Gavara Clemente, R.; Higueras-Contreras, L.; Lopez-Carballo, G.; Hernández-Muñoz, P. (2015). Reversible Covalent Immobilization of Cinnamaldehyde on Chitosan Films via Schiff Base Formation and Their Application in Active Food Packaging. Food and Bioprocess Technology. 8(3):526-538. doi:10.1007/s11947-014-1421-8.

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

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Abstract

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

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

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

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

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

Keywords

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1 Materials

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

2.2 Film preparation

2.2.1. Chitosan film preparation

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

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

2.2.2. Chitosan films reacted with cinnamadehyde

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

2.2.3. Treatments applied to films

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

2.3 Characterization of chitosan films modified with cinnamaldehyde

2.3.1. Elemental analysis

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

2.3.2. Optical properties

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

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

2.3.3. Swelling

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

2.3.4. Contact angle

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

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

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

2.3.6. Release studies

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

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

2.4 Antimicrobial activity

2.4.1. Culture strains

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

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

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

2.4.3. Antimicrobial assays in milk

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

2.5 Sensory analysis

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

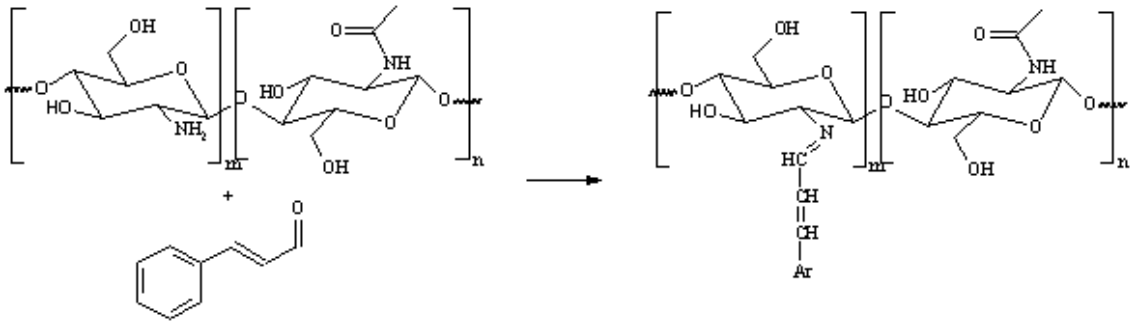
preference test samples are ordered from 1 to 5, 1 to assign the sample to greater acceptance and 5 the lowest. Data analysis was performed with the program Compusense ® five release 5.0 (Compusense Inc., Guelph, Ontario, Canada).

2.6 Data analyses

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

3. RESULTS AND DISCUSSION

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



Scheme 1. Nucleophilic reaction between chitosan and cinnamaldehyde

3.1 Characterization of chitosan films modified with cinnamaldehyde

3.1.1. Elemental analysis

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

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

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

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 ºC, 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 ºC, 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 ºC, 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 ºC, 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 ºC, 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]

[a-b] Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$)

[1]Oxygen percentage values calculated by mass balance

[2] Theoretical values

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

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

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

where (C / N) is the ratio of carbon and nitrogen of the chitosan derivative, $(C / N)_0$ is the ratio of carbon and nitrogen of chitosan, and n is the number of carbon introduced into the modified chitosan. The DS values, also included in Table 1, indicated an excellent reaction efficiency. Cinnamaldehyde molecules had been reacted with more than 70 % of the amine groups present in the matrix of chitosan. After the treatments, the DS values presented a slight reduction, which is due to the reversibility of the Schiff base reaction. This reduction increases with the increase of the treatment temperature, being significant for the treatments at 95 and 121 °C. It is noteworthy that even after a retorting-like process, more than 50 % of reacted cinamaldehyde remained in the film, implying a large reservoir of cinnamaldehyde.

3.1.2. Optical properties

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

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

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

L* value decreased with the treatments. CS were practically transparent, while CScin presented a significant loss of transparency (low L*). This decrement is more relevant with the thermal treatment probably due to elevated water sorption in the matrix during processing that after cooling is not completely removed, creating small water droplets within the matrix that diffracts the light. This observation has been reflected in studies with hydrophilic matrices (Aucejo, Catala, & Gavara, 2000). A decrease in L* parameter is also observable when pure CS film is heated, reducing the brightness and increasing the yellowish tone of the samples. Retorted CS film presented the following colour parameters: L* = 88.6 ± 0.8, a* = 2.38 ± 0, 1, b* = 23.65 ± 1.2; C_{ab}* = 23.7 ± 1.1; h_{ab} = 84.2 ± 0.4 (not included in Table 2).

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 ± 01^{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 ºC, 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 ºC,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 ºC, 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 ºC, 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 ºC, 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}

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

Prepared chitosan samples were also analysed by UV-visible spectroscopy (data not shown). Cinnamaldehyde in ethanol presented the three chracteristic absorption bands at 195, 221 and 292 nm. CScin samples before and after the diverse treatments presented large absorbances in these wavelenght but also presented a band between 415 and 485 nm, feature adscribed to the formation of the Schiff base and the congugated 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 65ºC 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 ºC, 30 min	237.07 ± 6.12 [a]	110.68 ± 5.89 [a]	29.07 ± 0.37 [b]	15.80 ± 1.01 [b]
65 ºC, 30 min	152.76 ± 13.62 [b]	88.27 ± 10.90 [b]	30.04 ± 2.21 [b]	17.78 ± 3.62 [b]
72 ºC, 15 min	176.56 ± 10.25 [b]	81.27 ± 14.25 [b]	31.37 ± 2.51 [b]	17.74 ± 4.10 [b]
95 ºC, 10 min	167.28 ± 13.55 [b]	78.27 ± 10.36 [b]	31.33 ± 1.46 [b]	17.72 ± 3.10 [b]
121 ºC, 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

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

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

3.1.4. Contact angle (CA)

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

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

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

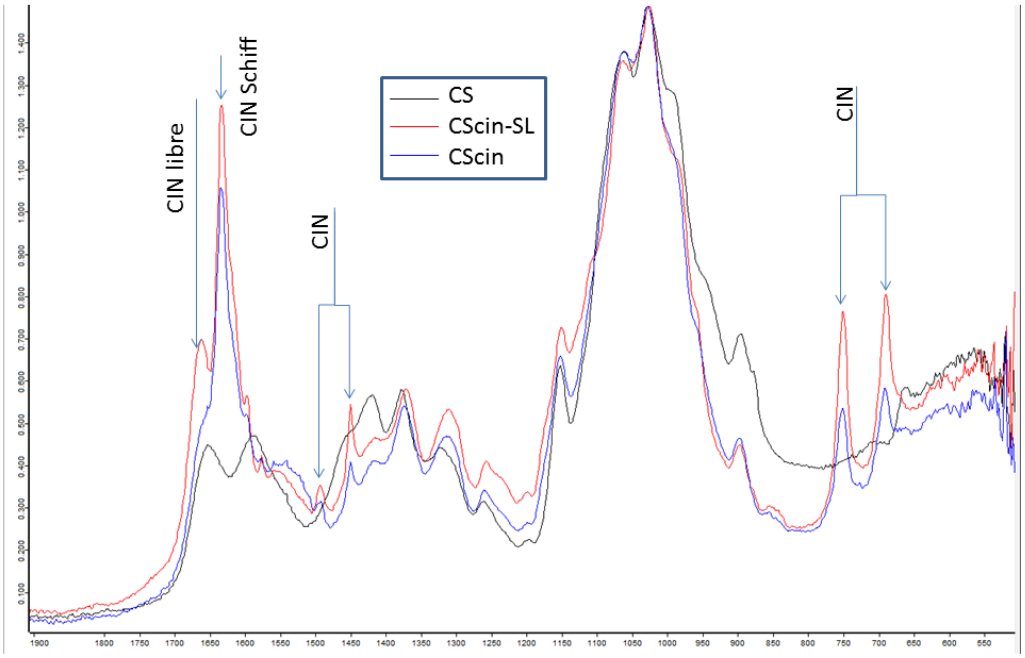
[a] Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$)

Two factors can be responsible for the discrepancy between contact angle and swelling results. The first one is the type of test. Swelling provides information on the effect on samples after long-time exposure to water, that is, polymer relaxation due to water intake occurs within the time of experiment. On the contrary, contact angle provides information on the films surface and at short time (1 min). In the case of the chitosan materials developed, which are hydrophilic and absorbent, when the liquid penetrates into the substrate, the polymer changes and so does the contact angle. Some authors document measured contact angles after hours of the droplet exposure, because in some cases even after a long time, the equilibrium has not been reached. Nevertheless, immediate contact angle is required in food packaging to understand properties such as antifogging, or printability.

The second factor, is that in swelling tests, the morphology and chemistry of the polymer matrix, that is the core of the film, is the main responsible for swelling characteristics, whilst contact angle tests exclusively evaluates the surface, film skin. Since aldehyde treatment is a matrix crosslinking process, the most relevant effect should be anticipated on the matrix core, especially, when films are submitted to three washing steps and free agent in the surface was fully eliminated.

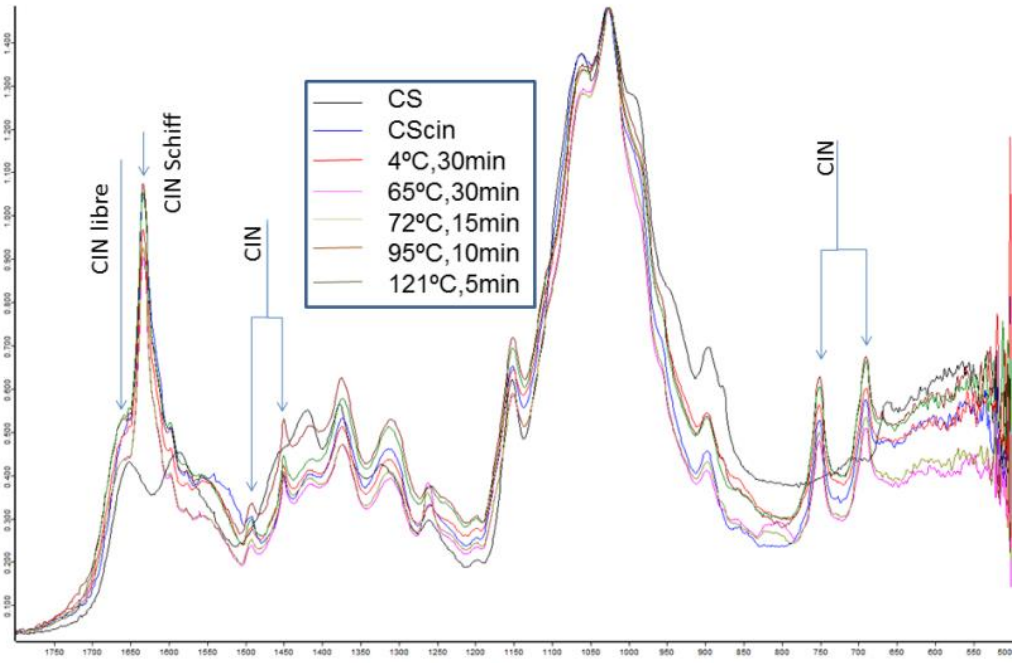
3.1.5. FTIR-ATR

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

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

3.1.6. Release of cinnamaldehyde

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

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

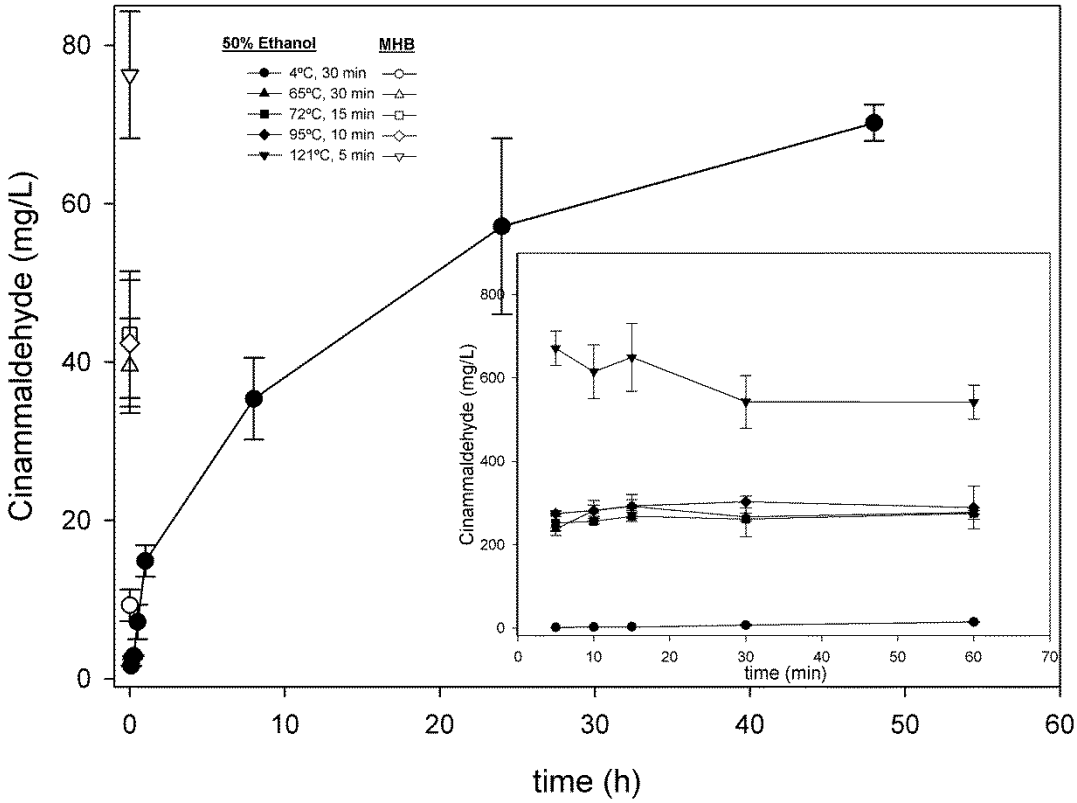


Figure 3. Cinnamaldehyde **concentration** released from films into MHB during thermal treatments (white symbols) and time evolution of cinamaldehyde released into ethanol 50% at 23ºC after the thermal processes.

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

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

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

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

3.2 Antimicrobial activity of the CScin films

3.2.1. In vitro study

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

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

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

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

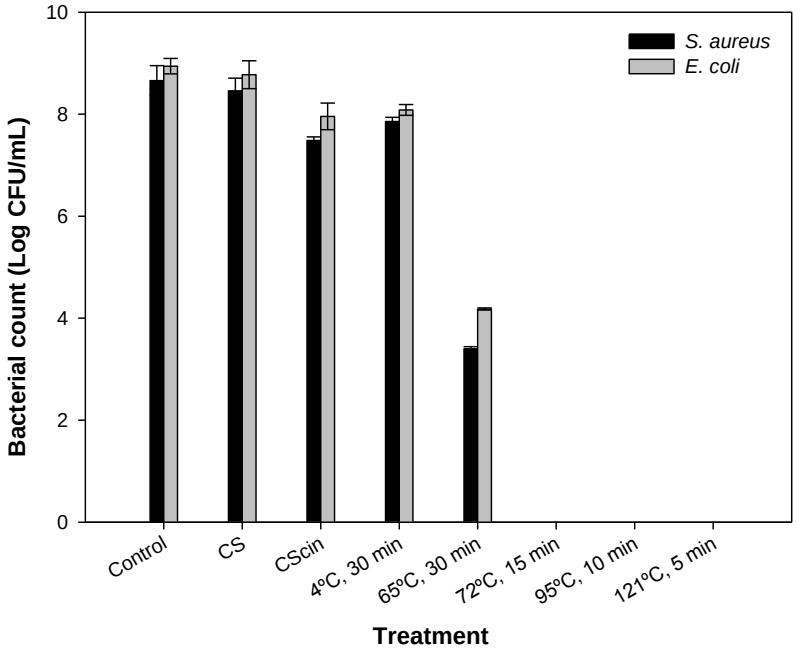


Fig. 4 Antimicrobial effect of chitosan film modified with cinnamaldehyde and subjected to different preservation treatments against *S. aureus* and *E. coli*

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

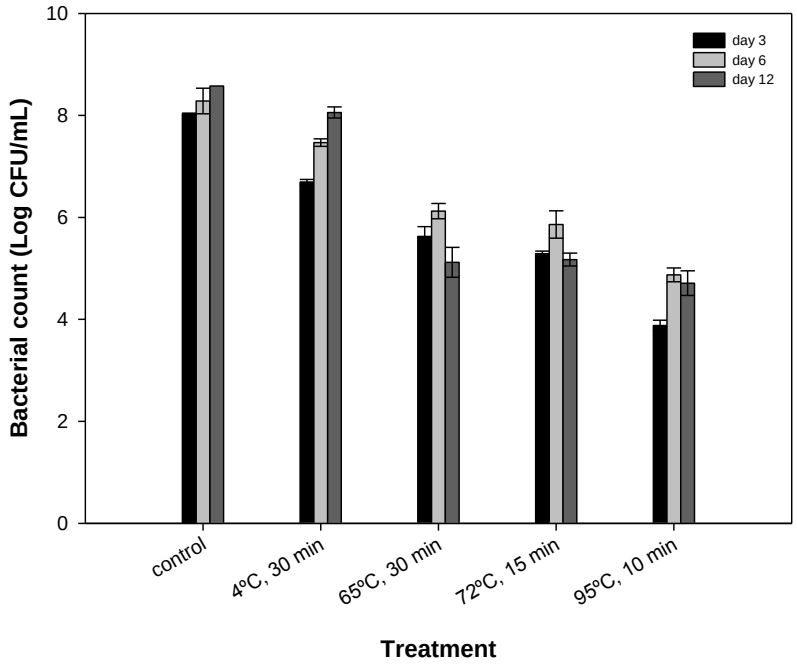
554 enough as to largely inhibit microbial growth. More severe heat treatments for short
555 times are much more effective, films even providing a bactericidal effect.

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 differents
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 ºC (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 ºC 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 poblation 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 couls
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**

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

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

After applying the Tukey test gives the following results:

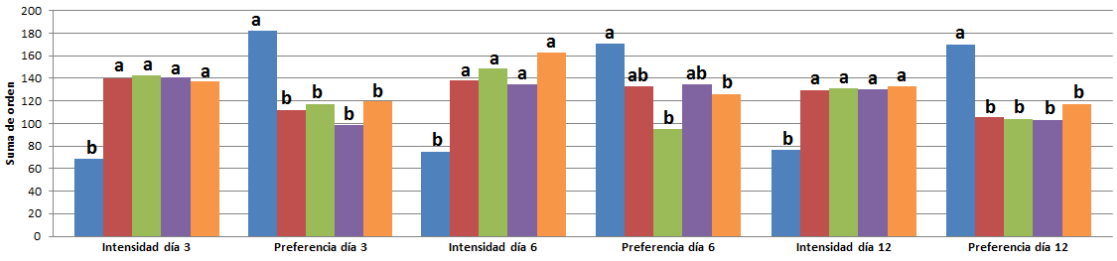


Fig. 6. Evaluación sensorial de muestras de leche sin película (azul) y en contacto con películas de quitosano modificado con cinamaldehído y tratamiento inicial de 4 ºC 30 min (rojo), 65 ºC 30 min (verde), 72 ºC 15 min (violeta) y 95 ºC 10 min (naranja)

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

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

safety of such products and subsequeltly lengthen the the shelf life due to the antimicrobial capacity but additionaly provides a flavour of a high acceptance by consumer.

4. Conclusion

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

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

Acknowledgments

The authors wish to thank the financial support provided by the Spanish Ministry of Science and Innovation (projects AGL2009-08776, AGL2012-39920-C03-01) and CSIC (JAE-Predoc L.H. fellowship), as well as the English correction performed by Mr. Tim Swillens.

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