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Additional Information

1 **Antioxidant edible films based on chitosan and starch containing polyphenols from**  
2 **thyme extracts**

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4 Gonzalez-Martinez, C.

5

6 **Abstract**

7

8 The aim of this study was to analyse the antioxidant activity of different polymeric matrices  
9 based on chitosan and starch, incorporating a thyme extract (TE) rich in polyphenols. TE  
10 provided the films with remarkable antioxidant activity. When mixed with chitosan, the  
11 polyphenols interacted with the polymer chains, acting as crosslinkers and enhancing the  
12 tensile behaviour of films. The opposite effect was observed when incorporated into the starch  
13 matrix. All the films became darker, more reddish and less transparent when TE was  
14 incorporated. These colour changes were more marked in starch matrices, which suggests that  
15 TE compounds were poorly encapsulated. The use of chitosan-based matrices carrying TE  
16 polyphenols is recommended as a means of obtaining antioxidant films, on the basis of their  
17 tensile response and greater antioxidant activity, which is associated with the polyphenol-  
18 chitosan interactions, contributing to a better protection of the functionality of polyphenols  
19 during film formation and conditioning.

20

21 **Keywords:** tensile properties, colour, gloss, DPPH method, microstructure.

22

23 **1. Introduction**

24

25 Oxidative reactions in foods represent a serious deteriorative process, leading to significant  
26 waste. Oxidative reactions promote the discoloration and the development of rancidity and off-  
27 flavours, negatively affecting the appearance, nutritional value and quality of foodstuffs. To  
28 overcome these problems, the food industry often uses synthetic antioxidants, with dubious  
29 health properties, to prevent these undesirable processes. So, new natural antioxidant  
30 compounds are necessary to control oxidative reactions in foods. More and more attention is  
31 being paid to natural antioxidants for two reasons; they are presumed to be safe because they  
32 are naturally occurring and they are well accepted by consumers. In many cases, they are  
33 derived from plant sources, such as herbs and spices. Polyphenols in plant extracts are of  
34 special interest due to the synergistic action of different polyphenolic compounds (Dai, Chen &

35 Zhou, 2008; Trifkovic et al., 2014). Phenolic compounds may terminate the propagation  
36 reactions, induced by free radical intermediates, by reacting either directly with the free radical  
37 or by preventing hydroperoxides from decomposing into free radicals (Zhou, Wu, Yang & Liu,  
38 2005; Mozafari et al., 2006).

39 Thyme (*Thymus serpyllum L.*) is a rich source of polyphenols, especially its leaves (Gallego,  
40 Gordon, Segovia, Skowyrza & Almajano, 2013; Stojanovic et al., 2012). The quality of this genus  
41 of the family *lamiaceae* is generally determined by its essential oil content. However, the  
42 hydrophilic extracts of the herb also contain interesting compounds, such as caffeic acid and its  
43 oligomers, flavonoid glycosides, hydroquinone derivatives, terpenoids, biphenyl compounds,  
44 among others (Fecka & Turek, 2008). Rosmarinic acid (caffeic acid oligomer) is the main  
45 phenolic compound found in the aqueous tea infusion (Mihailovic-Stanojevic et al., 2013). The  
46 potential antioxidant activity of these thyme extracts makes them suitable to be used as  
47 possible substitutes for synthetic antioxidants in the food industry. These polyphenols could be  
48 incorporated within a film or coating applied to the food, which could release the antioxidant  
49 into the product or act on its surface, limiting the oxidative reactions of food components.

50 Currently, in the field of food packaging, there is great interest in the development of  
51 biodegradable active materials, which contribute both to a reduction in the use of synthetic  
52 plastic wastes and to a longer shelf-life of the food products by means of the incorporation of  
53 active substances, such as antioxidants and antimicrobials. Of the biodegradable polymers,  
54 polysaccharides such as starch or chitosan have good film-forming capacity (Bergo, 2008). Starch  
55 (S) films are low cost, flexible, transparent and highly impermeable to oxygen, but exhibit a high  
56 degree of water sensitivity which negatively affects barrier and mechanical properties.

57 Improvement strategies are based on the mixture with other components, which allows films  
58 with better properties to be obtained. Chitosan (CH) is a cationic hydrocolloid with interesting  
59 film-forming properties (Elsabee & Abdou, 2013). It presents a great potential for a wide range  
60 of food applications due to its biocompatibility, non-toxicity and low cost of application  
61 (Sánchez-González, et al. 2011; Krochta & Mulder-Johnston, 1997). Furthermore, numerous  
62 studies have shown the antioxidant capacity (Yen, Yang & Mau, 2008) and the antimicrobial  
63 activity of chitosan (Dutta, Tripathi, Mehrotra & Dutta, 2009; Kumar, Muzzarelli R., Muzzarelli C.,  
64 Sashiwa & Domb, 2000), associated both with its positive charges, which can interfere with the  
65 negatively charged residues of macromolecules on the microbial cell surface, causing membrane  
66 leakage (Siripatrawan & Harte, 2010). Previous studies have reported that the S-CH blends  
67 exhibited an improved mechanical response compared to pure S films while providing  
68 antimicrobial activity at low cost (Bonilla, Atarés, Vargas & Chiralt, 2013). Tannic Acid (TA) is a

69 polyphenol naturally found in some green leaves, which exhibits interesting properties due to its  
70 multiple phenolic groups that can interact with macromolecules, such as chitosan (Aelenei,  
71 Popa, Novac, Lisa & Balaita, 2009). Thus, TA acts as a cross-linker, leading to a more rigid and  
72 compact chitosan matrix and improving the films' physical properties (Rivero, García & Pinotti,  
73 2010).

74 The aim of this study was to evaluate the physical and antioxidant properties of biodegradable  
75 films composed of different matrices made up of chitosan and pea starch enriched with thyme  
76 extract. Moreover, the addition of TA as a crosslinking agent to the chitosan matrices was  
77 studied.

78

## 79 **2. Materials and methods**

80

### 81 **2.1 Raw materials**

82

83 High molecular weight chitosan (Batch MKBH57816V) and tannic acid from Sigma-Aldrich,  
84 Madrid, Spain, pea starch (Batch W469V) supplied by Roquette Laisa SA Benifaió, Valencia,  
85 Spain, glacial acetic acid and glycerol from Panreac Química SLU, Castellar del Vallès,  
86 Barcelona, Spain, were used to prepare the films.

87 Methanol, Folin-ciocalteu and 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) from Sigma-Aldrich,  
88 Madrid, Spain and phosphorus pentoxide ( $P_2O_5$ ), sodium carbonate ( $Na_2CO_3$ ) and sodium  
89 chloride (NaCl) , provided by Panreac Química SLU, Castellar del Vallès, Barcelona, Spain, were  
90 used to carry out the different analyses.

91

### 92 **2.2 Thyme extract preparation**

93

94 Thyme extract (TE) was prepared by a conventional water extraction method with boiling  
95 distilled water. 10 g of dry thyme leaves, provided by Serpylli herba (Serbia) (Batch 29660913),  
96 were added to 200 ml of hot water (100°C), stirring for 30 minutes. The resulting infusion was  
97 vacuum filtrated using filter paper (Stojanovic et al., 2012) and lyophilized in a freeze drier  
98 Alpha 1–2 (Martin Christ, GmbH, Osterode am Harz, Germany) under vacuum pressure. Thyme  
99 extract powders were kept packaged under vacuum and refrigeration conditions.

100

### 101 **2.3. Experimental design and preparation of the films**

102

103 Films containing TE were produced by means of a casting method. Two different FFDs based  
 104 on pure biopolymers (CH and S) and a mixture of both polymers (CH:S) were obtained. In those  
 105 formulations based on chitosan, tannic acid was also added as a cross-linking agent (Rivero et  
 106 al., 2010). As controls, films with no added TE were prepared (CH, CH:TA, S, CH:S, CH:S:TA)  
 107 (Table 1).

108 CH (2% w/w) was dispersed in an aqueous solution of acetic acid (2% v/w) under magnetic  
 109 stirring at 40°C and 150 rpm for 24 h. Glycerol was added in a polymer:glycerol ratio of 1:0.2,  
 110 (w/w). To obtain the CH:TA film forming dispersion, TA was added to the CH dispersion in a  
 111 polymer:TA ratio of 1:0.04.

112 2% (w/w) pea starch solution in distilled water was prepared to obtain S dispersions by heating  
 113 at 95°C for 30 min to gelatinize the starch, under continuous stirring. Afterwards, glycerol was  
 114 added in the same ratio as that commented on above.

115 The CH and S dispersions prepared above were mixed in a CH:S ratio of 1:4 (w/w). In  
 116 formulations with TA, this was added in a polymer:TA ratio of 1:0.04 (w/w).

117 Dispersions containing TE were added using a polymer:TE ratio of 1: 0.15. All of the dispersions  
 118 were homogenized using rotor-stator equipment (Ultraturrax Yellow Line DL 25 Basic, IKA  
 119 Janke and Kunjel, Germany) for 4 min at 13500 rpm and degassed by means of a vacuum  
 120 pump. In order to obtain the films, these FFDs were poured into Teflon plates (15 cm  
 121 diameter) or petri dishes (8,7 cm diameter) resting on a level surface. The amount of polymer  
 122 always remained constant, the density per unit area being 56.62 g of polymer/m<sup>2</sup>.

123 After drying for 48 hours under controlled conditions (T=25°C and RH=50%) and prior to  
 124 further analysis, the films were conditioned in a desiccator at 25°C with a supersaturated NaCl  
 125 solution (aw= 0.75) till they reached constant weight.

126

127 **Table 1.** Total solids amount (g) per 100 g of the different film forming dispersions and  
 128 nomenclature used.

Formulation	CH	S	TA	TE	Glycerol
<b>CH</b>	1	-	-	-	0.2
<b>CH:TA</b>	1	-	0.04	-	0.2
<b>S</b>	-	1	-	-	0.2
<b>CH:S</b>	0.2	0.8	-	-	0.2
<b>CH:S:TA</b>	0.2	0.8	0.04	-	0.2
<b>CH:TE</b>	1	-	-	0.15	0.2
<b>CH:TA:TE</b>	1	-	0.04	0.15	0.2
<b>S:TE</b>	-	1	-	0.15	0.2
<b>CH:S:TE</b>	0.2	0.8	-	0.15	0.2
<b>CH:S:TA:TE</b>	0.2	0.8	0.04	0.15	0.2

129

## 130 2.4 Characterization of the films

131

### 132 2.4.1 Barrier properties

133

134 Water vapour permeability (WVP) was determined gravimetrically at 25°C and a RH gradient of  
135 75-100%, using a modification of the ASTM E96-95 gravimetric method (1995) for hydrophilic  
136 films. Payne permeability cups, 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau,  
137 Belgium), were filled with 5 mL of distilled water (100% RH). Six circular samples of each  
138 formulation (3.5 cm in diameter), without visible defects, were secured in the cups. The cups  
139 were placed in pre-equilibrated cabinets containing saturated solutions of sodium chloride to  
140 generate 75% RH inside the cabinet and with a fan on the top of the cup in order to reduce the  
141 resistance to water vapour transport. The shiny side of the films was exposed to the  
142 atmosphere at the lowest RH (75%). The cups were weighed periodically using an analytical  
143 balance (ME36S, Sartorius, Germany) ( $\pm 0.00001$  g) at intervals of 1.5 h for 24 h after the steady  
144 state had been reached. The slope of the weight loss versus time was plotted and the water  
145 vapour transmission rate (WVTR) and water vapour permeability were calculated according to  
146 Vargas, Albors, Chiralt & González-Martínez (2009).

147 The oxygen permeability (OP) was determined by following the ASTM Standard Method  
148 D3985-05 (2010). Three replicates of each formulation were measured using Ox-Tran  
149 equipment (Model 1/50, Mocon, Minneapolis, USA), where sample areas of 50 cm<sup>2</sup> were  
150 exposed to nitrogen and oxygen flows at 25 °C and 75% of RH. Oxygen permeability was  
151 calculated by dividing the oxygen transmission by the difference in oxygen partial pressure  
152 between the two sides of the film, and multiplying it by the average film thickness.

153

### 154 2.4.2 Tensile properties

155

156 Mechanical properties were determined using a Universal Testing Machine (Stable Micro  
157 System TA. XT plus, Haslemere, England) following the ASTM standard method (D882.ASTM  
158 D882, 2001). Twelve pre-conditioned film pieces were cut (25 mm × 100 mm) and mounted in  
159 the film's extension grip of the equipment and stretched at 50 mm·min<sup>-1</sup> until breaking. Force-  
160 distance curves were obtained and transformed into stress-strain curves. The mechanical  
161 behaviour was analysed in terms of: elastic modulus (EM), tensile strength (TS) and percentage  
162 of elongation at break (%E).

163

164 *2.4.3 Optical properties: colour, internal transmittance and gloss*

165

166 The optical properties (transparency and colour coordinates) were determined in triplicate by  
167 measuring the reflection spectrum of the samples from 400 to 700 nm using a MINOLTA  
168 spectrophotometer, model CM-3600d (Minolta CO., Tokyo, Japan). Measurements were taken  
169 on the side of film which was in contact with air during the drying.

170 The transparency was measured by means of the internal transmittance ( $T_i$ ), applying the  
171 Kubelka-Munk theory of the multiple dispersion of reflection spectrum (Hutchings, 1999) given  
172 the reflection spectra of both black and white backgrounds.

173 The CIEL\*a\*b\* colour coordinates were obtained from the reflectance of an infinitely thick  
174 layer of the material by considering illuminant D65 and observer 10°. Psychometric  
175 coordinates Chroma ( $C_{ab}^*$ ) and hue ( $h_{ab}^*$ ) were also determined (Bonilla, Talón, Atarés, Vargas  
176 & Chiralt, 2013).

177 Finally, gloss was determined at six random points of two films following the ASTM method  
178 ASTM D523 (1999), using a flat surface gloss meter at an angle of 60° (Multi-Gloss 268, Konica  
179 Minolta, Langenhagen, Germany). Gloss measurements were taken over a standard black  
180 matte surface in duplicate. Results were expressed as gloss units, relative to a highly polished  
181 surface of standard black glass with a value close to 100.

182

183 *2.4.4 Microstructural evaluation*

184

185 The microstructure of the films, previously conditioned at 25°C and 75% RH, was analysed in a  
186 JEOL JSM-5410 electron microscope (Japan) by using the scanning electron technique (SEM).  
187 SEM observations were carried in the cross-sections of the films. Thus, samples were  
188 previously dehydrated in a desiccator with phosphorus pentoxide, immersed in liquid N<sub>2</sub> and  
189 cryofractured. SEM micrographs were obtained in duplicate by mounting the samples on  
190 copper stubs and gold coating, using an accelerating voltage of 15 kV (x1500).

191

192 *2.4.5 Antioxidant activity*

193

194 The antioxidant capacity of the films was determined by using a 2,2-Diphenyl-1-picryl-hydrazyl  
195 (DPPH) reduction method, following the methodology described by Brand-Williams, Cuvelier,  
196 & Berset (1995). In this method, the stable free radical, DPPH<sup>·</sup>, is directly obtained by  
197 dissolving the compound in an organic medium. In the radical form, this molecule shows

198 absorbance at 515 nm, which disappears after accepting an electron or hydrogen radical from  
199 antioxidant compounds (Özcelik, Lee & Min, 2003).  
200 For this purpose, films containing TE (8,7 cm diameter) were dissolved in 150 ml of 1% acetic  
201 acid solution using the Rotor Stator (2 min at 8000 rpm) and magnetic stirring for 20 min at  
202 25°C in dark bottles. Blank films with added TA were also evaluated, as it is a naturally  
203 occurring polyphenol with reported antioxidant activity (Lopes, Schulman & Hermes-Lima,  
204 1999); Ferguson, 2001; Wu et al., 2004). In every case, 0.05 to 0.3 mL of the different  
205 appropriately diluted samples were mixed with a proper amount of methanol solution of  
206 DPPH· (0.022 g·L<sup>-1</sup>) in order to obtain a final volume of 4 ml.  
207 The decrease in absorbance at 25°C was determined in triplicate by using a spectrophotometer  
208 (ThermoScientific spectrophotometer Evolution 201 UV-visible) at 515 nm. Measurements  
209 were taken every 15 min until the reaction reached a plateau. The DPPH· concentration (mM)  
210 in the reaction medium was calculated from the calibration curve (equation 1) determined by  
211 linear regression (R<sup>2</sup> = 0.999). The percentage of remaining DPPH· (%DPPH'<sub>rem</sub>) was calculated  
212 following equation 2.

213  
214 
$$Abs_{515nm} = 12.21604 \cdot [DPPH^{\bullet}] + 0.00336 \quad (\text{equation 1})$$

215 
$$\% [DPPH]_{rem} = \frac{[DPPH^{\bullet}]_{t=30}}{[DPPH^{\bullet}]_{t=0}} \cdot 100 \quad (\text{equation 2})$$

216  
217 Where, [DPPH']<sub>t=30</sub> is the concentration of DPPH' at steady state and [DPPH']<sub>t=0</sub> is the  
218 concentration at the beginning of the reaction.

219 From these values, the parameter EC<sub>50</sub> was determined so as to measure the antioxidant  
220 activity of the films. The EC<sub>50</sub> antioxidant parameter indicates the amount necessary to reduce  
221 the initial concentration of DPPH' to 50% once the stability of the reaction was reached. Thus,  
222 the EC<sub>50</sub> values were obtained by plotting % [DPPH']<sub>rem</sub> versus the mass ratio of film to DPPH'  
223 (kg film/mol DPPH').

224 The antioxidant activity of the pure lyophilized thyme extract (TE), TA and CH was also  
225 determined, using the same methodology, in order to evaluate the contribution of these  
226 compounds to the total antioxidant activity of films.

227

#### 228 2.4.6 Statistical analysis

229



230 Statistical analyses of the results were performed through an analysis of variance (ANOVA)  
231 using Statgraphics Centurion XVI software (Manugistics Corp., Rockville, Md.). Fisher's least  
232 significant difference (LSD) procedure was used at the 95% confidence level.

233

### 234 **3. Results and discussion**

235

#### 236 3.1 Physical properties of films

237

##### 238 *3.1.1 Barrier properties*

239

240 The water vapour and oxygen permeabilities of the films are shown in Table 2. Pure chitosan  
241 and CH:TA films presented greater values of WVP and OP than the starch films, in agreement  
242 with the results reported by Kowalczyk, Kordowska-Wiater, Nowak & Baraniak (2015) and  
243 Bonilla et al. (2013). Blend CH:S films exhibited intermediate behaviour. The effect of TA and  
244 TE incorporation on the barrier properties of the films followed the same trends. These  
245 properties were affected by the water plasticisation level of the matrix due to its high water  
246 affinity, as is commented on bellow.

247 Tannic acid (TA) has been reported to act as a cross-linker in the presence of chitosan (CH),  
248 providing enhanced mechanical and water barrier properties. Thus, Rivero et al. (2010)  
249 reported that the addition of TA to CH films decreased the WVP values of the CH films  
250 conditioned at 20°C and 65% RH. This was not observed in the films conditioned at 75% RH,  
251 probably due to their greater equilibrium moisture content (around 22%, data not shown). In  
252 this case, the water plasticization effect of the polymeric matrix seems to mitigate the cross  
253 linking effect of TA.

254 The addition of TE significantly decreased the WVP values of the films with lower water  
255 content that is, pure starch films and starch blends, around 14% (g water/100 g film), where  
256 the effect of the water plasticization was less notable. No effect was observed when  
257 incorporated in pure CH and CH-TA films, in all likelihood due to its high water content (around  
258 22%) and to the low concentration of TE incorporated (ratio of CH:TE was 1:0,15). Siripatrawan  
259 & Harte (2010) reported a significant decrease in the WVTR of chitosan films, equilibrated at  
260 25°C and 50% RH, when adding green tea (GT), also rich in polyphenolic compounds, but only  
261 when incorporated at high concentrations (CH:GT ratio of 2:5). As regards the oxygen  
262 permeability (OP), CH based films showed the highest OP values, probably due to the greater

263 water uptake capacity of chitosan versus starch, which gave rise to a more plasticized, open  
 264 matrix (Bonilla et al., 2013).  
 265 The addition of TA improved the oxygen barrier property of the films, which is consistent with  
 266 the development of a tighter more closed structure. Nevertheless, the incorporation of TE was  
 267 only effective ( $p < 0.05$ ) when incorporated into low moisture content films (pure S and S  
 268 blends).

269

270 **Table 2.** Water vapour and oxygen permeability and water content of the films containing or  
 271 not thyme extract (TE) equilibrated at 25°C and 75% RH.

FORMULATION	WVP [ $g \cdot mm / (h \cdot m^2 \cdot KPa)$ ]		OP [ $cc \cdot \mu m / (h \cdot m^2 \cdot KPa)$ ]	
	CONTROL FILMS	FILMS + TE	CONTROL FILMS	FILMS + TE
CH	11.3 (1.7) <sup>b,1</sup>	11.6 (1.8) <sup>c,1</sup>	11.95 (1.14) <sup>c,1</sup>	14 (4) <sup>b,2</sup>
CH:TA	12 (2) <sup>b,1</sup>	11.1 (1.3) <sup>c,1</sup>	8.6 (0.4) <sup>b,1</sup>	11 (2) <sup>b,2</sup>
S	8.3 (1.5) <sup>a,2</sup>	6.9 (0.4) <sup>a,1</sup>	4.2 (1.4) <sup>a,1</sup>	4.1 (0.3) <sup>a,1</sup>
CH:S	9.6 (0.3) <sup>a,2</sup>	8.8 (1.3) <sup>b,1</sup>	6.6 (1.9) <sup>ab,2</sup>	4.3 (1.7) <sup>a,1</sup>
CH:S:TA	9.3 (0.4) <sup>a,2</sup>	7.27 (1.09) <sup>ab,1</sup>	4.8 (1.6) <sup>a,2</sup>	3.9 (0.5) <sup>a,1</sup>

272 \* Different letters in the same column indicate significant difference among formulations ( $p < 0.05$ )

273 \* Different numbers in the same row indicate significant difference between the film with and without TE ( $p < 0.05$ )

274

### 275 3.1.2 Tensile properties

276

277 The tensile strength (TS) and the deformation at break (%E) are the maximum stress and  
 278 elongation that a film can withstand before it breaks up, respectively. The elastic modulus  
 279 (EM) is related to the stiffness of the material at low strains. These mechanical parameters are  
 280 useful for the purposes of describing the mechanical properties of the film, and are closely  
 281 related to its structure (Mc Hugh & Krochta, 1994). Figure 1 shows the typical stress–strain  
 282 curves of pure S and CH films and the S:CH blend, where the effect of both the different  
 283 polymeric matrix (Figure 1A) and the addition of TE on the mechanical behaviour of the  
 284 samples (Figure 1B) can be observed. The incorporation of TA led to mechanical changes  
 285 similar to those produced by TE, but more pronounced (Figure 1). Pure chitosan behaved as a  
 286 rigid material, while pure S exhibited more viscoelastic behaviour (Figure 1). The CH:S blend  
 287 exhibited a better mechanical response than pure starch, becoming harder and more  
 288 stretchable. In every case, the incorporation of TE markedly decreased the elongation at break  
 289 of the films.

290 Table 3 shows the values of the mechanical parameters obtained. The mechanical behaviour of  
 291 the films greatly depended on the type and concentration of active incorporated, the kind of  
 292 polymeric matrix and the specific interactions between components (Ahmad, Benjakul,

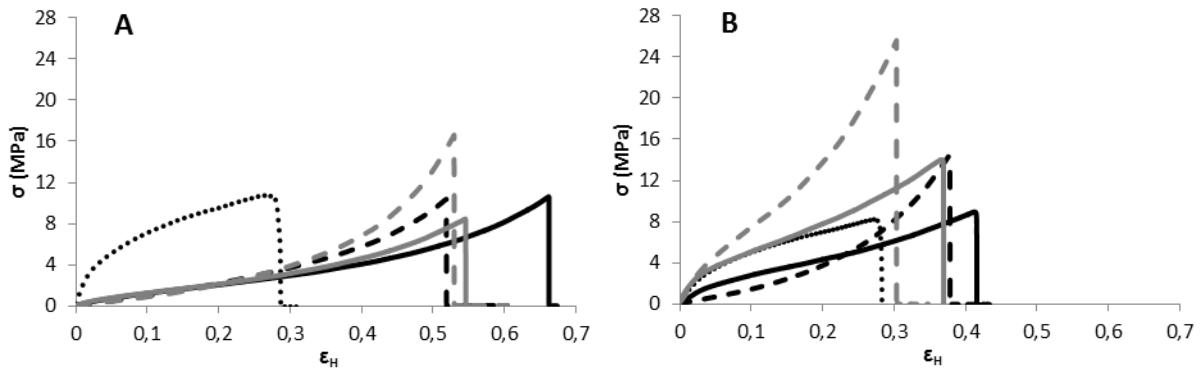
293 Prodpran & Agustini, 2012), which determine the effective attraction forces between  
294 polymeric chains. Pure CH based films presented significantly ( $p < 0.05$ ) greater extensibility  
295 (higher % $\epsilon$ ) than pure starch films, coinciding both with other studies (Silva-Weiss, Bifani, Ihl,  
296 Sobral & Gómez-Guillén, 2013). Silva-Weiss et al., 2013; Siripatrawan & Harte, 2010) and with  
297 its higher plasticisation level.

298 The addition of TA to pure CH-based films produced a significant increase in the elastic  
299 modulus (EM) and the resistance to break (TS) due to the reinforcement effect of the  
300 crosslinker (Rivero et al., 2010), resulting in stiffer and stronger films but, consequently,  
301 slightly less stretchable (lower %E). The same effects were observed when TE was added to  
302 chitosan based films due to the presence of polyphenols which can establish interactions with  
303 the chitosan chains, similarly to what occurred in the case of TA. These mechanical effects  
304 were more marked in films incorporating both TE and TA. Interactions between chitosan and  
305 polyphenolic compounds from green tea (Siripatrawan & Harte, 2010) and Indian gooseberry  
306 extract (Mayachiew & Devahastin, 2010), as well as catechin (Zhang & Kosaraju, 2007) and  
307 gallic acid (Curcio et al., 2009), have also been observed. It has been reported that the  
308 interaction mechanisms of different polyphenols with chitosan are due to electrostatic  
309 interactions (ionic complexations in acidic conditions), ester linkages and hydrogen bonds  
310 (Silva-Weiss et al., 2013; Rivero et al., 2010). Silva-Weiss et al., (2013) reported similar  
311 mechanical behaviour when working with chitosan films incorporating murta leaf extracts, rich  
312 in polyphenolic compounds (Hauser et al., 2016; Silva-Weiss, Bifani, Ihl, Sobral & Gómez-  
313 Guillén, 2014).

314 On the other hand, when TE was incorporated into pure starch films, they became less rigid  
315 (lower EM) and less resistant to fracture (lower TS) and the extensibility was not affected  
316 ( $p > 0.05$ ). CH blend films (CH:S and CH:S:TA) presented similar behaviour to pure CH films, but  
317 the tensile strength was not enhanced, in all likelihood due to the presence of starch which  
318 limits phenol-CH interactions.

319  
320  
321  
322  
323

324 **Figure 1.** Typical stress-train curve of starch (···), chitosan (---), chitosan with TA (---), the  
 325 blend starch-chitosan (—) and starch-chitosan with TA (—) films (A) and films incorporating  
 326 thyme extract (B).



327

328 **Table 3.** Mechanical properties of the films containing or not thyme extract (TE). Elasticity  
 329 modulus (EM), Tensile strength (TS) and percentage of elasticity (%E). Mean and (standard  
 330 deviation).

FORMULATION	EM		TS (MPa)		%E	
	CONTROL FILMS	FILMS + TE	CONTROL FILMS	FILMS + TE	CONTROL FILMS	FILMS + TE
<b>CH</b>	8.1 (0.5) <sup>a,1</sup>	14 (3) <sup>a,2</sup>	11 (3) <sup>a,1</sup>	13 (4) <sup>c,1</sup>	70 (4) <sup>c,2</sup>	39 (9) <sup>b,1</sup>
<b>CH:TA</b>	8.4 (0.5) <sup>a,1</sup>	102 (17) <sup>d,2</sup>	15 (3) <sup>b,1</sup>	23 (6) <sup>d,2</sup>	64 (4) <sup>b,2</sup>	35 (4) <sup>ab,1</sup>
<b>S</b>	147 (51) <sup>b,2</sup>	103 (36) <sup>d,1</sup>	9.8 (0.9) <sup>a,2</sup>	7.3 (0.9) <sup>a,1</sup>	29 (4) <sup>a,1</sup>	30 (9) <sup>a,1</sup>
<b>CH:S</b>	17 (3) <sup>a,1</sup>	51 (9) <sup>b,2</sup>	9.5 (1.7) <sup>a,2</sup>	8.2 (1.2) <sup>ab,1</sup>	90 (11) <sup>e,2</sup>	47 (6) <sup>c,1</sup>
<b>CH:S:TA</b>	18 (2) <sup>a,1</sup>	82 (31) <sup>c,2</sup>	10 (2) <sup>a,1</sup>	11 (3) <sup>bc,1</sup>	79 (7) <sup>d,2</sup>	36 (6) <sup>ab,1</sup>

331 \* Different letters in the same column indicate significant difference among formulations ( $p < 0.05$ )

332 \* Different numbers in the same row indicate significant difference between the film with and without TE ( $p < 0.05$ )

333

### 334 3.1.3 Optical properties

335

336 Table 4 shows the colour parameters of lightness ( $L^*$ ), chrome ( $C^*$ ) and hue ( $h^*$ ) and the  
 337 values of the internal transmittance at 460 nm ( $T_i$ ) of the different films, related to the  
 338 transparency of the films. Pure chitosan matrices exhibited similar lightness values to starch  
 339 but lower internal transmittance values, in agreement with their denser packed chain  
 340 structure (Sánchez-González, Cháfer, Chiralt & González-Martínez, 2010).

341 The effect of the addition of polyphenols (both from TA and TE) on the optical properties of  
 342 the films can also be observed in Table 4. Their incorporation provoked significant ( $p < 0.05$ )  
 343 changes, leading to darker, more reddish (lower  $L^*$  and  $h^*$  values, respectively) and more  
 344 opaque films (lower  $T_i$  values) than the control. Similar results were found by Siripatrawan &  
 345 Harte, (2010), when working with chitosan based films and green tea extract. These effects can  
 346 be explained by the light selective absorption of polyphenols (from TE and TA) at low

347 wavelengths that imparts a reddish colour to the films, thus decreasing the hue and Ti values  
 348 at low wavelengths. In the case of the chitosan films, the crosslinking process in the presence  
 349 of polyphenols significantly reduced the luminosity values. In general, the addition of  
 350 polyphenols from TA or TE gave rise to a more saturated colour in the films (higher C\*).  
 351 On the other hand, pure starch films containing TE presented the lowest luminosity, Ti and hue  
 352 values in coherence with the development of more brownish colours, related to the oxidation  
 353 of polyphenols during the drying and storage of the films. This underlines the protective effect  
 354 of the chitosan matrix against the oxidation of TE polyphenols, which were less affected by  
 355 light oxidation. Several authors previously reported the formation of reversible polyphenols-  
 356 chitosan complexes by means of the establishment of interactions between the hydroxyl  
 357 groups of the polyphenols and the amine functionality of the chitosan molecule (Popa,  
 358 Aelenei, Popa, & Andrei, 2000; Liang et al., 2011; Dudhani & Kosaraju, 2010; Kosaraju, D'ath &  
 359 Lawrence, 2006), leading to the encapsulation of these compounds. Because of the  
 360 encapsulation process, the core material is usually protected against the undesirable effects of  
 361 light and oxygen, among others (Shahidi & Han, 1993; Desai & Jin-Park, 2005; Fang & Bhandari,  
 362 2010).  
 363 In general, gloss values at 60° of all the films were very similar, with an average value of 28 ± 7.  
 364 No significant changes (p > 0.05) were observed when incorporating the polyphenols (data not  
 365 shown).

366  
 367 **Table 4.** Luminosity (L\*), chroma (C\*) and hue (h\*) and internal transmittance at 460 nm (Ti)  
 368 values of the films containing or not thyme extract (TE).

FORMULATION	L*		Cab*		hab*		Ti (460nm)	
	CONTROL FILMS	FILMS + TE	CONTROL FILMS	FILMS + TE	CONTROL FILMS	FILMS + TE	CONTROL FILMS	FILMS + TE
CH	72 (2) <sup>bc,2</sup>	48 (1) <sup>b,1</sup>	21.3 (0.3) <sup>e,1</sup>	31.9 (0.3) <sup>c,2</sup>	90.0 (0.3) <sup>d,2</sup>	73.7 (0.8) <sup>c,1</sup>	0.771 (0.003) <sup>b,2</sup>	0.31 (0.05) <sup>b,1</sup>
CH:TA	61 (3) <sup>a,2</sup>	49 (2) <sup>b,1</sup>	15.9 (0.7) <sup>d,1</sup>	30 (1) <sup>b,2</sup>	73.8 (1.7) <sup>b,1</sup>	72 (3) <sup>b,1</sup>	0.706 (0.012) <sup>a,2</sup>	0.38 (0.03) <sup>c,1</sup>
St	76 (8) <sup>c,2</sup>	37 (2) <sup>a,1</sup>	1.2 (0.3) <sup>a,1</sup>	20 (2) <sup>a,2</sup>	61 (9) <sup>a,1</sup>	59 (3) <sup>a,1</sup>	0.856 (0.004) <sup>e,2</sup>	0.18 (0.04) <sup>a,1</sup>
CH:St	80 (1) <sup>d,2</sup>	55 (1) <sup>c,1</sup>	10 (1) <sup>b,1</sup>	34 (1) <sup>d,2</sup>	95.8 (0.8) <sup>e,2</sup>	79.0 (0.7) <sup>d,1</sup>	0.832 (0.005) <sup>d,2</sup>	0.540 (0.014) <sup>d,1</sup>
CH:St:TA	71 (2) <sup>b,2</sup>	56 (1) <sup>c,1</sup>	14.2 (0.3) <sup>c,1</sup>	31 (1) <sup>c,2</sup>	80.1 (1.6) <sup>c,2</sup>	78,1 (0.4) <sup>d,1</sup>	0.786 (0.005) <sup>c,2</sup>	0.534 (0.018) <sup>d,1</sup>

369 \* Different letters in the same column indicate significant difference among formulations (p<0.05)  
 370 \*Different numbers in the same row indicate significant difference between the film with and without TE (p<0.05)  
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### 376 3.2 Film microstructure

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378 The microstructure of the films is affected by the spatial organization of their different  
379 components and by how they interact during the drying process. Their microstructure provides  
380 information about the arrangement of the different compounds and permits a better  
381 understanding of the film's physical properties (Vargas, Perdonés, Chiralt, Cháfer & González-  
382 Martínez, 2011).

383 Figure 2 shows the cross-section SEM micrographs of the films, where remarkable differences  
384 can be observed. Thus, chitosan films presented a heterogeneously-fractured surface,  
385 probably due to the presence of more ordered and crystalline regions. On the other hand,  
386 although starch films exhibited a smoother appearance, the presence of crystalline regions at  
387 the top and bottom of the films can also be observed. The S-CH blend presented a more  
388 homogenous, smoother structure, indicating a good compatibility between both polymers. The  
389 reduction in the amount of crystalline regions in the chitosan films when mixed with  
390 gelatinised starch has been previously reported by Xu, Kim, Hanna & Nag (2005).

391 In pure chitosan films, polyphenols (TA or TE) led to a more dense structure with a more  
392 regular packaging of polymer chains, which could be related with the described interactions  
393 between chitosan and polyphenols. This contributes to the formation of a more compact  
394 matrix, with enhanced mechanical resistance in agreement with the results obtained from the  
395 mechanical assays. In the blended films, the microstructure was only modified when using TA.  
396 In this case, the film structure became less smooth, exhibiting areas with more brittle fracture  
397 in agreement with the crosslinking action. The incorporation of TE into starch or CH-S films was  
398 observed to have no notable effect on the film microstructure.

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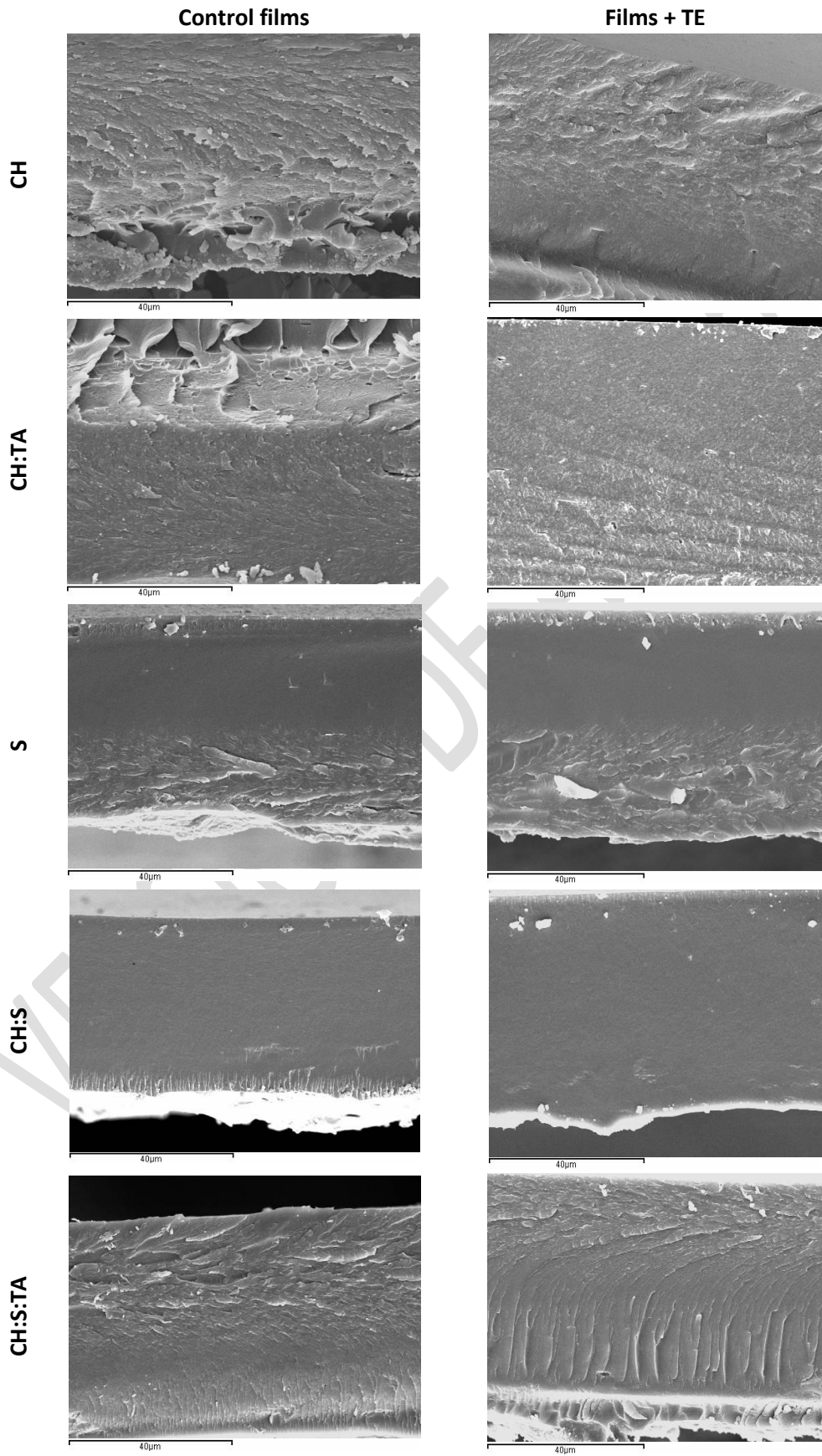
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**Figure 2.** SEM micrographs of films containing or not thyme extract (TE) (x1500).



### 412 3.3. Antioxidant activity

413

414 From the absorbance data both at zero reaction time and after 30 minutes (when the steady  
415 state of the reaction was achieved), the  $EC_{50}$  parameter (mass of sample/mol DPPH, necessary  
416 for the purposes of reducing the initial DPPH concentration to 50%) was calculated for every  
417 sample. Thus, the lower the  $EC_{50}$  values, the greater the antioxidant activity of the tested  
418 sample.

419 This value was also determined for the phenolic ingredients (tannic acid and lyophilized thyme  
420 extract) so as to take into account the contribution of these components to the total  
421 antioxidant activity of the films. The  $EC_{50}$  value for TA was  $0.022 \pm 0.002$  kg TA/mol DPPH (or  
422  $0.0131 \pm 0.0013$  mol TA/mol DPPH). This pointed to its high antioxidant activity, more potent  
423 than resveratrol, ascorbic acid, caffeic acid, or BHT, but close to that of gallic acid (0.7, 0.27,  
424 0.1, 0.08 mol antioxidant/mol DPPH, respectively) (Brand-Williams et al., 1995; Pastor,  
425 Sánchez-González, Chiralt, Cháfer & González-Martínez, 2013). This high antioxidant capacity  
426 has been correlated with its multiple phenolic groups (Aelenei et al., 2009). On the other hand,  
427 thyme extracts exhibited a remarkable antioxidant capacity (of around  $0.26 \pm 0.02$  kg TE/mol  
428 DPPH), but lower than that of TA. Although CH has been reported to exert some antioxidant  
429 activity (Vargas & González-Martínez, 2010), it was not able to reduce the initial DPPH  
430 concentration to 50%. Both tannic acid and thyme extract were found to react quickly with  
431 DPPH (fast kinetic behaviour), taking around 30 minutes to reach the steady state.

432 In Table 5, the  $EC_{50}$  values of the films with and without polyphenols are shown. In films  
433 without TE, DPPH reductions of only 50% were obtained in those films containing tannic acid,  
434 in agreement with its potent antioxidant activity. In these films, the DPPH reductions were  
435 more marked in the formulations with chitosan-starch mixture, probably because tannic acid is  
436 more available as there is less chitosan to interact with, compared to the pure CH formulation.  
437 The  $EC_{50}$  values found were in range of those reported by Pastor et al. (2013) for  
438 methylcellulose and chitosan based films containing resveratrol (of around 2-20 kg film/mol  
439 DPPH).

440 All of the films containing TE exhibited antioxidant activity, although as shown in Table 5, the  
441 formulations were found to have significant differences ( $p < 0.05$ ). In accordance with previous  
442 data, the films containing both tannic acid and TE (CH:TA:TE and CH:S:TA:TE) showed the  
443 greatest antioxidant power (0.7 and 0.8 kg film/mol DPPH, respectively). TA-free films (CH, S  
444 and CH:S) with TE also exhibited remarkable antioxidant activity, especially those films  
445 formulated with chitosan. Taking into account that all the films contained the same amount of



446 TE, these differences in the films' antioxidant activity once again underline the protective  
 447 effect of the chitosan matrix against polyphenol oxidation during the drying/storage of the  
 448 films, as commented on above. The blend film (S-CH with TE) exhibited a lower antioxidant  
 449 capacity than the CH film with TE, coinciding with the less effective encapsulating ability of the  
 450 blend than the pure CH films.

451 The values obtained for EC<sub>50</sub> were also expressed in terms of the g of TE per DPPH moles (by  
 452 taking into account the amount of TE in each film sample). These values reflect that, in pure  
 453 starch films, some losses of antioxidant activity occurred, since these values are significantly  
 454 higher (p<0.05) than the ones obtained for pure TE (0.26 kg TE/mol DPPH). This could be due  
 455 to some degradation of TE during the drying of the films caused by the poor protective effect  
 456 of starch. On the other hand, in CH+TE films, the EC<sub>50</sub> values were lower (p<0.05) than in pure  
 457 TE. This could indicate that the chitosan is preventing the oxidation of polyphenols occurring  
 458 during the handling of the compound since, when TE powder was dissolved, degradation  
 459 occurred to some extent. In order to clarify this hypothesis, the EC<sub>50</sub> value of a water solution  
 460 containing the same amount of chitosan and TE as CH+TE films (0.22% and 0.034%  
 461 respectively) was measured. The EC<sub>50</sub> value obtained for this system was 0.148 Kg TE/mol  
 462 DPPH; this is very close to that of the CH+TE film, thus pointing to the protective effect of CH  
 463 against phenol oxidation. Nevertheless, the fact that chitosan can also contribute to the total  
 464 antioxidant activity of the film or dispersion cannot be neglected.

465

466 **Table 5.** Antioxidant activity of films containing or not thyme extract (TE), expressed in terms  
 467 of EC<sub>50</sub> values.

FORMULATION	EC <sub>50</sub> (kg film/mol DPPH)		EC <sub>50</sub> (kg TE/mol DPPH)
	CONTROL FILMS	FILMS + TE	FILMS + TE
<b>CH</b>	-	1.56 (0.03) <sup>b</sup>	0.141 (0.003) <sup>b</sup>
<b>CH:TA</b>	1.31 (0.07) <sup>b,1</sup>	0.713 (0.017) <sup>a,2</sup>	0.0637 (0.0013) <sup>a,2</sup>
<b>S</b>	-	3.5 (0.3) <sup>d</sup>	0.42 (0.05) <sup>d</sup>
<b>CH:S</b>	-	2.07 (0.06) <sup>c</sup>	0.227 (0.005) <sup>c</sup>
<b>CH:S:TA</b>	1.061 (0.008) <sup>a,1</sup>	0.90 (0.03) <sup>a,2</sup>	0.095 (0.003) <sup>a,2</sup>

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\* Different letters in the same column indicate significant difference among formulations (p<0.05)

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\*Different numbers in the same row indicate significant difference between the film with and without TE (p<0.05)

470

#### 471 **4. Conclusions**

472

473 The use of thyme extract (TE) was analysed as a source of natural polyphenols for the purposes  
 474 of the development of antioxidant films for food preservation. This extract was efficiently  
 475 incorporated into starch and chitosan film matrices. The films presented some changes in their

476 microstructural and physical properties due to the incorporation of TE, which depended on the  
477 type of matrix. The best results were obtained when thyme extract was added to chitosan  
478 based films, due to the cross-linking effect that occurs among the polyphenols and the  
479 chitosan, promoting a better tensile response (greater resistance at break and higher degree of  
480 stiffness). Nevertheless, films became less stretchable and more opaque due to the structural  
481 changes provoked by polyphenols in the matrix. These films also exhibited remarkable  
482 antioxidant activity after film formation and conditioning. The results obtained highlighted the  
483 possibility of using these natural compounds for coating purposes in order to promote the  
484 shelf life of the products sensitive to oxidative processes.

485

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