showed the lowest  
609 increase of antioxidant values which is negligible as compared to heated reducing sugar  
610 containing films. However, heated glucose containing films, especially F2-110-24 h showed  
611 the better increase of antioxidant potential comparing to the blank film F2. In addition, the  
612 heated films containing COS1 (F3 group) exhibited the better antioxidant activities, for all the  
613 tests, followed by F2 and F4. Where, the film F3-110-24 h reached 100% for ABTS and DPPH  
614 radical scavenging activities,  $2.44 \pm 0.07$  for reducing power test and  $179,325 \pm 1.65$   $\alpha$ -  
615 tocopherol ( $\mu\text{mol/ml}$ ) for the total antioxidant activity.

616           The antioxidant activity was enhanced subsequently to the development of MR. This  
617 increase was directly correlated to the incorporated reducing sugars in the film matrix, the  
618 temperature and the heating time. Different mechanisms could explain the antioxidant activity  
619 of MRP. The high scavenging activity of crosslinked films could be a result of the reduction of  
620 reactive amino groups, through MR development, generating intermediate and final brown  
621 melanoidins that were able to donate hydrogen atoms (Akar, Küçük, & Doğan Akar, 2017; Li  
622 et al., 2014; Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007). Moreover, Chen et al.  
623 (2019) and Li et al. (2014) reported that primary MRP and finally brown heterocyclic  
624 compounds were responsible to the improved reducing power of the MR crosslinked films.  
625 Furthermore, Kchaou et al. (2018) suggested that MRP could react with Mo (VI) to convert it  
626 to more stable molecules, Mo (V) by donating electrons, in total antioxidant activity test.

#### 627 **4. Conclusion**

628 Different reducing sugars types (glucose and COS), temperature and heating times were  
629 studied to induce Maillard reaction (MR) crosslinking of chitosan-based films. The rate of MR  
630 was better at higher temperature and exposure times and for glucose containing films, followed  
631 by films conjugated by COS1 and COS2, as shown by browning index values, **Ultraviolet** and  
632 **Fourier-transform infrared** spectra, water resistance properties and thermal stability  
633 degradation. However, crosslinked films mechanical properties were enhanced for  
634 chitosan/COS films and reduced for chitosan/glucose films, which is correlated with SEM  
635 results. Moreover, MR development enhanced significantly the antioxidant potential, via four  
636 different mechanisms, of all the crosslinked films as compared to blank films thanks to the  
637 formation of highly active MR products. Among all treated films, the most actives were  
638 chitosan/COS1 crosslinked films, followed by chitosan/glucose and chitosan/COS2 films.  
639 These films possess interesting functional and antioxidant properties that were suitable for food  
640 packaging and biomedical applications.

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**Conflict of interest**

The authors declare that there are no conflicts of interest.

**Credit authorship contribution statement**

**Sawsan Affes:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing.

**Hana Maalej:** Supervision, Conceptualization, Resources, Writing - Review & Editing.

**Suming Li:** Project administration, Investigation.

**Rania Abdelhedi:** Statistical analysis.

**Rim Nasri:** Project administration, Investigation.

**Moncef Nasri:** Supervision, Resources, Visualisation, Writing - Review & Editing.

### **Figures captions**

**Figure 1:** UV-vis spectra and DTGA profiles chitosan-based films, before and after (0, 6 and 24 h) thermal treatment through Maillard reaction at 90 °C and 110 °C, without (**A, E**) and with reducing sugars addition: chitosan-glucose (**B, F**), chitosan-COS1 (**C, G**) and chitosan-COS2 (**D, H**) based films, respectively.

**Figure 2:** FTIR spectra of chitosan (**A**) chitosan-glucose (**B**) chitosan-COS1 (**C**) and chitosan-COS2 (**D**) films before and after heating at different heating times and temperatures. X-ray diffractograms of control and MR-treated chitosan-based films, during 24 h at 90 and 110 °C, without (**E**) and with the addition of different reducing sugars: glucose (**F**), COS1 (**G**) and COS2 (**H**).

**Figure 3:** Section and surface SEM micrographs of control films and heated films at 110 °C during 24 h, except of the film F2 which is tested after treatment at 90 °C during 6 h.

**Figure 4:** Antioxidant activities of chitosan-based films conjugated or not with different reducing sugars through MR, as a function of temperature (90 and 110 °C) and heating time (0, 6, 24 h), by means of ABTS<sup>+</sup> (**A**) and DPPH (**B**) radicals-scavenging activities (%), reducing power (OD 700 nm) (**C**) and total antioxidant activity ( $\alpha$ -tocopherol ( $\mu\text{mol/ml}$ )) (**D**). Values are means  $\pm$  standard deviation ( $n = 3$ ). Means with different letters (A-M) indicate significant difference ( $p < 0.05$ ).

Fig. 1

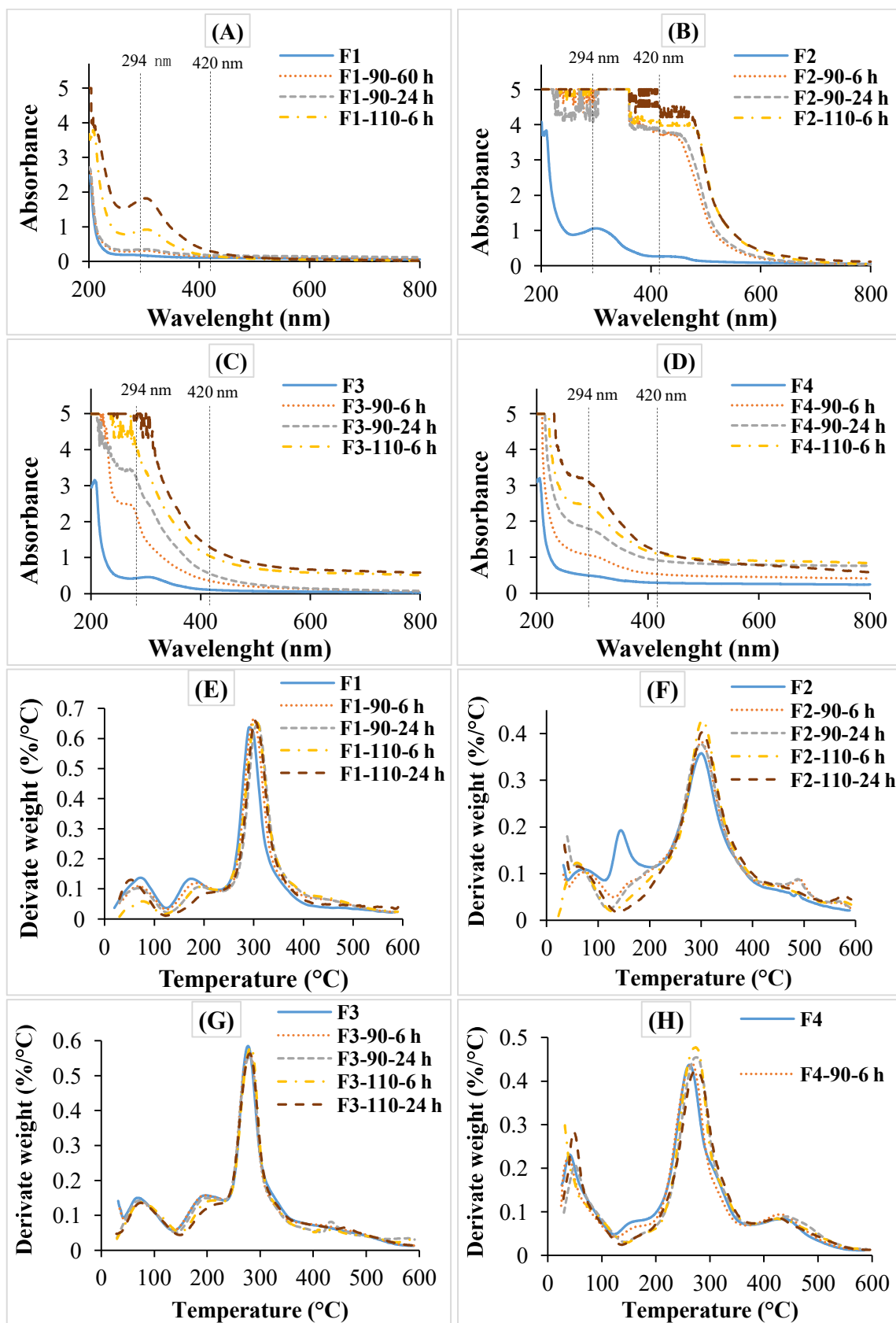
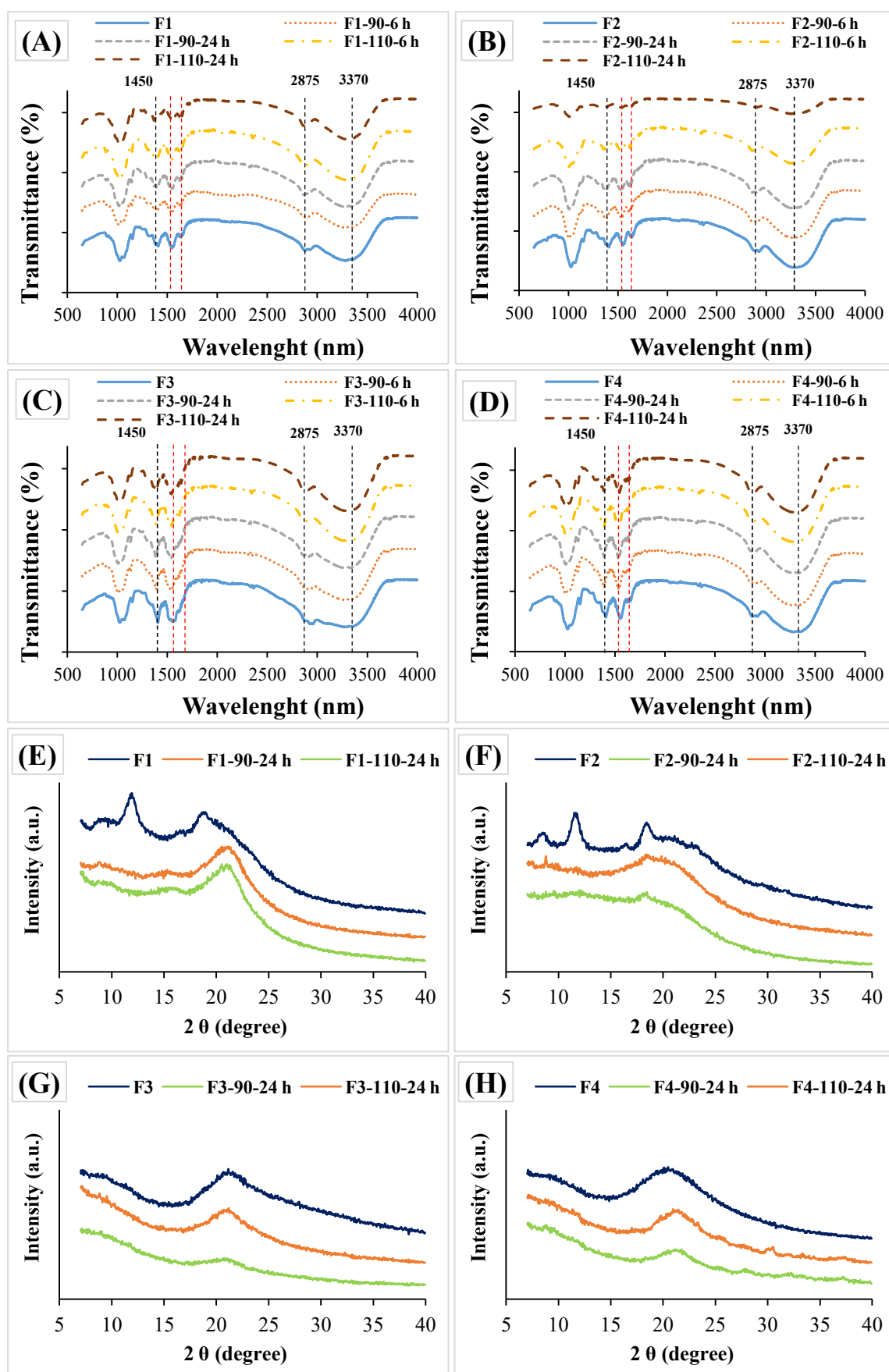


Fig. 2





**Fig. 3**

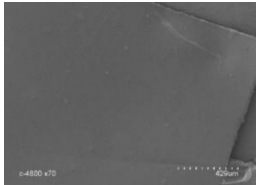
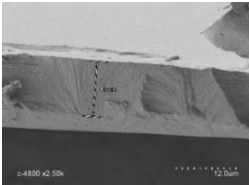
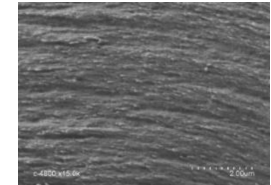
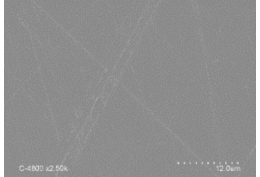
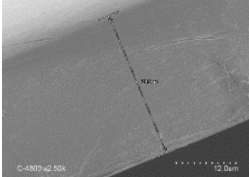
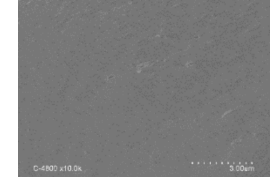
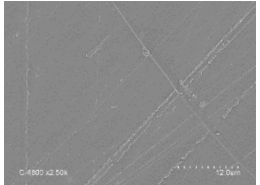
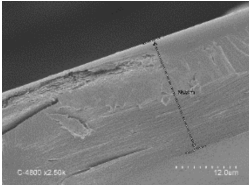
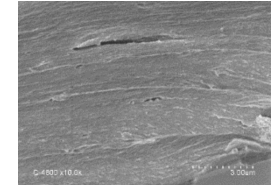
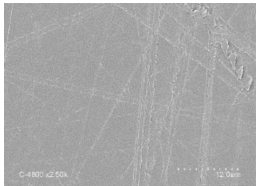
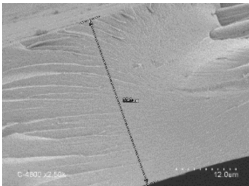
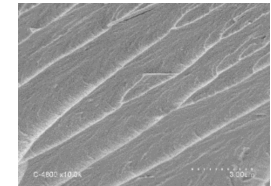
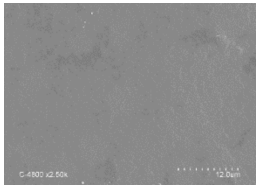
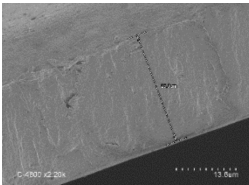
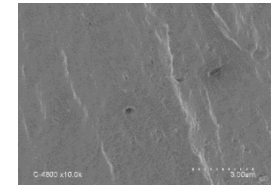
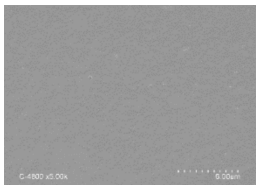
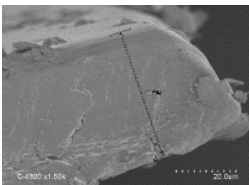
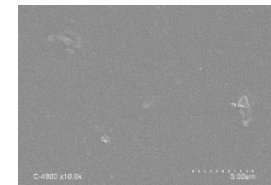
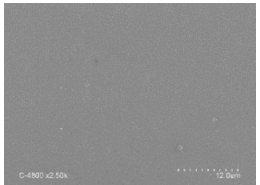
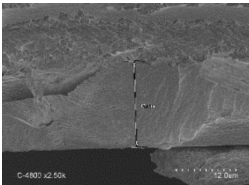
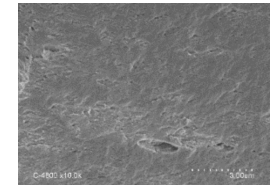
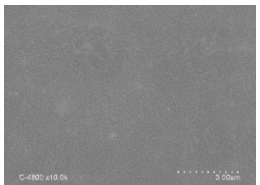
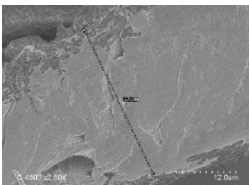
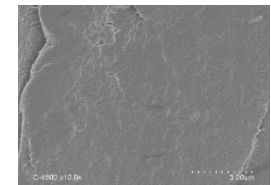
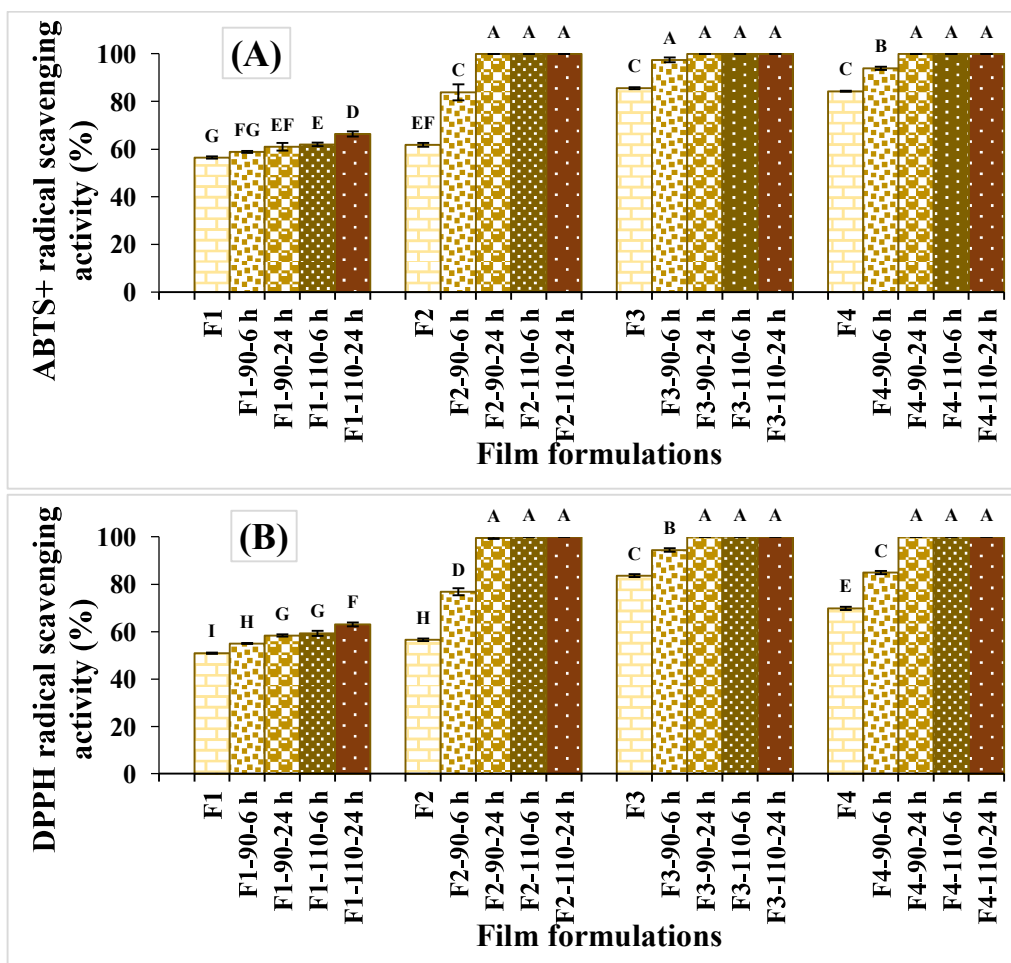
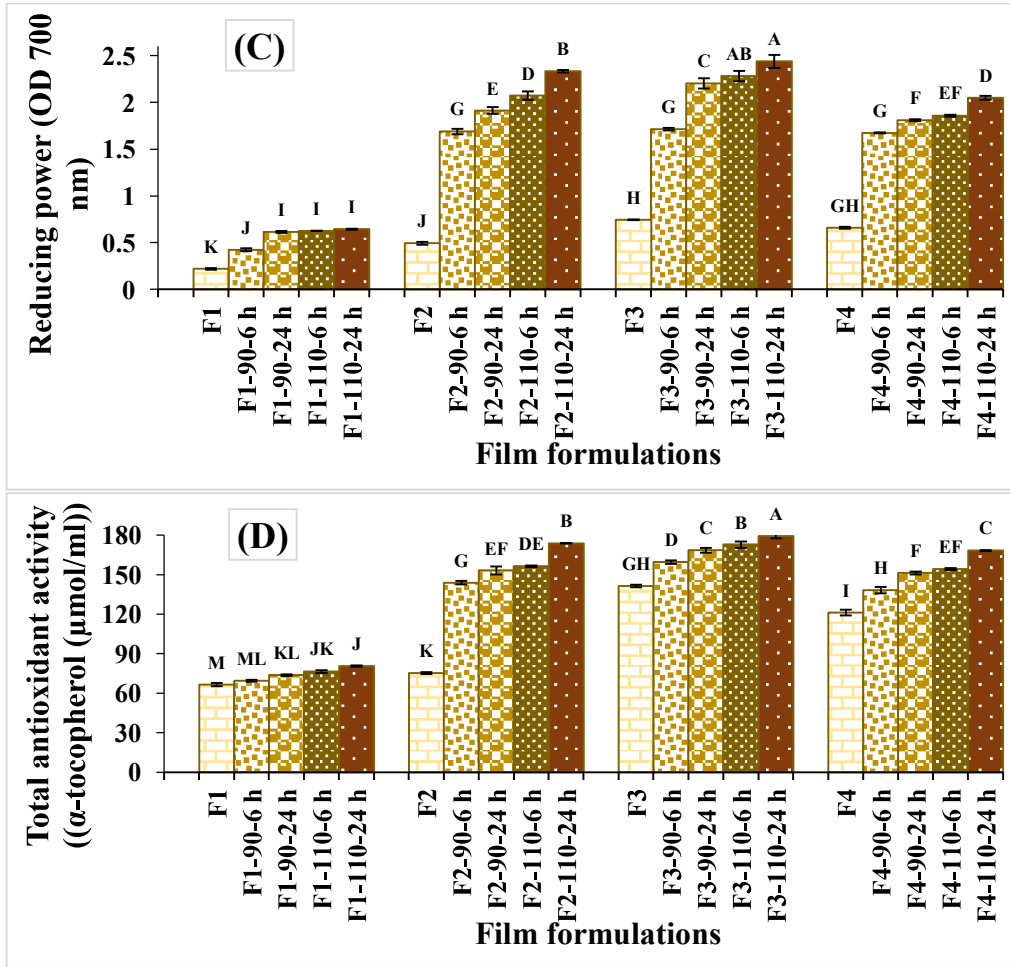
<b>Film</b>	<b>Surface</b>	<b>Section</b>	
<b>F1</b>			
			
<b>F2</b>			
			
<b>F3</b>			
			
<b>F4</b>			
			

Fig. 4





**Table 1:** Color parameters ( $L^*$ ,  $a^*$  and  $b^*$ ), total color change ( $\Delta E^*$ ) and browning index (BI) of the different films.

Films	$L^*$	$a^*$	$b^*$	$\Delta E^*$	BI
<b>F1</b>	$28.36 \pm 1.09$ <sup>AB</sup>	$-0.45 \pm 0.05$ <sup>A</sup>	$-0.46 \pm 0.07$ <sup>M</sup>	-	-
<b>F1-90-6 h</b>	$27.54 \pm 0.43$ <sup>ABCD</sup>	$-0.55 \pm 0.02$ <sup>AB</sup>	$0.52 \pm 0.01$ <sup>L</sup>	$1.32 \pm 0.26$ <sup>H</sup>	$0.40 \pm 0.03$ <sup>H</sup>
<b>F1-90-24 h</b>	$27.30 \pm 0.85$ <sup>ABCD</sup>	$-0.60 \pm 0.03$ <sup>AB</sup>	$0.64 \pm 0.04$ <sup>L</sup>	$1.68 \pm 0.51$ <sup>H</sup>	$0.70 \pm 0.11$ <sup>H</sup>
<b>F1-110-6 h</b>	$26.40 \pm 0.82$ <sup>CDE</sup>	$-0.77 \pm 0.05$ <sup>BC</sup>	$0.71 \pm 0.02$ <sup>L</sup>	$2.35 \pm 0.70$ <sup>H</sup>	$0.67 \pm 0.23$ <sup>H</sup>
<b>F1-110-24 h</b>	$24.54 \pm 0.43$ <sup>GH</sup>	$-0.88 \pm 0.02$ <sup>CD</sup>	$1.22 \pm 0.12$ <sup>K</sup>	$4.20 \pm 0.34$ <sup>G</sup>	$2.30 \pm 0.39$ <sup>H</sup>
<b>F2</b>	$25.97 \pm 1.33$ <sup>DEF</sup>	$-0.64 \pm 0.19$ <sup>B</sup>	$2.03 \pm 0.01$ <sup>J</sup>	$3.60 \pm 0.90$ <sup>G</sup>	-
<b>F2-90-6 h</b>	$16.93 \pm 0.60$ <sup>J</sup>	$-1.11 \pm 0.05$ <sup>EF</sup>	$6.20 \pm 0.14$ <sup>D</sup>	$13.25 \pm 0.58$ <sup>C</sup>	$38.65 \pm 2.65$ <sup>C</sup>
<b>F2-90-24 h</b>	$14.88 \pm 0.40$ <sup>K</sup>	$-1.29 \pm 0.08$ <sup>FGH</sup>	$9.20 \pm 0.08$ <sup>B</sup>	$16.61 \pm 0.38$ <sup>B</sup>	$81.60 \pm 4.03$ <sup>B</sup>
<b>F2-110-6 h</b>	$14.68 \pm 0.21$ <sup>K</sup>	$-1.41 \pm 0.07$ <sup>GHI</sup>	$9.36 \pm 0.14$ <sup>B</sup>	$16.87 \pm 0.25$ <sup>B</sup>	$84.94 \pm 3.53$ <sup>B</sup>
<b>F2-110-24 h</b>	$12.96 \pm 0.16$ <sup>L</sup>	$-1.68 \pm 0.08$ <sup>J</sup>	$10.26 \pm 0.14$ <sup>A</sup>	$18.80 \pm 0.22$ <sup>A</sup>	$120.85 \pm 5.27$ <sup>A</sup>
<b>F3</b>	$27.83 \pm 0.13$ <sup>ABC</sup>	$-0.95 \pm 0.02$ <sup>DE</sup>	$1.41 \pm 0.02$ <sup>K</sup>	$2.00 \pm 0.09$ <sup>H</sup>	-
<b>F3-90-6 h</b>	$24.78 \pm 0.65$ <sup>EFG</sup>	$-1.07 \pm 0.05$ <sup>E</sup>	$3.90 \pm 0.05$ <sup>G</sup>	$5.68 \pm 0.66$ <sup>F</sup>	$13.35 \pm 1.70$ <sup>EF</sup>
<b>F3-90-24 h</b>	$22.26 \pm 0.20$ <sup>H</sup>	$-1.26 \pm 0.04$ <sup>FG</sup>	$5.46 \pm 0.04$ <sup>E</sup>	$8.54 \pm 0.14$ <sup>E</sup>	$22.77 \pm 0.03$ <sup>D</sup>
<b>F3-110-6 h</b>	$22.30 \pm 0.07$ <sup>H</sup>	$-1.26 \pm 0.01$ <sup>FG</sup>	$5.48 \pm 0.01$ <sup>E</sup>	$8.53 \pm 0.08$ <sup>E</sup>	$22.85 \pm 0.90$ <sup>D</sup>
<b>F3-110-24 h</b>	$19.69 \pm 0.47$ <sup>I</sup>	$-1.54 \pm 0.03$ <sup>IJ</sup>	$6.70 \pm 0.03$ <sup>C</sup>	$11.30 \pm 0.34$ <sup>D</sup>	$33.79 \pm 0.91$ <sup>C</sup>
<b>F4</b>	$28.68 \pm 0.23$ <sup>A</sup>	$-1.35 \pm 0.01$ <sup>GHI</sup>	$1.39 \pm 0.29$ <sup>K</sup>	$2.10 \pm 0.22$ <sup>H</sup>	-
<b>F4-90-6 h</b>	$26.57 \pm 0.04$ <sup>BCD</sup>	$-1.39 \pm 0.02$ <sup>GHI</sup>	$2.58 \pm 0.02$ <sup>I</sup>	$3.65 \pm 0.03$ <sup>G</sup>	$5.99 \pm 0.15$ <sup>H</sup>
<b>F4-90-24 h</b>	$24.47 \pm 0.02$ <sup>GH</sup>	$-1.47 \pm 0.01$ <sup>HI</sup>	$3.38 \pm 0.36$ <sup>H</sup>	$5.56 \pm 0.26$ <sup>F</sup>	$9.84 \pm 1.58$ <sup>FG</sup>
<b>F4-110-6 h</b>	$24.16 \pm 0.06$ <sup>G</sup>	$-1.47 \pm 0.02$ <sup>HI</sup>	$3.42 \pm 0.13$ <sup>H</sup>	$5.81 \pm 0.13$ <sup>F</sup>	$10.18 \pm 0.56$ <sup>FG</sup>
<b>F4-110-24 h</b>	$21.89 \pm 0.22$ <sup>H</sup>	$-1.51 \pm 0.01$ <sup>IJ</sup>	$4.69 \pm 0.31$ <sup>F</sup>	$8.34 \pm 0.36$ <sup>E</sup>	$18.01 \pm 1.87$ <sup>DE</sup>

Values are means  $\pm$  standard deviation ( $n = 3$ ). Means with different letters (A-M) and within a column indicate significant difference ( $p < 0.05$ ).  $\Delta E^*$  was the change of color measured as compared to F1.

**Table 2:** Water content (WC, %), water solubility (WS, %), water contact angle (WCA, °), thickness ( $\mu\text{m}$ ) and mechanical properties (TS, % and EAB, MPa) of the different films.

Films	Water resistance properties				Mechanical properties		
	WC (%)	WS (%)	WCA (°)		Thickness ( $\mu\text{m}$ )	TS (MPa)	EAB (%)
			T <sub>10 s</sub>	T <sub>20 s</sub>			
<b>F1</b>	12.28 ± 0.16 <sup>B</sup>	12.12 ± 1.01 <sup>CDE</sup>	108.60 ± 2.24 <sup>A</sup>	106.93 ± 2.26 <sup>A</sup>	29.4 ± 1.5 <sup>A</sup>	17.99 ± 0.25 <sup>A</sup>	15.26 ± 0.74 <sup>A</sup>
<b>F1-90-6 h</b>	10.93 ± 0.23 <sup>E</sup>	10.71 ± 0.04 <sup>HI</sup>	96.54 ± 1.78 <sup>B</sup>	94.18 ± 1.73 <sup>B</sup>	28.00 ± 2.0 <sup>A</sup>	18.29 ± 0.01 <sup>A</sup>	15.35 ± 0.26 <sup>A</sup>
<b>F1-90-24 h</b>	10.33 ± 0.33 <sup>FG</sup>	9.33 ± 0.50 <sup>JK</sup>	95.70 ± 0.62 <sup>B</sup>	95.10 ± 0.94 <sup>B</sup>	30.0 ± 4.0 <sup>A</sup>	18.44 ± 0.73 <sup>A</sup>	15.50 ± 0.85 <sup>A</sup>
<b>F1-110-6 h</b>	10.13 ± 0.38 <sup>G</sup>	8.85 ± 0.27 <sup>KL</sup>	95.30 ± 1.98 <sup>B</sup>	94.11 ± 1.77 <sup>B</sup>	30.0 ± 2.0 <sup>A</sup>	18.49 ± 0.23 <sup>A</sup>	15.71 ± 0.12 <sup>A</sup>
<b>F1-110-24 h</b>	9.31 ± 0.19 <sup>HI</sup>	7.92 ± 0.07 <sup>L</sup>	94.64 ± 0.67 <sup>B</sup>	92.93 ± 1.06 <sup>B</sup>	32.0 ± 4.0 <sup>A</sup>	18.60 ± 0.30 <sup>A</sup>	15.93 ± 0.10 <sup>A</sup>
<b>F2</b>	14.07 ± 0.04 <sup>A</sup>	16.19 ± 0.32 <sup>B</sup>	85.09 ± 3.06 <sup>C</sup>	82.38 ± 3.16 <sup>C</sup>	30.0 ± 1.5 <sup>A</sup>	14.05 ± 0.66 <sup>A</sup>	12.80 ± 0.44 <sup>A</sup>
<b>F2-90-6 h</b>	11.45 ± 0.16 <sup>CD</sup>	11.83 ± 0.31 <sup>DEFG</sup>	71.13 ± 2.07 <sup>D</sup>	69.19 ± 2.33 <sup>D</sup>	30.0 ± 3.0 <sup>A</sup>	-	-
<b>F2-90-24 h</b>	11.06 ± 0.06 <sup>DE</sup>	11.01 ± 0.09 <sup>FGHI</sup>	70.49 ± 0.91 <sup>D</sup>	68.44 ± 0.78 <sup>D</sup>	31.0 ± 1.2 <sup>A</sup>	-	-
<b>F2-110-6 h</b>	10.15 ± 0.05 <sup>G</sup>	10.81 ± 0.22 <sup>GHI</sup>	69.33 ± 2.15 <sup>D</sup>	68.24 ± 2.43 <sup>D</sup>	32.0 ± 5.0 <sup>A</sup>	-	-
<b>F2-110-24 h</b>	9.60 ± 0.10 <sup>HI</sup>	9.97 ± 0.23 <sup>IJ</sup>	67.84 ± 0.74 <sup>D</sup>	66.30 ± 0.80 <sup>D</sup>	33.0 ± 2.0 <sup>A</sup>	-	-
<b>F3</b>	13.98 ± 0.08 <sup>A</sup>	18.56 ± 0.06 <sup>A</sup>	65.50 ± 2.25 <sup>D</sup>	58.31 ± 2.46 <sup>E</sup>	30.0 ± 2.0 <sup>A</sup>	15.76 ± 0.66 <sup>A</sup>	14.52 ± 0.1 <sup>A</sup>
<b>F3-90-6 h</b>	12.60 ± 0.10 <sup>B</sup>	12.69 ± 0.18 <sup>CD</sup>	44.81 ± 2.38 <sup>F</sup>	37.28 ± 1.65 <sup>G</sup>	30.0 ± 2.0 <sup>A</sup>	16.83 ± 0.35 <sup>A</sup>	14.94 ± 0.23 <sup>A</sup>
<b>F3-90-24 h</b>	11.70 ± 0.10 <sup>C</sup>	11.97 ± 0.03 <sup>CDEF</sup>	42.84 ± 1.78 <sup>FG</sup>	36.69 ± 1.91 <sup>GH</sup>	31.0 ± 3.0 <sup>A</sup>	17.19 ± 0.12 <sup>A</sup>	16.05 ± 0.05 <sup>A</sup>
<b>F3-110-6 h</b>	11.47 ± 0.04 <sup>CD</sup>	11.82 ± 0.08 <sup>DEFG</sup>	39.74 ± 2.59 <sup>FG</sup>	34.35 ± 2.20 <sup>GH</sup>	31.0 ± 2.0 <sup>A</sup>	17.25 ± 0.15 <sup>A</sup>	16.11 ± 1.40 <sup>A</sup>
<b>F3-110-24 h</b>	10.75 ± 0.01 <sup>EF</sup>	11.37 ± 0.54 <sup>EFGH</sup>	39.74 ± 2.21 <sup>FG</sup>	33.63 ± 2.96 <sup>GH</sup>	32.0 ± 4.0 <sup>A</sup>	17.95 ± 0.64 <sup>A</sup>	16.64 ± 1.09 <sup>A</sup>
<b>F4</b>	14.15 ± 0.05 <sup>A</sup>	19.00 ± 0.23 <sup>A</sup>	59.20 ± 2.04 <sup>E</sup>	50.10 ± 2.54 <sup>F</sup>	32.0 ± 3.0 <sup>A</sup>	15.95 ± 1.07 <sup>A</sup>	14.79 ± 0.17 <sup>A</sup>
<b>F4-90-6 h</b>	12.73 ± 0.03 <sup>B</sup>	13.01 ± 0.11 <sup>C</sup>	42.47 ± 2.27 <sup>FG</sup>	35.05 ± 2.75 <sup>GH</sup>	32.0 ± 1.0 <sup>A</sup>	16.09 ± 1.28 <sup>A</sup>	14.98 ± 0.07 <sup>A</sup>
<b>F4-90-24 h</b>	11.76 ± 0.04 <sup>C</sup>	12.24 ± 0.14 <sup>CDE</sup>	40.93 ± 1.43 <sup>FG</sup>	34.69 ± 1.24 <sup>GH</sup>	33.0 ± 2.0 <sup>A</sup>	16.23 ± 1.20 <sup>A</sup>	15.29 ± 0.51 <sup>A</sup>
<b>F4-110-6 h</b>	11.54 ± 0.04 <sup>C</sup>	12.15 ± 0.25 <sup>CDE</sup>	38.18 ± 0.89 <sup>G</sup>	32.45 ± 0.77 <sup>GH</sup>	34.0 ± 3.0 <sup>A</sup>	16.49 ± 0.66 <sup>A</sup>	15.83 ± 0.60 <sup>A</sup>
<b>F4-110-24 h</b>	10.87 ± 0.10 <sup>E</sup>	11.91 ± 0.09 <sup>DEF</sup>	36.82 ± 1.30 <sup>G</sup>	30.40 ± 0.30 <sup>H</sup>	34.0 ± 2.0 <sup>A</sup>	16.87 ± 0.93 <sup>A</sup>	16.24 ± 0.01 <sup>A</sup>

Values are means ± standard deviation (n = 3). Means with different letters (A-L) and within a column indicate significant difference ( $p < 0.05$ ).

**Supplementary material**

**Table S1:** Maximal degradation temperature ( $T_{\max}$ , °C), weight loss ( $\Delta w$ , %), and residual weight at 600 °C ( $R$ , %) as function of degradation temperatures, based on the TGA thermograms of chitosan based films conjugated or not with glucose and COS through Maillard reaction (MR) at 90 °C and 110 °C and as function of time (0, 6 and 24 h).

Films	$T_{\max}$ (°C)	Weight loss (%)			R (%)
		$\Delta w_1$	$\Delta w_2$	$\Delta w_3$	
<b>F1</b>	297.00	9.67	10.6	48.3	31.43
<b>F1-90-6 h</b>	298.09	7.61	10.54	50.57	31.28
<b>F1-90-24 h</b>	298.15	7.20	8.8	51.8	32.20
<b>F1-110-6 h</b>	303.33	7.33	8.06	52.21	32.40
<b>F1-110-24 h</b>	303.50	6.54	6.96	51.49	35.01
<b>F2</b>	300.00	12.18	12.41	44.94	30.47
<b>F2-90-6 h</b>	300.91	8.14	4.20	54.03	33.63
<b>F2-90-24 h</b>	301.00	7.89	4.12	54.52	33.41
<b>F2-110-6 h</b>	301.12	7.74	-	58.14	34.13
<b>F2-110-24 h</b>	301.10	7.35	-	58.03	34.63
<b>F3</b>	277.30	12.56	11.98	46.09	31.37
<b>F3-90-6 h</b>	279.72	11.76	9.91	45.2	33.13
<b>F3-90-24 h</b>	279.75	11.17	9.07	45.3	34.46
<b>F3-110-6 h</b>	282.14	10.58	8.82	46.14	34.47
<b>F3-110-24 h</b>	283.33	10.37	6.97	46.68	35.89
<b>F4</b>	267.62	13.86	5.42	47.95	31.78
<b>F4-90-6 h</b>	270.06	13.25	3.64	48.13	34.95
<b>F4-90-24 h</b>	271.09	12.64	3.13	49.00	35.30
<b>F4-110-6 h</b>	273.67	12.13	2.20	50.05	35.62
<b>F4-110-24 h</b>	274.00	12.07	2.17	50.10	35.66