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Additional Information

1 **Reversible Covalent Immobilization of Cinnamaldehyde on Chitosan Films via**
2 **Schiff Base Formation and Their Application in Active Food Packaging**

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10
11 **Abstract**

12 In this work, active packaging films for antimicrobial food with materials derived from
13 renewable resources and biodegradable nature have been developed and
14 characterized. Chitosan was chosen as polymeric matrix and cinnamaldehyde as
15 antimicrobial active agent. Cinnamaldehyde was anchored to the matrix by nucleophilic
16 reaction with the formation of a Schiff base, with a degree of substitution of 70 %. The
17 obtained films have been processed at different temperatures and times simulating
18 typical preservation processes of the food industry and their effects on the release of
19 cinnamaldehyde and the properties of the film were analyzed.

20 The antimicrobial effect of the films was tested against pathogen bacteria (*S. aureus*
21 and *E. coli*) and pasteurized milk inoculated with *L. monocytogenes*.

22 Sensory evaluation by a panel of untrained judges was carried out to determine
23 whether the release of the active component into pasteurized milk changed its odor
24 appreciably and if so, to estimate whether this modification was acceptable by
25 consumers.

26 The results show that all films with cinnamaldehyde showed antimicrobial effect against
27 bacteria studied model, being more effective against Gram positive bacteria. The films
28 provided a highly effective antimicrobial effect with both mild but sustained heat
29 treatment or short but more intense heat treatments, being possible to achieve a high
30 reduction of microbial load or even complete. The application of the films developed in
31 pasteurized milk inhibits the growth of *L. monocytogenes* for 12 days under
32 refrigeration conditions which may lengthen the security of such products. Sensory
33 analysis of pasteurized milk in contact with the films has shown that cinnamon smell
34 does not cause any rejection among potential consumers, being preferred over the
35 control sample.

36 The use of chitosan films with anchored cinnamaldehyde would extend the shelf life or
37 of milk products due to the antimicrobial capacity and the added value of a high
38 acceptance by consumers.

39 **Keywords**

40 Antimicrobial films, chitosan, cinnamaldehyde, foodborne pathogens, antimicrobial
41 active packaging.

42

43

44 **1. Introduction**

45 Chitosan is a natural, biocompatible, biodegradable, biorenewable, biofunctional,
46 polysaccharide that is finding attractive applications in several industrial areas. In
47 packaging technology, chitosan produces highly transparent films with excellent gas
48 and organic compound barrier characteristics which can be an alternative to oil-derived
49 barrier polymers and that can be used to provide barrier to other polymeric films and
50 porous materials such as fibre-based paper (Gallstedt & Hedenqvist, 2006). Chitosan,
51 employed as a delivery system, is finding applications in a variety of technological
52 areas, such as agrochemistry, pharmacy, biomedicine, textiles and active food
53 packaging. The development of antimicrobial materials and their application in the
54 design of active packaging is focusing considerable expectation in the food industry,
55 since food safety is an area of great concern. Although there are many studies in the
56 literature that focus on the use of chitosan films as antimicrobials in contact with food,
57 the use of chitosan films for the release of antimicrobials has received much less
58 attention (Higueras, Lopez-Carballo, Cerisuelo, Gavara, & Hernandez-Munoz, 2013).

59 Chitosan films can be used as matrix for the release of antimicrobial essential oils,
60 although the incorporation of hydrophobic compounds is problematic because of the
61 hydrophilic nature of chitosan. Recent studies have reported the incorporation in
62 chitosan of previously encapsulated essential oils (Abreu, Oliveira, Oliveira, Paula, &
63 de Paula, 2012; Higueras, et al., 2013; Hosseini, Zandi, Rezaei, & Farahmandghavi,
64 2013). Another alternative procedure could be the exploitation of chitosan reactive
65 groups. Chitosan chain contains amino and hydroxyl substituents which can be used to
66 chemically alter its properties under mild reaction conditions (Li, Liu, Tian, Liu, & Fan,
67 2007). The presence of amino groups allows several chemical modifications, including
68 the formation of a Schiff base by reaction with aldehydes or ketones (Wang, Lian,
69 Wang, Jin, & Liu, 2012). The reaction of chitosan with aromatic aldehydes to produce
70 the corresponding Schiff bases has been described recently (Cavalheiro, dos Santos,
71 & Dockal, 2005; Guinesi & Gomes Cavalheiro, 2006; Guo, et al., 2007). According to
72 Kurita, Mori, Nishiyama, and Harata (2002), the binding of carbonyl groups to the
73 chitosan macromolecule results in Schiff bases whose degree of substitution is
74 dependent on the stoichiometric amount of aldehyde used in the reaction (Kurita, Mori,
75 Nishiyama, & Harata, 2002).

76 Cinnamaldehyde is an aromatic aldehyde and the main component of cinnamon bark
77 extract (Holley & Patel, 2005). Cinnamaldehyde is a well-known natural antimicrobial
78 compound, active against a wide spectrum of foodborne pathogens. Among the
79 potential mechanism of action, cinnamaldehyde molecules might react with aminoacids
80 forming Schiff base adducts. The organoleptic effect of essential oils is one of the most

81 important factors that limit their application as antimicrobial agents to real food
82 products, even though their efficiency has been widely described in *in vitro* tests
83 (Belletti, Lanciotti, Patrignani, & Gardini, 2008). Therefore, any food application of this
84 agent should consider the potential sensory impact which could result in non-
85 acceptance by the consumer.

86

87 There are some studies focused on the synthesis of Schiff base from chitosan and the
88 potential antimicrobial activity of the obtained derivatives (Abreu, et al., 2012; Jin, Wang,
89 & Bai, 2009; Wang, et al., 2012). Nevertheless, literature shows no reports on the
90 formation of Schiff base between chitosan and cinnamaldehyde with potential
91 application in the promotion of food safety.

92 The aim of this study was to develop an efficient method to synthesize the Schiff base
93 of chitosan with cinnamaldehyde and to produce films which could present
94 antimicrobial activity. The developed films have been subjected to different
95 temperatures simulating typical preservation processes applied in food industry, and
96 the cinnamaldehyde release and the properties of the film were analyzed. The
97 antimicrobial and sensory effects were also evaluated.

98

99 **2. Materials and methods**

100

101 **2.1 Materials**

102 Low molecular weight chitosan with a degree of acetylation of 15 -25 % was supplied
103 by Sigma (Barcelona, Spain). *Trans*-cinnamaldehyde and acetic acid were provided by
104 Aldrich (Steinheim, Germany). Sodium hydroxide and ethanol 96 % (v/v) were
105 purchased from Panreac (Barcelona, Spain) and hydrochloric acid 37% from Merck
106 (Darmstadt, Germany). Ortho-phosphoric acid / sodium hydroxide pH 3 buffer and
107 potassium dihydrogen phosphate / di-Sodium hydrogen phosphate pH 7 buffer were
108 purchased from Scharlab (Barcelona, Spain). Water was obtained from a Milli-Q Plus
109 purification system (Millipore, Molsheim, France).

110

111 **2.2 Film preparation**

112 **2.2.1. Chitosan film preparation**

113 A 1.5 % chitosan (w/w) solution in a 0.5% (w/w) acetic acid solution was prepared and
114 filtrated to eliminate impurities. Chitosan acetate films with 55 ± 5 μm average
115 thickness were obtained by casting on polystyrene plates at 37 °C and 22% relative
116 humidity for 48 h. 1.7 x 1.7 cm cut samples from these films were neutralized with 0.1

117 M sodium hydroxide for 24 h at 37 °C to make them insoluble in water. After
118 neutralization, films were washed with water (pH= 5.5-6) and dried at 37 °C. Finally, the
119 film samples were stored in an amber glass desiccator at 22 °C and 0 % RH prior to
120 use. Some of these films were used as chitosan control films (CS) others were reacted
121 with cinnamadehyde.

122 2.2.2. Chitosan films reacted with cinnamadehyde

123 A cinnamaldehyde solution was prepared by adding 4 g of cinnamaldehyde to 75
124 mL of acidified (200 µL of HCl) ethanol. Cut neutralized films (2 g) were hydrated with
125 water (pH=5.5-6) for 2 h and stirred at room temperature. Then, the water excess was
126 removed by gently rubbing with paper tissue and films were immediately exposed to
127 the cinnamaldehyde solution in a shaking bath at 60 °C during 24 h. Finally, reacted
128 films were washed three times by dipping in ethanol for 1, 2 and 24 h. Finally, these
129 films (CScin) were stored in an amber glass desiccator at 22 °C and 0 % RH prior to
130 use. The films thickness ($55 \pm 5 \mu\text{m}$) was individually measured with a digital Mitutoyo
131 micrometer (Metrotec, San Sebastian, Spain).

132 2.2.3. Treatments applied to films

133 Developed films were subjected to different time and temperatures treatments
134 simulating various preservation processes. A 0.25 g film portion was put into a glass
135 vial with 10 mL of Mueller Hinton Broth (MHB) (Scharlab, Barcelona, Spain) buffer
136 solution at pH 7 or pasteurized whole milk. Vials were then submitted to different
137 temperature/time treatments: a) 30 min at 4 °C in a cooling chamber to simulate
138 refrigeration conditions; b) 30 minutes at 65 °C, 15 minutes at 72 °C, 10 minutes at 95
139 °C in thermostatic bath with agitation to simulate pasteurization treatments; and c) 5
140 minutes at 121 °C in autoclave to simulate retorting processes.

141

142 **2.3 Characterization of chitosan films modified with cinnamaldehyde**

143 2.3.1. Elemental analysis

144 Carbon, hydrogen, nitrogen, oxygen and sulfur elemental analyses were performed on
145 a CE instruments (Thermo-Fisher) EA 1110 CHNS-O elemental analyzer (University of
146 Barcelona, Spain). Samples were analyzed in triplicate. Results are expressed as
147 average value \pm standard deviaton

148

149 2.3.2. Optical properties

150 The colour of the diverse chitosan-based films was measured with a CR-300 Minolta
151 Chroma meter® (Minolta Camera Co., Ltd., Osaka, Japan). The film samples were
152 placed on a white standard plate; the results were expressed in accordance with the
153 CIELAB system with reference to illuminant D65 and a visual angle of 10°. The
154 measurements were performed through a 6.4-mm-diameter diaphragm containing an
155 optical glass, monitoring L*, a*, b*, chroma ($C^*_{ab} = (a^{*2}+b^{*2})^{1/2}$) and hue ($h_{ab} = \arctan$
156 (b^*/a^*)). The samples were measured in triplicate and each sample was analysed by
157 eight measurements in different locations of the film sample.

158 The absorbance spectrum of each film was obtained between 400–800 nm using an
159 Agilent 8453 UV-visible spectrophotometer (Agilent, Barcelona, Spain).

160

161 2.3.3. Swelling

162 To observe the water gain capacity and the swelling of the manufactured films, 1.7 x
163 1.7 cm samples were immersed in buffered aqueous media at pH 3 and at pH 7 for 48
164 h, and changes in films weight, length and thickness were recorded. First,, the films
165 subjected to the different treatments were stored in a desiccator with sodium pentoxide,
166 Sigma (Barcelona, Spain) for 48 h, and then weighed and dimensionally measured.
167 Then, films were placed in vials with 25 mL of buffer and the properties measured at 2,
168 24 and 48 h previously removing water excess. The experiment was performed in
169 triplicate. The results were expressed in water absorption percentage, and increments
170 in thickness and area.

171

172 2.3.4. Contact angle

173 After each treatment (see section 2.2.3), film samples were left under pressure
174 between two glass sheets for 48 h to increase film flatness, and then stored for 48 h in
175 a desiccator with sodium pentoxide at 0 % RH. The contact angle was measured using
176 a goniometer OCA 15EC (DataPhysics Instruments GmbH, Filderstadt, Germany). A 2
177 µL water droplet was dispensed onto the sample surface and the drop image was
178 recorded during 2 min. The contact angle at 60 s was estimated by using the
179 instrument SCA20 embedded software module. The experiment was performed in
180 triplicate.

181

182 2.3.5. Attenuated total reflectance – Fourier transform infrared spectroscopy (ATR-
183 FTIR)

184 The developed films were analyzed by ATR-FTIR. Samples were placed in a Golden
185 Gate single reflection diamond ATR accessory (Teknokroma, Barcelona, Spain) and
186 the spectra were recorded with a Bruker Tensor 27 FTIR spectrometer (Bruker
187 Española S.A., Barcelona, Spain). The resolution was 4 cm⁻¹ in the range of 4000 to
188 600 cm⁻¹ and 128 scans were recorded per test. Results were recorded in triplicate and
189 analysed with the instrument OPUS vs. 2.06 software.

190 2.3.6. Release studies

191 To know the amount of cinnamaldehyde released from the chitosan matrix during the
192 microbiological assays, the concentration of cinamaldehyde in Mueller Hinton Broth
193 MHB was determined. Immediately after each preservation treatment, the liquid
194 medium was transferred to a new vial and the amount of cinamaldehyde measured by
195 UV-Vis spectroscopy at 221 nm.

196 A study of the release of cinnamaldehyde from the films was carried out by determining
197 the specific migration from the polymer into ethanol 50 %, the food simulant specified in
198 European law (EC Regulation 10/2011). A 1.7 x 1.7 cm film sample was introduced in a
199 glass vial with 7 mL of ethanol 50 % closed tightly with PTFE septum and aluminum
200 caps to constitute a sample. After the diverse treatments (section 2.2.3.), three vial
201 samples per treatment and exposure time (5, 10, 15, 30 min and 1, 8, 24 and 48 hours)
202 were opened and the liquid analyzed by UV-vis spectroscopy at 221 nm wavelength.

203 **2.4 Antimicrobial activity**

204

205 2.4.1. Culture strains

206 *Staphylococcus aureus* CECT 86, *Escherichia coli* CECT 434 and *Listeria*
207 *monocytogenes* CECT 934 were obtained from the Spanish Type Culture Collection
208 (Valencia, Spain). Strains were stored in Tryptone Soy Broth (TSB, Scharlab) with 20
209 % glycerol at -80 °C until needed. For experimental use, the stock cultures were
210 maintained by regular subculture on agar Tryptone Soy Agar (TSA, Scharlab) slants at
211 4 °C and transferred monthly.

212

213 2.4.2. Antimicrobial effect of the released cinnamadehyde against *Staphylococcus*
214 *aureus* and *Escherichia coli*

215 Before analysis, a loopful of each strain was transferred to 10 mL of TSB and
216 incubated at 37 °C for 18 h to obtain early-stationary phase cells. Cell cultures of each
217 microorganism in stationary phase, with an optical density of 0.9 at 600 nm, were
218 diluted in TSB and incubated at 37 °C until exponential phase with an optical density of
219 0.2 at 600 nm (10^5 UFC/mL). 0.25g of chitosan films with cinnamaldehyde were put in
220 contact with 10 mL of Mueller Hinton Broth MHB (Scharlab, Spain) and subjected at
221 different preservation treatments (see section 2.2.3). Control films of neutralized
222 chitosan without cinnamaldehyde were analyzed as well in every experiment. Liquid
223 medium in contact with the films were recovered after treatment and cooled down to
224 room temperature. Then , 100 μ L of *cell culture* in exponential phase (10^5 CFU/mL)
225 were added and the tubes were incubated at 37 °C for 18 h. Depending on the turbidity
226 of the tubes, serial dilutions with peptone water were carried out and plated in Petri
227 dishes with 15 mL of culture medium TSA. Colonies were counted after incubation at
228 37 °C for 18h. The result was expressed in log of CFU/mL. All analyses were carried
229 out in triplicate.

230

231 2.4.3. Antimicrobial assays in milk

232 The antimicrobial activity of the films was tested in a commercial pasteurized milk. To
233 do this, the procedure described in section 2,4,2 was followed by using milk instead of
234 MHB and inoculating *Listeria monocytogenes* in exponential phase. Sterilized tubes
235 with 10 mL of milk were inoculated in sterilized conditions with 100 μ L of *L.*
236 *monocytogenes* in exponential phase (10^5 CFU/mL). The tubes were then kept at 4 °C
237 for 12 days, and antimicrobial assays were performed on days 3, 6 and 12. Serial
238 dilutions with peptone water were made and plated in Listeria Palcam agar (Merck).
239 Plates were incubated at 37 °C for 48 h. All experiments were carried out in triplicate.

240

241 **2.5 Sensory analysis**

242 Sensory test on milk exposed to the films and after the preservation treatments were
243 carried out on 3rd, 6th and 12th days by an untrained panel (44 judges). The tests were
244 done in a standardized test room (ISO 8589-2007). Samples of milk were placed in
245 hermetic sealed transparent tubes and identified by three digit codes. They were asked
246 to smell the sample and describe the intensity of the perceived cinnamon aroma and
247 preference in terms of smell. The odour intensity was indicated in a 1 to 5 scale being
248 1 the lowest intensity perceived smell of cinnamon and 5 the most intense. For the

249 preference test samples are ordered from 1 to 5, 1 to assign the sample to greater
250 acceptance and 5 the lowest. Data analysis was performed with the program
251 Compusense ® five release 5.0 (Compusense Inc., Guelph, Ontario, Canada).

252

253 **2.6 Data analyses**

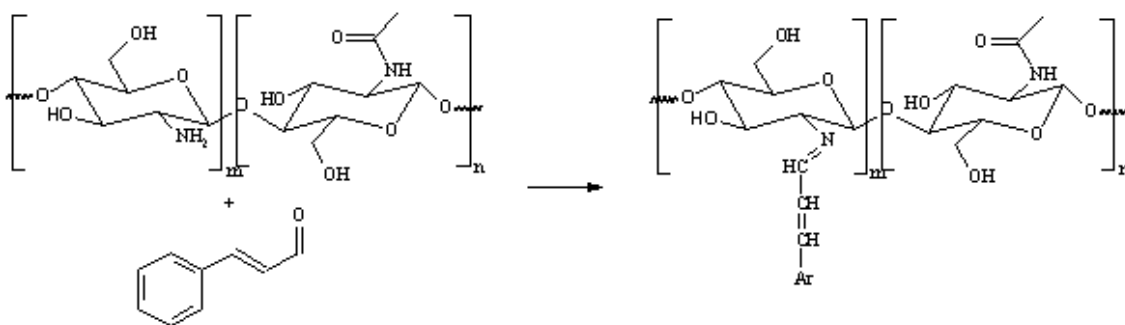
254 Statistical test were performed using the SPSS® Statistics computer program, version
255 19.0 (SPSS Inc., Chicago, IL, USA). One-way of variance was carried out. Differences
256 between pairs of means were assessed on the basis of confidence intervals using the
257 Tukey-b test. Moreover, comparisons between two samples were analyzed by
258 Student's t test. The level of significance was $p \leq 0.05$. The data are represented as
259 average \pm standard deviations. The data were analyzed and plotted using the Sigma-
260 plot 10.0 software (Sytat Software Inc., Richmond, CA).

261

262 **3. RESULTS AND DISCUSSION**

263

264 All cast CS films were transparent, without discontinuities and with an average
265 thickness of $55 \pm 5 \mu\text{m}$. 1.7×1.7 film samples were modified by nucleophilic reaction
266 with cinnamaldehyde (CScin) as indicated in squeme 1. In contrast to previous reports
267 where cinnamaldehyde is added to the film forming solution and then the film is casted,
268 the film, since other authors have developed chitosan films with incorporated by direct
269 addition of the aldehyde to the film forming solutions (Jin, et al., 2009; Wang, et al.,
270 2012), in the present method chitosan films are obtained and then cinnamaldehyde is
271 anchored, The advantage of this novel method is that the film is manufactured and
272 neutralized prior to the incorporation of the agent, this way reducing the partial loss of
273 the added cinnamaldehyde during film drying due to its high volatility. Three
274 consecutive washes largely reduces the presence of free cinnamaldehyde on film
275 surface, only remaining those molecules chemically anchored to the chitosan chains or
276 physically embedded in the polymer matrix. Obtained films were subjected to different
277 preservation processes representing those of the food processing industry and their
278 effects on cinnamaldehyde release of and film properties were analyzed.



279

280 **Scheme 1.** Nucleophilic reaction between chitosan and cinnamaldehyde

281

282 **3.1 Characterization of chitosan films modified with cinnamaldehyde**

283 3.1.1. Elemental analysis

284 The elemental composition of chitosan films before and after reaction with
 285 cinnamaldehyde, and after the diverse preservation processes are collected in Table
 286 1. The degree of acetylation (DA) for the untreated sample was calculated with the
 287 following equation (Kasaai, Arul, Chin, & Charlet, 1999; Lago, et al., 2011):

$$288 \quad DA = \frac{(C/N) - 5.145}{6.861 - 5.145} \times 100 \quad (1)$$

289 The degree of acetylation of the chitosan was 20.3 %, in agreement with that
 290 indicated by the supplier for low molecular weight chitosan (15 -25 % degree of
 291 acetylation).

292

Table 1 Elemental analysis of films

Samples	N%	C%	H%	O ¹ %	Substitution grade (%)
CS	7.33 ± 0.07 ^d	40.22 ± 0.07 ^a	7.18 ± 0.04 ^d	45.28 ± 0.18 ^d	
CScin	6.03 ± 0.04 ^{b,c}	53.93 ± 0.08 ^e	6.64 ± 0.01 ^a	33.40 ± 0.13 ^a	72.11 ± 0.20 ^a
4 °C, 30 min	5.58 ± 0.30 ^a	51.48 ± 0.19 ^b	6.77 ± 0.04 ^c	36.17 ± 0.53 ^c	65.46 ± 0.59 ^{ab}
65 °C, 30 min	5.76 ± 0.27 ^{a,b}	52.73 ± 0.41 ^c	6.80 ± 0.05 ^b	34.71 ± 0.73 ^b	65.09 ± 0.78 ^{ab}
72 °C, 15 min	5.97 ± 0.09 ^{a,b,c}	53.07 ± 0.11 ^{c,d}	6.75 ± 0.03 ^{a,b}	34.22 ± 0.23 ^{a,b}	59.32 ± 0.28 ^{ab}
95 °C, 10 min	6.15 ± 0.16 ^{b,c}	51.82 ± 0.45 ^b	6.92 ± 0.06 ^b	35.11 ± 0.67 ^b	53.55 ± 0.66 ^b
121 °C, 5 min	6.29 ± 0.06 ^c	53.37 ± 0.03 ^{d,e}	6.95 ± 0.09 ^a	33.40 ± 0.18 ^a	52.17 ± 0.17 ^b
Cin		81.82 ²	6.06 ²	12.12 ²	72.11 ± 0.20 ^a

293 ^{a-b} Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$)

294 ¹Oxygen percentage values calculated by mass balance

295 ²Theoretical values

296

297 As expected from the elemental composition of cinamaldehyde, the reacted films
298 experimented an increase in C percentage, and a decrease in nitrogen and oxygen.
299 This was observed when CS values are compared with the rest of samples,
300 independently of the treatment.

301 To facilitate the comparison among reacted samples, the percentage of chitosan
302 amine groups that reacted with cinamaldehyde, or degree of substitution (DS) was
303 estimated following the work of Inukai et al 1998 (Inukai, Chinen, Matsuda, Kaida, &
304 Yasuda, 1998):

$$305 \quad DS = \frac{(C/N) - (C/N)_0}{n} \quad (2)$$

306

307 where (C / N) is the ratio of carbon and nitrogen of the chitosan derivative, (C / N)₀ is
308 the ratio of carbon and nitrogen of chitosan, and n is the number of carbon introduced
309 into the modified chitosan. The DS values, also included in Table 1, indicated an
310 excellent reaction efficiency. Cinnamaldehyde molecules had been reacted with more
311 than 70 % of the amine groups present in the matrix of chitosan. After the treatments,
312 the DS values presented a slight reduction, which is due to the reversibility of the Schiff
313 base reaction. This reduction increases with the increase of the treatment temperature,
314 being significant for the treatments at 95 and 121 °C. It is noteworthy that even after a
315 retorting-like process, more than 50 % of reacted cinamaldehyde remained in the film,
316 implying a large reservoir of cinnamaldehyde.

317

318 3.1.2. Optical properties

319 Table 2 shows the color coordinates L *, a * and b * as well as the chroma (C*) and hue
320 (h_{ab}) of individual films prepared CS, CScin and CScin with different time-temperature
321 treatments. Significant differences have been observed between CS and CScin films,
322 the latter presenting higher C* values. The formation of a Schiff base between the free
323 amino group of chitosan and the aldehyde group of cinnamaldehyde, gives rise to a
324 conjugated double bond chitosan-cinnamaldehyde. This feature is responsible for the
325 increase in C* (Jin, et al., 2009), and the corresponding colour change from colorless to
326 orange which was visually perceptible.

327 When, the colour of thermally treated CScin samples was examined, changes were
328 observable in all colour parameters. Hue of the CS cin samples (81.2°) decreases with
329 the treatment as a consequence of the partial loss of the retained cinnamaldehyde. In

330 general terms, the films evolved from reddish (lower T treatments) to yellow (more
 331 severe treatments). With respect to chroma, no differences on C* values were
 332 observed between reacted samples and those subjected to low temperature treatments
 333 (4 °C and 65 °C). The samples treated at higher temperatures, 72 °C for 15 min, 95 °C
 334 for 10 min and 121 °C for 5 min significantly differ on C*. The C* values for samples
 335 treated at 72 °C and 95 °C showed higher colour intensity than CScin films. However
 336 this parameter decreased for the retorted sample at 121 °C for 5 min. This may be due
 337 to an increased release of cinnamaldehyde from the film during the treatment as a
 338 consequence of a reversion of the anchored reaction.

339 L* value decreased with the treatments. CS were practically transparent, while CScin
 340 presented a significant loss of transparency (low L*). This decrement is more relevant
 341 with the thermal treatment probably due to elevated water sorption in the matrix during
 342 processing that after cooling is not completely removed, creating small water droplets
 343 within the matrix that diffracts the light. This observation has been reflected in studies
 344 with hydrophilic matrices (Aucejo, Catala, & Gavara, 2000). A decrease in L* parameter
 345 is also observable when pure CS film is heated, reducing the brightness and increasing
 346 the yellowish tone of the samples. Retorted CS film presented the following colour
 347 parameters: L* = 88.6 ± 0.8, a* = 2.38 ± 0.1, b* = 23.65 ± 1.2; C_{ab}* = 23.7 ± 1.1; h_{ab} =
 348 84.2 ± 0.4 (not included in Table 2).

349 **Table 2.** Colour parameter values of chitosan (CS) and chitosan/ cinnamaldehyde (CS/cin) films

Films	L*	a*	b*	C _{ab} *	h _{ab}
CS	91.5 ± 0.2 ^a	-1.9 ± 0.1 ^a	9.0 ± 0.2 ^a	9.2 ± 0.2 ^a	101.8 ± 0.1 ^a
CScin	74.2 ± 0.4 ^b	13.4 ± 0.1 ^b	86.6 ± 1.2 ^b	87.6 ± 1.2 ^b	81.2 ± 0.1 ^b
4 °C, 30 min	73.5 ± 0.2 ^b	14.4 ± 0.3 ^b	87.7 ± 0.1 ^b	88.8 ± 0.3 ^b	80.7 ± 0.2 ^{b,c}
65 °C, 30 min	74.0 ± 0.9 ^b	14.5 ± 1.0 ^b	88.2 ± 1.5 ^b	89.4 ± 1.3 ^b	80.7 ± 0.7 ^{b,c}
72 °C, 15 min	72.2 ± 1.5 ^c	16.2 ± 0.5 ^c	90.4 ± 1.1 ^c	91.9 ± 1.0 ^c	79.9 ± 1.0 ^c
95 °C, 10 min	69.1 ± 0.8 ^d	20.2 ± 1.4 ^d	94.0 ± 1.1 ^d	96.2 ± 0.8 ^d	77.9 ± 0.9 ^d
121 °C, 5 min	53.2 ± 0.8 ^e	38.7 ± 0.6 ^e	73.3 ± 0.2 ^e	82.9 ± 0.1 ^e	62.2 ± 0.4 ^e

350 ^{a-e} Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$)

351
 352
 353

354 Prepared chitosan samples were also analysed by UV-visible spectroscopy (data not
 355 shown). Cinnamaldehyde in ethanol presented the three characteristic absorption bands
 356 at 195, 221 and 292 nm. CScin samples before and after the diverse treatments
 357 presented large absorbances in these wavelenght but also presented a band between
 358 415 and 485 nm, feature adscribed to the formation of the Schiff base and the
 359 conjugated dienes .

360

361 3.1.3. Film swelling

362 Chitosan is a hydrogel with a high capacity for incorporating water into its matrix. In
363 order to study the effect of cinnamaldehyde on the water sorption capacity, the films
364 were immersed into two buffered media at pH 3 and 7. The results after 24 hours are
365 shown in Table 3.

366 At pH 3, the sorption of water in all chitosan-base materials was very high due to
367 hydrophilicity of chitosan biopolymer when the amino groups are protonated, as occurs
368 at pH below the pKa of chitosan (pKa = 6.3). This ionization produces electrostatic
369 repulsion between polymer segments allowing film swelling and large water gain which
370 subsequently practically doubled the film area after immersion. However, the reaction
371 with cinnamaldehyde reduced the amount of sorbed water and the area increment,
372 effect which is significant in films treated at 65°C or higher temperatures.

373 An explanation for this difference among samples could be that at room (or
374 refrigeration temperatures) the Schiff base could be rapidly reversed at pH 3, while
375 more severe thermal treatments (65 °C, 72 °C and 95 °C) may promote the
376 crosslinking of the polymer, improving matrix structure cohesion, and consequently
377 restricting the swelling capacity of the biopolymeric matrix. This matrix stabilization is
378 much more obvious in the films treated at retorting temperatures, when both weight
379 and surface increments were reduced to half of those of the original chitosan film.

380

381 **Table 3.** Water Sorption of films at pH 3 and pH 7 for 24 hours. Weigth and area increase (%)

Films	pH 3		pH 7	
	weigth increase (%)	area increase (%)	weight increase (%)	area increase (%)
CS	231.67 ± 1.60 ^a	119.90 ± 3.41 ^a	155.83 ± 0.86 ^a	99.80 ± 1.87 ^a
CScin	235.23 ± 6.23 ^a	116.68 ± 5.34 ^a	36.98 ± 0.96 ^b	15.40 ± 1.57 ^b
4 °C, 30 min	237.07 ± 6.12 ^a	110.68 ± 5.89 ^a	29.07 ± 0.37 ^b	15.80 ± 1.01 ^b
65 °C, 30 min	152.76 ± 13.62 ^b	88.27 ± 10.90 ^b	30.04 ± 2.21 ^b	17.78 ± 3.62 ^b
72 °C, 15 min	176.56 ± 10.25 ^b	81.27 ± 14.25 ^b	31.37 ± 2.51 ^b	17.74 ± 4.10 ^b
95 °C, 10 min	167.28 ± 13.55 ^b	78.27 ± 10.36 ^b	31.33 ± 1.46 ^b	17.72 ± 3.10 ^b
121 °C, 5 min	91.47 ± 14.23 ^c	52.41 ± 6.68 ^c	34.07 ± 1.57 ^b	17.89 ± 4.61 ^b

382 ^{a-c} Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$)

383

384 In contrast, at pH 7, the CS samples presented much lower values of water sorption
385 than in acidic conditions. This decrease in the swelling of the matrix is due to the

386 unprotonated state of the amine groups ($\text{pH} > \text{pKa}$ of chitosan). After the reaction with
 387 cinnamaldehyde, the water uptake and the swelling effect largely decreased in all
 388 samples. No significant differences were observed between samples submitted to the
 389 diverse thermal treatments. At this pH, the Schiff base is not significantly reversed
 390 maintaining the cohesion of the matrix and thus, reducing water sorption and swelling.

391 This study was also conducted at 48 hours with relevant results. At pH 3, the integrity
 392 of film was loss and samples could not be handled nor measured. On the contrary, no
 393 significant differences were observed at pH 7 between 24 and 48 h (data not shown).

394

395 3.1.4. Contact angle (CA)

396 The surface properties of the films were determined by contact angle analysis. The
 397 contact angle or angle humectancy is defined as the angle between the surface of a
 398 liquid (in this work, water) and the tangent line at the point of contact with the substrate.
 399 The value of the contact angle depends mainly on the relationship between the
 400 adhesive forces between the liquid and the solid and the liquid cohesive forces.
 401 Chitosan matrix was modified with a hydrophobic molecule such as cinnamaldehyde,
 402 and, therefore, higher contact angles and lower wettability could be anticipated. As
 403 Table 4 shows, the contact angle values for samples reacted with cinnamaldehyde
 404 presented slightly higher values, from 78° to 82° for CScin and samples treated at low
 405 to medium temperature. Films treated at 95 and 121 °C presented values closer to
 406 those of CS film probably because as the temperature of the treatment increases, more
 407 cinnamaldehyde is released, especially near the film surface. Nevertheless, contact
 408 angle differences were not statistically significant.

409

410 **Table 4.** Contact angle of the films in water before / after chemical modification and before /
 411 after preservation treatment

Samples	CA(M)[°]
CS	78.03 ± 2.01^a
CScin	81.17 ± 2.12^a
4 °C, 30 min	82.63 ± 2.56^a
65 °C, 30 min	82.73 ± 0.59^a
72 °C, 15 min	81.06 ± 0.89^a
95 °C, 10 min	79.54 ± 0.78^a
121°C, 5 min	78.18 ± 2.54^a

412 ^a Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$)

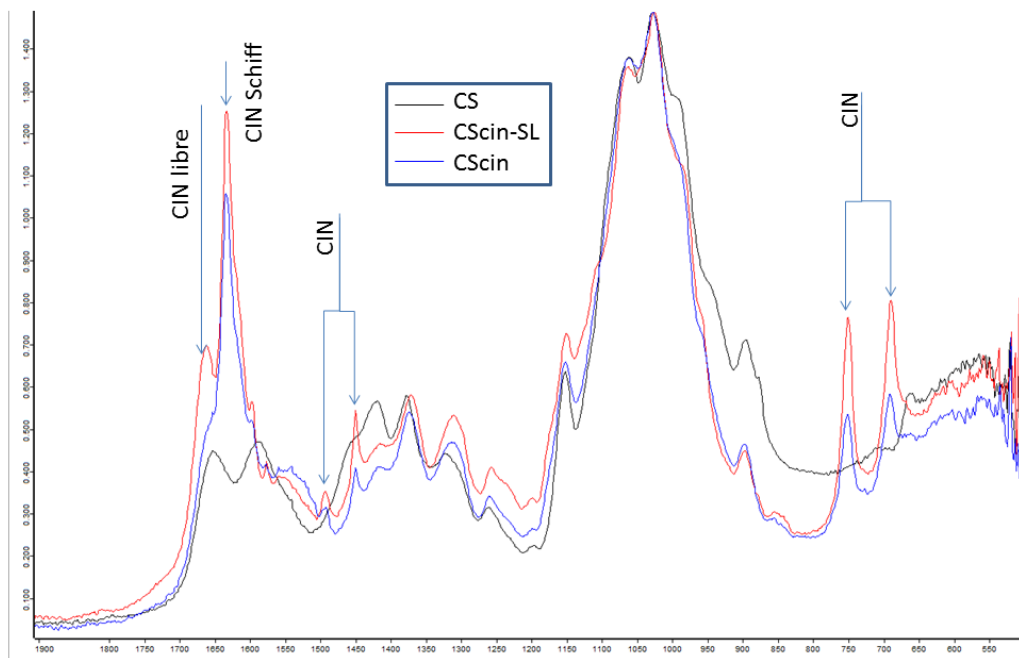
413

414 Two factors can be responsible for the discrepancy between contact angle and swelling
415 results. The first one is the type of test. Swelling provides information on the effect on
416 samples after long-time exposure to water, that is, polymer relaxation due to water
417 intake occurs within the time of experiment. On the contrary, contact angle provides
418 information on the films surface and at short time (1 min). In the case of the chitosan
419 materials developed, which are hydrophilic and absorbent, when the liquid penetrates
420 into the substrate, the polymer changes and so does the contact angle. Some authors
421 document measured contact angles after hours of the droplet exposure, because in
422 some cases even after a long time, the equilibrium has not been reached.
423 Nevertheless, immediate contact angle is required in food packaging to understand
424 properties such as antifogging, or printability.
425 The second factor, is that in swelling tests, the morphology and chemistry of the
426 polymer matrix, that is the core of the film, is the main responsible for swelling
427 characteristics, whilst contact angle tests exclusively evaluates the surface, film skin.
428 Since aldehyde treatment is a matrix crosslinking process, the most relevant effect
429 should be anticipated on the matrix core, especially, when films are submitted to three
430 washing steps and free agent in the surface was fully eliminated.

431

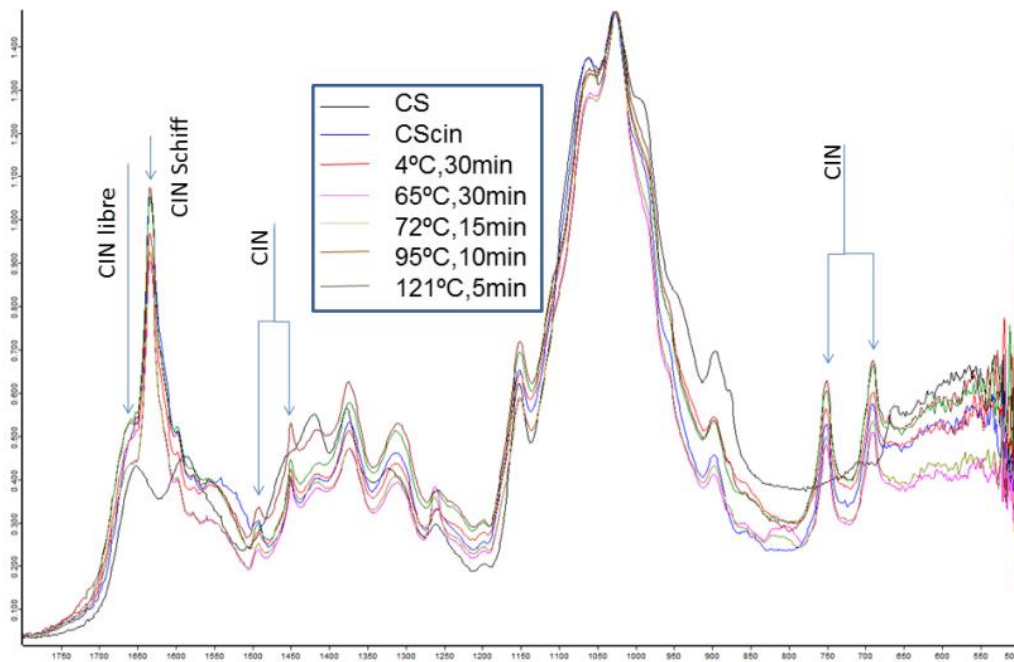
432 3.1.5. FTIR-ATR

433 FTIR-ATR spectra were recorded from the diverse films obtained in this work. Figure 1
434 shows the FTIR-ATR spectra of a sample of CS and CScin before and after the
435 washing procedure. The spectra have been maximized respect to the peak of chitosan
436 at 1025 cm^{-1} . Compared to CS spectra, the CScin films spectra presented distinctive
437 features at 690 , 751 , 1451 and 1492 cm^{-1} , which correspond to the phenolic group of
438 cinnamaldehyde. The 1660 cm^{-1} peak corresponding to the stretching of the C=O
439 bondaldehyde group is present in the unwashed sample. However, this band appears
440 as a shoulder in the washed sample, indicating that the free cinnamaldehyde is
441 practically eliminated after washing. In both, washed and unwashed CScin films, a
442 strong band at 1633 cm^{-1} can be observed which is assigned to the stretching of the
443 imine group (C = N) of the Schiff base. Also, a part of the cinamaldehyde bonded to
444 the chitosan is also released, probably because of a partial reversion of the Schiff-base
445 reaction.



446

447 **Fig. 1.** FTIR-ATR spectra of chitosan (CS) and chitosan modified with cinnamaldehyde before
 448 (CScin-SL) and after washing (CScin) with ethanol.
 449



450

451 **Fig. 2.** FTIR-ATR spectra of CS and CScin films after the different preservation treatments.

452 After washing, films were exposed to different thermal treatments. Figures 2 compares
453 the FTIR-ATR spectra for the different samples, including that of pure CS, using the
454 1025 cm^{-1} band as reference. During treatments, there is a partial release of
455 cinnamaldehyde because of the reversibility of the reaction, Nevertheless, there is a
456 large percentage of cinnamaldehyde still anchored to the chitosan matrix even after the
457 more severe treatment (121 °C, 5 min).

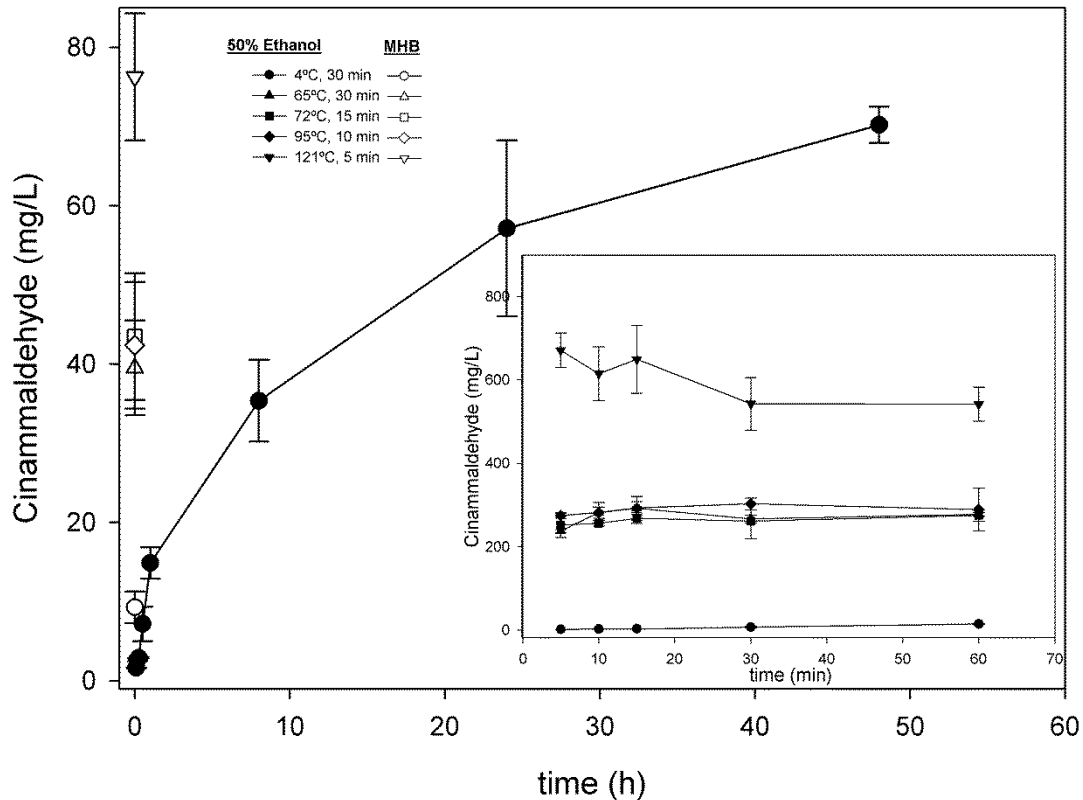
458

459 3.1.6. Release of cinnamaldehyde

460 Two experiments were made to evaluate the cinnamaldehyde released by films
461 exposed to the different treatments: a) release to MHB during the treatment, and b)
462 release to 50% ethanol after 1h of the treatment. In this latter, the results could indicate
463 whether agent release continued after the thermal process. The results of both
464 experiments are presented together in figure 3.

465 In the first test, films were immersed in liquid culture medium Mueller Hinton (MHB) and
466 subjected to preservation treatment. Immediately after, the films were removed from
467 the liquid and analysed by UV-vis spectroscopy. 5 cinnamaldehyde solutions in MHB
468 were also analysed for calibration. As can be seen in figure 3 the concentrations of
469 cinnamaldehyde in the broth increased with the treatment temperature applied to the
470 films. At refrigeration temperature, the release was significantly lower than moderated
471 thermal treatments. No differences were observed between sample processed
472 between 65 for 30 min and 95°C 10 for min. The severe retorting process also resulted
473 in a greater release of the agent into the liquid medium.

474



475 **Figure 3. Cinnamaldehyde concentration** released from films into MHB during thermal
 476 treatments (white symbols) and time evolution of cinnamaldehyde released into ethanol 50% at
 477 23°C after the thermal processes.
 478

479

480 The second experiment was carried out in fatty food simulant (ethanol 50 %, which
 481 simulates a food emulsion). After heat treatment, the samples were stored at room
 482 temperature and liquid aliquots were extracted at several times during one hour (48
 483 hours for the refrigerated sample) after heat treatment. As figure 3 reveals, the
 484 concentration changes with different profiles. The sample processed at low
 485 temperature, presents after the treatment a very low release (ca. 1 mg/L) but the
 486 amount release increases with time following a exponential increase to maximum
 487 profile, reaching ca. 70 mg/L after 48 h. . The treatments at 65, 72 and 95°C yielded
 488 much higher values of release, 280 mg/L, without differences between treatments.
 489 Also, it should be noticed that the cinnamaldehyde released does not change
 490 significantly with time during the 1-hour storage, indicating that probably, all the free
 491 cinnamaldehyde present in the matrix due to the reversion of the Schiff reaction has
 492 been release during the treatments. The films submitted to the sterilization treatment
 493 released the highest concentration of cinnamaldehyde, with values ca. 700 mg/L after
 494 treatment. This high concentration is consistent with the increased effect of heat

495 treatment on the reversion of the Schiff base. However, the concentration of the agent
496 presented a decreasing trend during storage. Since the measured concentration
497 indicates the cinnamaldehyde molecules that already had moved out of the film, a
498 rebuilt of the Schiff base is certainly unexpected. Most probably, the decrease in
499 concentration was due to the condensation of the volatile in the walls and septum of
500 the vial and even cinnamaldehyde sorption in the film caused by a change in the
501 partition equilibrium constant of cinnamaldehyde with temperature.

502 Another relevant feature is the large difference in released agent observed between the
503 two liquids. In our opinion, MHB is an aqueous media which causes film swelling and
504 therefore increases the diffusion rate of any substance through the matrix. This effect
505 explains the higher concentration of agent observed in MHB at 4°C. On the contrary,
506 after the hot treatments (65°C and above), the release was greater into the ethanolic
507 media. This might be caused by the higher solubility of cinnamaldehyde in this
508 simulant.

509 The release results show that the films were activated by temperatures ≥ 65 °C
510 reaching high concentrations of cinnamaldehyde in the medium. Films store at
511 refrigeration produce a sustained release over time. The data obtained indicate that the
512 developed films can be used as reservoir able to release sustained cinnamaldehyde
513 over time and as coadjuvant of preservation treatments.

514 515 **3.2 Antimicrobial activity of the CScin films**

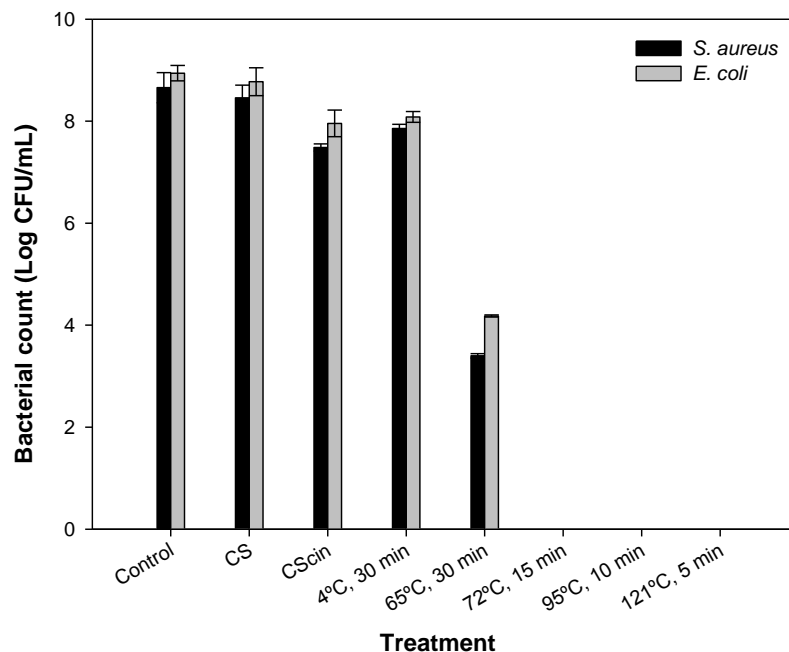
516 517 **3.2.1. In vitro study**

518
519 The antimicrobial activity of the developed films was studied against a Gram positive
520 bacteria, *Staphylococcus aureus*, and a gram negative bacteria, *Escherichia coli*. First,
521 the *in vitro* effectiveness of the films exposed to various preservation treatments in
522 MHB liquid medium was determined.

523 Figure 4 shows the effectiveness of CScin before and after preservation treatments.
524 Chitosan is a known antimicrobial agent, amino groups charged positively interact with
525 negatively charged membrane of bacteria, altering the permeability and disrupting the
526 DNA replication (Coma, et al., 2002; Zivanovic, Chi, & Draughon, 2005). However, the
527 results showed that the prepared chitosan film did not present a relevant antimicrobial
528 activity, as could be expected since the chitosan films were neutralized and,
529 subsequently, the amino groups were not protonated (Arachchi, Shahidi, & Jeon, 1999;
530 Foster & Butt, 2011).

531 All CScin films subjected to the different preservation treatments showed antimicrobial
532 activity against both tested microorganisms. Films were more effective against Gram
533 positive bacteria than Gram negative bacteria, as was anticipated since Gram negative
534 bacteria have an outer membrane that generally provides more resistant to damage
535 caused by chemical agents.

536 CScin and CScin films subjected to a storage temperature of 4 °C for 30 min showed a
537 reduced antimicrobial activity (1 log reduction). These results are in agreement with the
538 release study showed in the previous section (figure 2). Films not activated by
539 temperature released cinnamaldehyde very slowly due to the slow reversibility of the
540 Schiff base at low temperatures. CScin films after treatment at 65 °C for 30 min
541 showed a great log reduction, 5.66 ± 0.04 against *S. aureus*, and 4.76 ± 0.02 against
542 *E. coli*. Finally, it was observed that the films treated at 72 °C for 15 min, 95 °C for 10
543 min and 121 °C for 5 min produced bactericidal effect. Therefore, the antimicrobial
544 activity is related to the active agent released during the different treatments. The films
545 subjected to higher temperatures released more active agent, and so did their
546 antimicrobial capacity.



547

548 **Fig. 4** Antimicrobial effect of chitosan film modified with cinnamaldehyde and subjected
549 to different preservation treatments against *S. aureus* and *E. coli*

550 In conclusion, the results of the antimicrobial study show that the developed films can
551 be very effective when submitted to a thermal treatment. At low temperatures, the films
552 developed presented a extended stability with very slow agent loss (or release). At mild
553 and sustained heat treatments (hot filling or mild pasteurization), the release is high

554 enough as to largely inhibit microbial growth. More severe heat treatments for short
555 times are much more effective, films even providing a bactericidal effect.

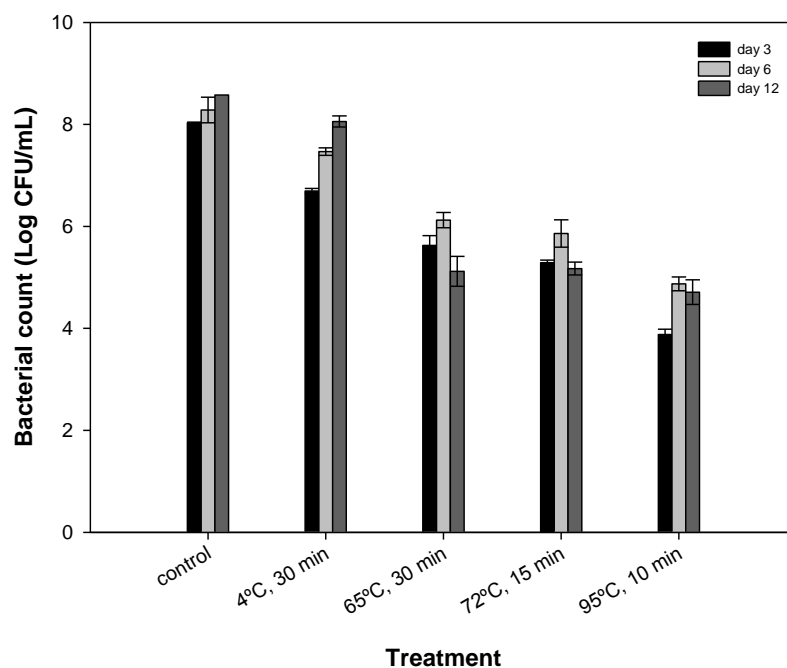
556

557 3.2.2. Study of the antimicrobial capacity of CScin films applied to inoculated food

558 Once the *in vitro* effectiveness of the developed films was verified, their antimicrobial
559 activity was examined on refrigerated and pasteurized whole milk having a high fat
560 content (3.6 %). CScin films were immersed in milk and subjected to different
561 preservation treatments and then milk was inoculated with *L. monocytogenes*, a
562 microorganism able to grow at low temperatures (Doyle MP, 2007). Previous studies
563 on the thermal behaviour of *L. monocytogenes* in foods showed that, the mean
564 minimum growth temperature was 1.1 °C (Junttila, Niemela, & Hirn, 1988) and that this
565 microorganism can survive pasteurization (Fleming, et al., 1985; Lovett, Francis, &
566 Hunt, 1987). The characteristics of refrigerated milk (pH close to neutrality, large
567 presence of nutrients) could also have favored the increase of the viable counts of *L.*
568 *monocytogenes* (Muriel Galet, et al., 2012).

569 As Fig. 5 shows, the more severe the temperature treatment was, the greater was the
570 reduction of bacterial growth, in good correlation with the data obtained *in vitro* assays
571 and in release tests. CScin films treated at 4 °C for 30 min yielded a log reduction of
572 1.34 at 3 days, 0.81 at 6 days and 0,52 at 12 days. Activation of the films by higher
573 temperatures resulted in more efficient antimicrobial activity. Thus, CScin films treated
574 at 95 ° C for 10 min showed a log reduction of 4.15 ± 0.02 at 3 days, 3.41 ± 0.02 at 6
575 days and 3.87 ± 0.07 after 12 days.

576 It was not possible to inoculate the samples treated at 121 °C for 5 min because milk
577 was coagulated after treatment. There are two possible reasons for this effect. It is
578 documented that certain aromatic compounds, such as cinnamaldehyde, may cause
579 conformational changes in proteins by binding (Damodaran & Kinsella, 1980; Kuhn,
580 Considine, & Singh, 2006). These changes in the structure protein and temperature
581 can produce a denaturation which involves the deployment and aggregation forming a
582 gel. Besides, this treatment can produce hydrolysed chitosan release which causes
583 milk coagulation due to coagulation and flocculation properties of chitosan (Renault,
584 Sancey, & Crini, 2009).



585

586 **Fig. 5.** Antimicrobial effect of chitosan film modified with cinnamaldehyde and subjected to
 587 different preservation treatments against *Listeria monocytogenes* in pasteurized milk

588 The lower antimicrobial activity of the films observed on milk (Fig. 5) compared to that
 589 in MHB medium (Fig. 4) can be explained because in the latter the experiment is more
 590 controlled and the use of the optimal culture medium for the microorganism magnifies
 591 any effect. However, milk is a complex food matrix which may interfere with the
 592 antimicrobial agent requiring higher concentrations to achieve the same effect
 593 (Gutierrez, Barry-Ryan, & Bourke, 2008). Similar differences between in vivo and in
 594 vitro antimicrobial activity of agents and active films have been reported previously
 595 (Belletti, et al., 2008; Burt, 2004; Muriel Galet, et al., 2012).

596 *L. monocytogenes* is an important pathogen microorganism involved in cases of
 597 septicemia and meningitis, especially in children, the elderly and immunosuppressed
 598 population by drugs or diseases. However, there are also cases of listeriosis in
 599 apparently healthy children and adults. In pregnant women can cause abortions or
 600 premature death of the fetus. Therefore, the developed films could improve safety for
 601 products susceptible to contamination with microorganisms such as *Listeria* and could
 602 also extend commercialization period, important advantage for a product with a shelf
 603 life of only three days under refrigeration.

604

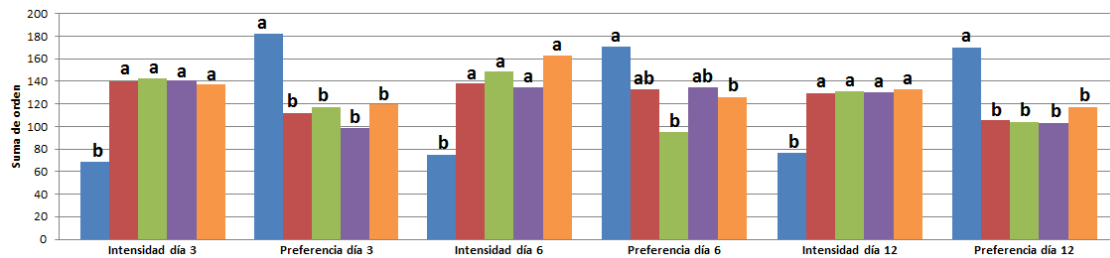
605 3.3 Sensory analysis

606 The use of essential oils in food may have a significant sensory impact that could result
 607 in non-acceptance by the consumer. For this reason, a sensory analysis was carried
 608 out by a panel of judges with the aim of determining whether the content of active
 609 component migrated to pasteurized milk modifies its aroma appreciably and if so
 610 determine whether this odor is accepted by consumers. Samples prepared for sensory
 611 analysis simulate a commercial product packaging that is in contact with the modified
 612 CScin and subjected to different heat treatments before marketing. The tests were
 613 conducted on 3rd, 6th and 12th days of refrigerated storage at 4 ° C. The samples were
 614 evaluated by a minimum of 40 random non-expert judges.

615 Friedman analysis indicated significant differences in the intensity of cinnamon odor
 616 perceived and acceptability in the three tests, since in all cases the value of F exceed
 617 the threshold level of significance of $p < 0.001$.

618
 619
 620

After applying the Tukey test gives the following results:



621

622 **Fig. 6.** Evaluación sensorial de muestras de leche sin película (azul) y en contacto con
 623 películas de quitosano modificado con cinamaldehído y tratamiento inicial de 4 °C 30
 624 min (rojo), 65 °C 30 min (verde), 72 °C 15 min (violeta) y 95 °C 10 min (naranja)

625 The ordering of the samples according to intensity of cinnamon odor after 3 days
 626 of storage showed no significant differences between the CScin samples but
 627 differences respect to the control sample (Fig.6). The same results were obtained in
 628 the tasting on 6th and 12th day.

629 Samples with cinnamaldehyde are preferred compared to the control without any
 630 significant differences between them after 3 and 12 days of storage. Sensory results
 631 obtained indicated that only the control sample was significantly different from the
 632 samples containing cinnamaldehyde, these being similar. Sensory analysis showed
 633 that panelists perceived the presence of the agent in the milk samples exposed to
 634 CScin films. Nevertheless, the panelists preferred the milk in contact with CScin films at
 635 the three tested periods,. Fresh pasteurized milk is a product whose shelf life is very
 636 short, 2-3 days once opened. The use of the developed films not only may increase the

637 safety of such products and subsequently lengthen the the shelf life due to the
638 antimicrobial capacity but additionally provides a flavour of a high acceptance by
639 consumer.

640

641 **4. Conclusion**

642 In this work, chitosan has been selected as a vehicle matrix for the controlled delivery
643 of cinnamaldehyde, a known antimicrobial agent, with the purpose of developing active
644 biodegradable packaging materials to improve food safety. Cinnamaldehyde was
645 succesfully anchored to the matrix by nucleophilic reaction on the amine chitosan
646 groups with the formation of a Schiff base, with a high degree of substitution, ca. 70 %.
647 This process additionally produces a matrix crosslinking which increases its water
648 stability.

649 The reversibility and the effect of temperature on Schiff-base reaction was used to
650 release the agent in food or food simulants. When the obtained films were processed in
651 simulated food processing conditions, the release of cinnamaldehyde increased with
652 the severity of the treatment. As a consequence of agent release, the films presented
653 antimicrobial effect against pathogen bacteria (*S. aureus* and *E. coli*) in *in vitro* assays
654 and inhibited the growth of *L. monocytogenes* in inoculated pasteurized milk, effect
655 which was enhanced by the thermal treatments. Although the release of the agent
656 caused a perceptible cinnamon aroma in milk, a panel of untrained considered this
657 effect as positive, being treated milk preferred over the control sample. The controlled
658 release of cinnamadehyde from the developed matrices could be optimized to maintain
659 an improved safety after package opening,

660

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666

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