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Optimization of the Film-Forming and Storage Conditions of Chitosan as an Antimicrobial Agent

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The aim of this work was to assess the antimicrobial capacity of chitosan-based films obtained by a dissolution and solvent evaporation (solvent casting) method at various temperatures (i.e., 37, 80, and 120 °C) on the growth of *Staphylococcus aureus* and *Salmonella* spp. bacteria. The effect of temperature (4, 23, 37 °C) and relative humidity (RH; 0, 75%) during storage on the biocide performance was also investigated. Color parameters and ATR-FTIR spectra were analyzed for each sample to investigate the relationship between structural and/or chemical alterations in the films during storage and biocide performance. The results indicated that films formed at 37 and 80 °C presented a significant inhibitory effect for both types of bacteria; however, when cast at 120 °C, the films ceased to exhibit antimicrobial properties. Curiously, chitosonium acetate films were seen to diminish to a large extent their biocide properties when stored at 23 °C and 75% RH for 2 months or alternatively when stored and 37 °C and 0% RH over the same period of time. In good agreement with this behavior the FTIR results indicated that under the previous conditions a significant fraction of the biocide carboxylate chemistry remained in the polymer after contact with the bacterial solution due to a strong reduction in cast film solubility. Because biopolymer active species migration from the film to the culture media is needed for the biomaterial to exhibit measurable antimicrobial effect, proper control of temperature and humidity during film formation and storage is necessary to design the optimum performance of chitosan as a biocide.

KEYWORDS: Chitosan; active packaging; coatings; antimicrobial properties; ATR-FTIR spectroscopy; color

INTRODUCTION

Current trends in consumer preferences toward mildly preserved, fresh, and healthy foodstuffs have triggered innovation in food, food coating, and food packaging applications, and a considerable amount of research work has been conducted toward the design of biocide edible systems with improved quality and safety. As a result, in recent years much attention has been paid to the development of antimicrobial active systems by means of the incorporation of antimicrobial substances into foods and on food or packaging coatings. Antimicrobial additives to foods or to food packaging systems can greatly benefit if selected from biobased resources due to, among other factors, edibility and low environmental toxicity. Thus, the use of bacterial starter cultures, biopreservatives, and plant or animal extracts as antimicrobial hurdles are perceived as a low risk to consumers.

Chitosan [β-(1,4)-2-amino-2-deoxy-D-glucose] is a biodegradable, biocompatible, nontoxic aminopolysaccharide obtained by deacetylation of chitin. This biopolymer shows excellent film- and coating-forming properties when cast from organic acidic water solutions (*1*). Moreover, it presents an inherent antimicrobial character against the growth of pathogen and spoilage bacteria that has already been widely demonstrated in a general range of foods such as bread, strawberries, juices, mayonnaise, milk, and rice cake (*2*−*7*). Nevertheless, the use of chitosan as an anti-infective biomaterial is also of relevance in other application areas such as in the biomedical and pharmaceutical fields (*8*−*10*). Although the exact mechanism by which chitosan exerts its antimicrobial activity is currently unknown, its polycationic nature seems to be a crucial factor. Thus, it has been proposed that the positively charged amino groups of the glucosamine units interact with negatively charged components in microbial cell membranes, altering their barrier properties (*11, 12*). Furthermore, it is worth mentioning that this compound presents better antimicrobial effects against Gram-positive bacteria than against Gram-negative bacteria (*13*−*16*). In relation to the mode of action of chitosan matrices, a direct relationship between their antimicrobial capacity and the

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release of glucosamine chains from the film to the medium has recently been demonstrated (*17*).

From an application viewpoint, the effectiveness of an antimicrobial agent (for instance, chitosan) when applied in or coated over foods or packaging materials may deteriorate during film formation, distribution, and storage. Hence, the chemical stability of an incorporated substance is likely to be affected by the forming process or the storage conditions until its use. Consequently, to obtain highly efficient biocides, all of these parameters should be assessed and optimized for the application. The properties of chitosan films obtained under specific formation and storage conditions in terms of mechanical and barrier properties have been investigated to some extent. Thus, it has been demonstrated that water vapor permeability and elongation at break of low molecular weight chitosan films decreased with storage time at environment conditions [23 °C and 50% relative humidity (RH)] (*18*). Also, it has been shown that a thermal treatment (120 °C, 3 h) of chitosan films led to a significant strengthening of the films and reduced their solubility in aqueous media (*19*). This last effect was also observed by other authors after storage of chitosan films at different conditions of temperature and relative humidity (*20, 21*). With regard to film-forming conditions, it has been demonstrated that infrared illumination drying showed to be faster and superior in preserving desirable physical characteristics such as water or oxygen barrier properties than those prepared by oven-drying or room temperature drying (*22*). Moreover, several published works have followed by infrared spectroscopy the chemical changes of chitosan salts during film formation and even at different storage conditions (*19, 21, 23−26*). Nevertheless, the optimal procedure to preserve the full biocide capacity of chitosan-based films with minimum losses of the activated antimicrobial species in the materials has not been fully investigated to date, and further research on this matter is required.

The aim of the present work was to evaluate the impact of certain essential aspects concerning the production and storage of chitosan-based films for their proper use as antimicrobial materials. For this purpose, the biocide properties of films obtained at different temperatures were analyzed and related to color changes and differences in their molecular structure. Finally, a correlation between biocide properties, biopolymer structure, and glucosamine migration to the culture media as a function of treatment was established.

EXPERIMENTAL PROCEDURES

Materials and Film-Forming Conditions. Chitosan polysaccharide with low molecular weight (83.3% degree of deacetylation and viscosity of 115 mPa s at 1% in 1% acetic acid as stated by the manufacturer) was purchased from Sigma-Aldrich (Spain). Chitosan dispersions were prepared in 1% (v/v) acetic acid to a final concentration of 1.5% (w/v) and stirred at 37 °C for approximately 3 h. Chitosonium acetate solution was filtered through polyester cloth to remove residues of insoluble particles and then autoclaved before film formation. Neat chitosonium acetate films of approximately 50 μm thickness were obtained by casting technique on glass Petri dishes at 37, 80, and 120 °C. When the influence of temperature (4, 23, and 37 °C) and relative humidity (0 and 75%) of storage was studied, films of ca. 25 μm thickness were also obtained by casting technique on polystyrene (PS) Petri dishes at 37 °C and were kept under the different conditions for various periods of time (15, 30, and 60 days). The thickness of the films was accurately measured using a micrometer. The mean of five values was considered for the values of this study.

Color Measurements. Color values were determined using a handheld Minolta Chroma meter CR300 (Minolta Camera Co., Ltd., Osaka, Japan) set to D65 illuminant/10° observer. Film specimens were placed on a white standard plate (calibration plate CR-A43; $L = 97.14$, $a = 0.29$, and $b = 1.76$), and the CIELAB color space was used to determine the parameters L^*, a^*, and b^*. L^* value range was from 0 (black) to 100 (white); a^* value range was from −80 (greenness) to 100 (redness); and b^* value range was from −80 (blueness) to 70 (yellowness). Three measurements were taken at random locations on the studied films. The films analyzed in this assay were those obtained at different temperatures (37, 80, and 120 °C) and those stored at the different values of temperature and relative humidity after 60 days of storage. To discuss the effects of storage conditions on the total color changes, ΔE^* was calculated as a global parameter (eq 1) using a just-formed chitosonium acetate film as the reference sample.

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (1)$$

FTIR Spectroscopy Measurements. ATR-FTIR spectra were collected at 24 °C and 40% RH by coupling the ATR accessory GoldenGate of Specac Ltd. (Orpington, U.K.) to Bruker (Rheinstetten, Germany) FTIR Tensor 37 equipment. Time-resolved experiments were collected by averaging five scans at 4 cm^{-1} resolution at predefined time intervals. The just-formed chitosonium acetate films and the samples stored for 60 days were analyzed according to this technique. A conditioning period (0% RH, 23 °C, 2 weeks) to eliminate the influence of water bands was applied when required.

Bacterial Strain and Growth Conditions. *Staphylococcus aureus* CECT 86 and *Salmonella* spp. CECT 554 were obtained from the Spanish Type Culture Collection (Valencia, Spain). These strains were stored in tryptone soy broth (TSB) 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) slants at 4 °C and transferred monthly. A loopful of bacteria was removed to 10 mL of TSB and incubated at 37 °C overnight. A 100 μL aliquot from the overnight culture was again transferred to TSB and grown at 37 °C to the midexponential phase of growth. This culture served as the inoculum for the susceptibility studies, starting with approximately 10^5 colony-forming units (CFU)/mL in the tests tubes. These CFU counts were accurately and reproducibly obtained by inoculation of 0.1 mL of the culture having an absorbance value of 0.2, as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy.

Susceptibility Tests. Biocide properties of the previously obtained films were evaluated by employing the macrodilution method described by the National Committee of Clinical Laboratory Standards (*27*) with some modifications. The tests were carried out by adding 40 and 60 mg of chitosonium acetate film samples for *S. aureus* and *Salmonella*, respectively, into 10 mL of MHB Mueller−Hinton broth (MHB; at pH 6.2; from Conda Laboratories, Madrid, Spain). A bacterial suspension of midlog phase culture of bacteria was inoculated in each test tube, starting with an initial inoculum size of approximately 5×10^5 CFU/mL, and incubated at 37 °C for 24 h. Then, 0.1 mL of each MHB sample was subcultivated on TSA plates. Finally, the plates were read after overnight incubation at 37 °C. The results were compared against a control sample (MHB solution, pH 6.2) without film. Three replicates per condition were analyzed. The antimicrobial effectiveness (AE) of the samples stored at the various conditions was also calculated by the relationship between the final bacterial counts using eq 2:

$$\mathrm{AE}\ (\%) = \frac{\log(\mathrm{CFU/mL})_c - \log(\mathrm{CFU/mL})_y}{\log(\mathrm{CFU/mL})_c - \log(\mathrm{CFU/mL})_x} \times 100 \qquad (2)$$

where c corresponds to the final counts of the control test and x and y correspond to the final counts when a just-formed film and a stored film were tested, in that order.

Effect of the Forming and Storage Conditions on the Solubility of Chitosonium Acetate Films. A standardized spectrophotometric method based on reaction with ninhydrin was used to determine the release and dissolution of chitosan from the film to the MHB medium. The exact methodology for this assay has been thoroughly developed and described in a previous work (*17*). For this assay, film specimens of 40 mg formed at the different temperatures and/or stored for 60

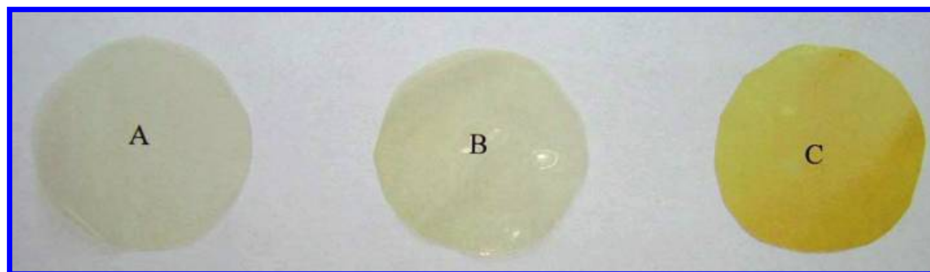

Figure 1. Typical pictures taken on films of ca. 50 μm formed at different casting temperatures: (**A**) 37 °C; (**B**) 80 °C; (**C**) 120 °C.

Figure 2. Typical pictures taken on films of ca. 25 μm stored for 60 days under different conditions: (**D**) control (a just formed film); (**E**) 4 °C, 0% RH; (**F**) 37 °C, 0% RH; (**G**) 23 °C, 0% RH; (**H**) 23 °C, 75% RH.

Table 1. Effect of the Casting Temperature on Color of Just-Formed Chitosonium Acetate Films[a]

color coordinate	temperature		
	37 °C	80 °C	120 °C
L^*	94.95 A	94.32 A	89.74 B
a^*	−1.10 A	−1.68 B	−2.21 C
b^*	9.16 A	13.22 A	33.69 B

[a] Mean values with different letters in the same row represent statistically significant difference at $p < 0.05$. Films presented ca. 50 μm thickness.

Table 2. Color Changes of Chitosonium Acetate Films Stored at Various Temperatures and Relative Humidities for 60 Days

sample[a]	color coordinates			
	L^*	a^*	b^*	ΔE
control[b]	94.92 A[c]	−0.12 A	6.85 A	
temperature (0% RH)				
4 °C	94.77 A	−0.04 B	7.77 B	0.94 A
23 °C	94.68 A	−0.31 C	8.97 C	2.14 B
37 °C	93.32 B	−0.77 D	13.19 D	6.57 C
relative humidity (23 °C)				
0%	94.68 A	−0.31 B	8.97 B	2.14 A
75%	92.91 B	−1.95 C	19.02 C	17.91 B

[a] All films were cast at 37 °C [b] The control sample corresponds to a just-formed chitosonium acetate film. [c] Mean values with different letters in the same column represent significant differences ($p < 0.05$) between the control and samples inside the same variable studied (T or RH). Films presented ca. 25 μm thickness.

days at the various specific conditions were analyzed in triplicate. The percentage of dissolution of the sample was also calculated from the values of released chitosan (mg/10 mL of nutrient medium).

Statistical Analysis. The statistical significance of differences in color changes and antimicrobial capacity between the test samples was determined using Xlstat-Pro (win) 7.5.3. (Addinsoft, New York). Data were ranked and statistical differences were evaluated on the ranks with a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison tests. In all cases, a value of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

It is desirable that edible/biodegradable films be transparent and colorless in the application for consumer acceptance. To analyze this attribute, the sample color changes were measured for the various samples. The film specimens were highly transparent, but some of them, although highly translucent, presented a slightly yellow appearance (**Figures 1** and **2**).

Table 1 reveals color changes as a function of film casting temperature (37, 80, 120 °C). As can be observed from the results, the films presented good transparency as indicated by high lightness values (L = 94.32−94.95), except when the forming process was performed at 120 °C, at which a significant decrease in this parameter was found (L = 89.74). Furthermore, when the casting temperature was increased, the subsequent films presented more yellow color and greenness as indicated by the significantly greater and lower values of b^* and a^*, respectively. **Table 2** shows the recorded values of color after 60 days of storage at the various temperature and relative humidity conditions used. From this table, the results indicate that the films also darkened significantly (as evidenced by decreasing values of L^*) when stored at a high relative humidity (75% RH) or temperature (37 °C). In addition, all of them increased significantly in yellowness and greenness, these changes being more acute at the just mentioned storage conditions (75% RH and 37 °C). ΔE was calculated from the

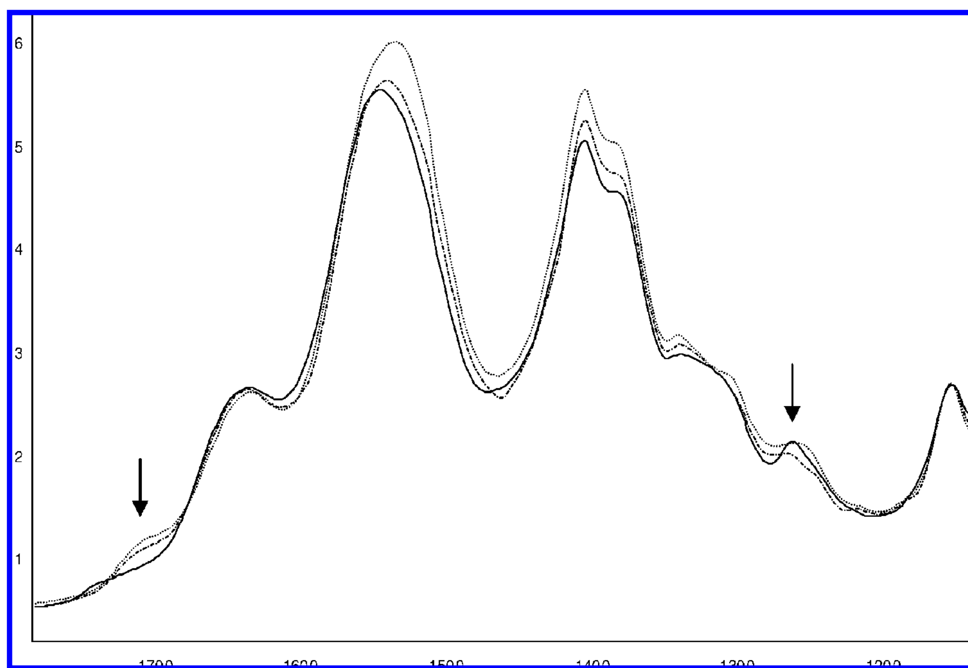

Figure 3. ATR-FTIR spectra of freshly formed chitosonium acetate films at different casting temperatures: 37 °C (- - -); 80 °C (— — —); 120 °C (—).

color parameter values as an indicator of the global changes after the storage period (eq 1). This value shows again more drastic changes with increasing temperature but, especially, when the films were stored in the presence of water.

As mentioned under Experimental Procedures, the thickness of the samples was different depending on the test. Thus, to evaluate the film-forming temperature conditions, samples of 50 μm were obtained given that it was not possible to obtain thinner samples at higher temperatures (i.e., 80 and 120 °C). On the other hand, to evaluate the effect of the storage conditions, chitosan films of ca. 25 μm were obtained at 37 °C because at this particular temperature it was possible to obtain such thin films simulating an internal coating of the packaging material.

Color changes were not comparable between the two different tests (i.e., film-forming conditions and storage conditions) because differences in thickness influence color parameters. Thus, the just-formed samples (cast at 37 °C) showed increases in yellowness and greenness in line with the increase of their thickness from 25 μm (in **Table 1**) to 50 μm (control in **Table 2**).

Larena et al. (*28*) also studied the color changes of chitosan films after a storage period and obtained a decrease in the membrane transmittance with temperature. The authors attributed these color changes to nonenzymatic browning reactions, which led to the presence of conjugated double bonds in the structure of the polymer. Similar conclusions were reported by Srinivasa et al. (*22*), who observed an increase in yellowness in oven-dried chitosan films. The same findings were reported Kam et al. (*21*) when chitosonium acetate films were heated for 2 h at 120 °C. In the latter work, color changes were more intense with higher amounts of acetic acid in the films, which suggests that color formation is a visible indicator factor underlying chemical alterations in the biomaterial.

In the present work, color changes must also originate from molecular alterations during the casting process or the storage conditions. In an attempt to monitor such variations, the chemical structure of some of the chitosonium acetate samples was studied by ATR-FTIR spectroscopy (see **Figures 3−6**). In all tested samples, a strong absorption band envelope was observed in the 1400−1700 cm^{-1} region with maxima at 1405 and 1548 cm^{-1}, which is associated with the formation in the biopolymer of the antimicrobial active carboxylate groups ($-NH_3^{+-}OOCH$) (*23, 34*). The dominant bands overlap other features such as amine (NH_2, 1590 cm^{-1}) and amide II (N−H bending, 1550 cm^{-1}) modes (*21, 24, 26*). Nevertheless, the amide I band at 1641 cm^{-1} (C=O, carbonyl stretching) can be easily discerned (*19, 21, 23, 26*). The latter band has also been associated with the presence of other contributory compounds, including water and imines (*29*). Thus, it has been assigned by some authors to have contributions from the C=N stretching modes of Schiff bases (*30, 31*) probably produced by condensation of amino groups with the carbonyl group of the glucose backbone. The absorption band at 1320 cm^{-1} is thought to have contributions from the amide III mode (*32*), which position seems to shift to 1335 cm^{-1} in the chitosan salt. Other authors have suggested that the amide III band is at 1270 cm^{-1}. This band is a contribution of species arising from the coupling of C−N stretching and N−H bending (*33*). The presence of the band at 1380 cm^{-1} is attributed to the C−H of CH_3 groups of the acetamide group, which demonstrates that chitosan is not completely deacetylated (*34*). **Table 3** gathers the abovementioned bands and their corresponding assignments.

To identify the initial differences between samples in terms of the intensity of the carboxylate bands related to the antimicrobial capacity of the materials, spectra were taken from the just-formed samples at different temperatures (37, 80, and 120 °C). **Figure 3** shows the spectra of these films before the conditioning period explained under Experimental Procedures (i.e., before equilibration at dry conditions to eliminate the solvent sorption influence from the spectra). From this figure, the high intensity of the bands corresponding to the antimicrobial carboxylate spectral features is observed. Furthermore, a shoulder could be detected at 1706 cm^{-1} for samples obtained at 37 or 80 °C, which indicates that these films still contained a certain amount of “free” acetic acid when measured (see arrow). This band is no longer discerned in the films formed at 120 °C due to more efficient thermally induced solvent

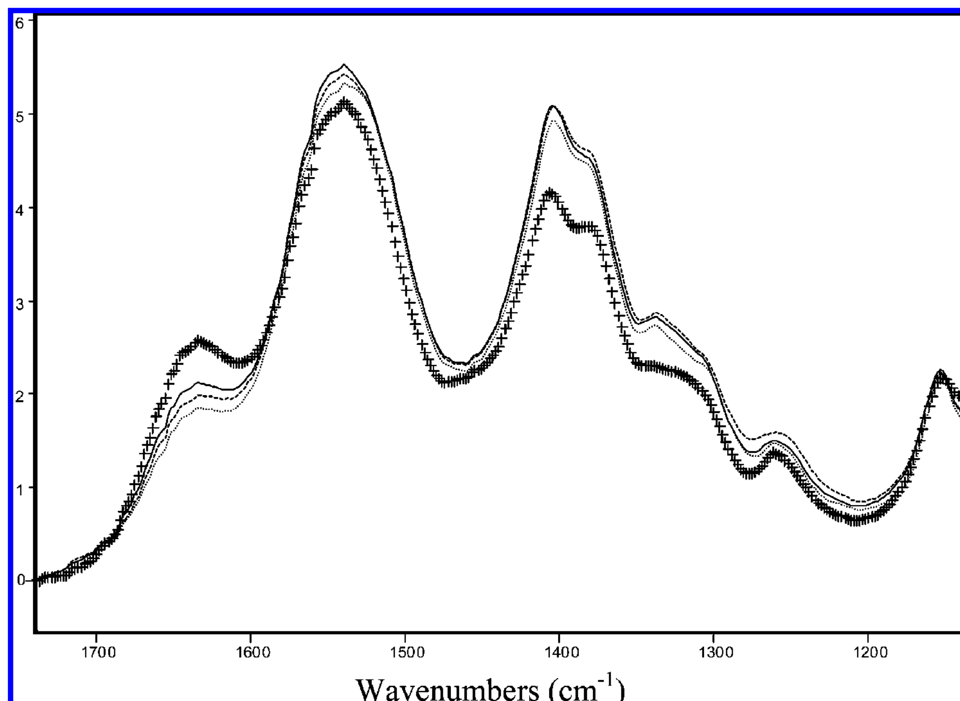

Figure 4. ATR-FTIR spectra of freshly formed chitosonium acetate films at different casting temperatures (37 °C, - - -; 80 °C, — — —; 120 °C, —) after the conditioning period (0% RH, 23 °C, 2 weeks). (+++) Spectrum of a conditioned film formed at 120 °C and immersed in water for 24 h.

Table 3. Assignment and Position of Some Chitosonium Acetate Bands of Interest

assignment	band position (cm^{-1})	ref
carboxylate groups	1405, 1548	Lagaron et al.(*23*) Fernandez-Saiz et al.(*24*) Zotkin et al.(*19*) Ritthidej et al.(*20*)
amine	1590	Osman and Arof(*26*)
amide I	1641	Lagaron et al.(*23*) Zotkin et al.(*19*) Osman and Arof(*26*) Kam et al.(*21*)
amide II	1550	Zotkin et al.(*19*) Kam et al.(*21*)
amide III	1320, 1270	Qin et al.(*32*) Erukhimovitch et al.(*33*)
acetamide	1380	Tsai et al.(*34*)

evaporation. In the latter sample an additional shoulder contribution is observed at ca. 1270 cm^{-1} (see arrow), which after the conditioning period is no longer a differentiating feature between samples, therefore relating the observed previous differences to particular interactions with the sorbed solvents (**Figure 4**). In a subsequent step, the effect of water or microbial solution contact with the film was studied in the same matrices by immersion of the samples in water for 24 h. After that, only the film formed at 120 °C showed a water-resistant behavior, and therefore, this was the only sample that allowed the FTIR spectrum to be taken as it did not change shape or size ("+" spectrum in **Figure 4**).

In this spectrum, the carboxylate bands at 1548 and 1405 cm^{-1} maintained a relatively high intensity, suggesting that the antimicrobial species do not extensively migrate into the medium even when these are potentially soluble. This behavior is in sharp contrast with a previous work (*23*), which demonstrated by ATR-FTIR that a chitosonium acetate film cast at controlled conditions (23 °C, 40% RH) showed an immediate release of a significant fraction of the carboxylate groups when put in contact with water, leading to a large redissolution of the matrix and a large drop in the above-mentioned FTIR bands. An explanation is that the samples formed at 120 °C underwent water resistance chemical alterations which could include some degree of cross-linking or other molecular entanglement processes, which prevent the release of the protonated glusosamine fractions from the film into the liquid phase. Interestingly, the "+" spectrum shows the apparent relative increase (compared to the normalization band at 1150 cm^{-1}) (*23*) of the band centered at ca. 1641 cm^{-1}, suggesting the increase of new amide and/or imide groups. Regardless of the exact molecular mechanisms undergone by the biopolymer, the molecular alterations suffered by the material result at the macroscopic scale in a film with a more yellow appearance and with a stronger water resistance than the other two samples prepared at milder temperature conditions.

Figure 5 shows the spectra of chitosonium acetate films cast at 37 °C and stored in a dry environment at various temperature conditions (4, 23, 37 °C) for 60 days. From the spectra a considerable decrease of the bands (i.e., 1548 and 1405 cm^{-1}) associated with the protonated antimicrobial species in the polymer can be easily discerned when the sample was stored at the highest temperature (37 °C). Demarger et al. (*25*) showed by transmission FTIR that the NH_3^+ groups decreased rapidly when the films were stored in a dry environment but that upon storage these groups diminished more slowly with time. In our work, the bands associated with the antimicrobial species decreased to a larger extent at a higher temperature, turning partially the antimicrobial chitosonium acetate film into the nonbiocide chitosan chemistry.

The evolution of the molecular structure of the chitosan matrices was also studied for samples stored for 60 days at two different relative humidities (i.e., 0 and 75%). **Figure 6** gathers the IR spectra of these samples after the additional conditioning period explained under Experimental Procedures. In this figure,

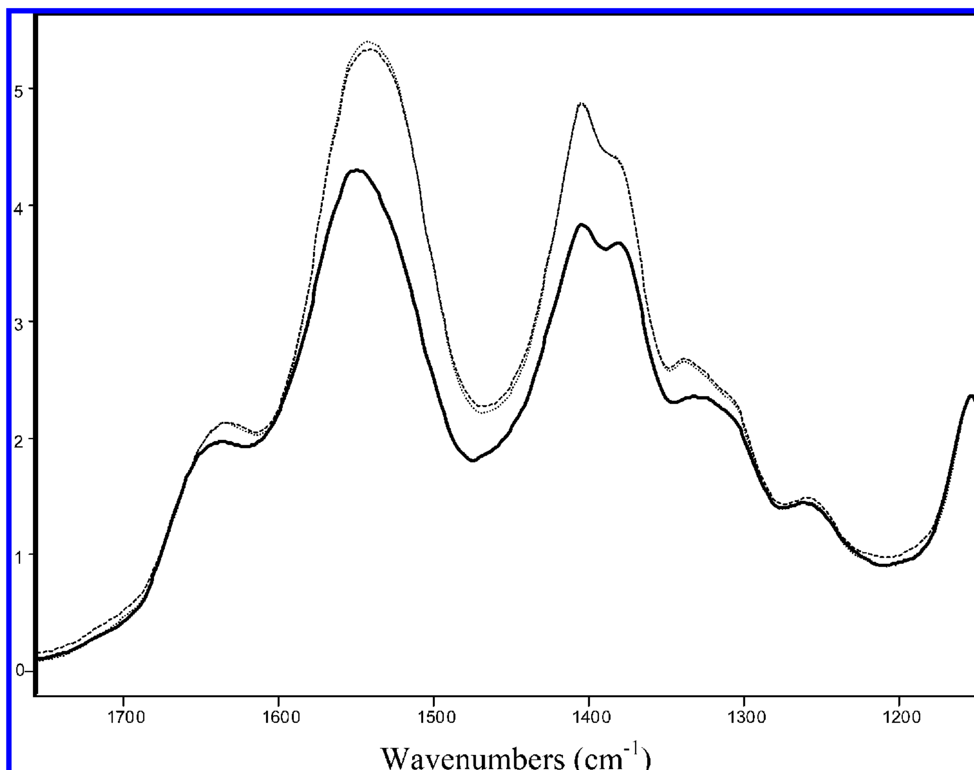

Figure 5. ATR-FTIR spectra of chitosonium acetate matrices stored at different temperatures [4 °C (- - -), 23 °C (— — —), 37 °C (—)] in a dry ambient for 60 days.

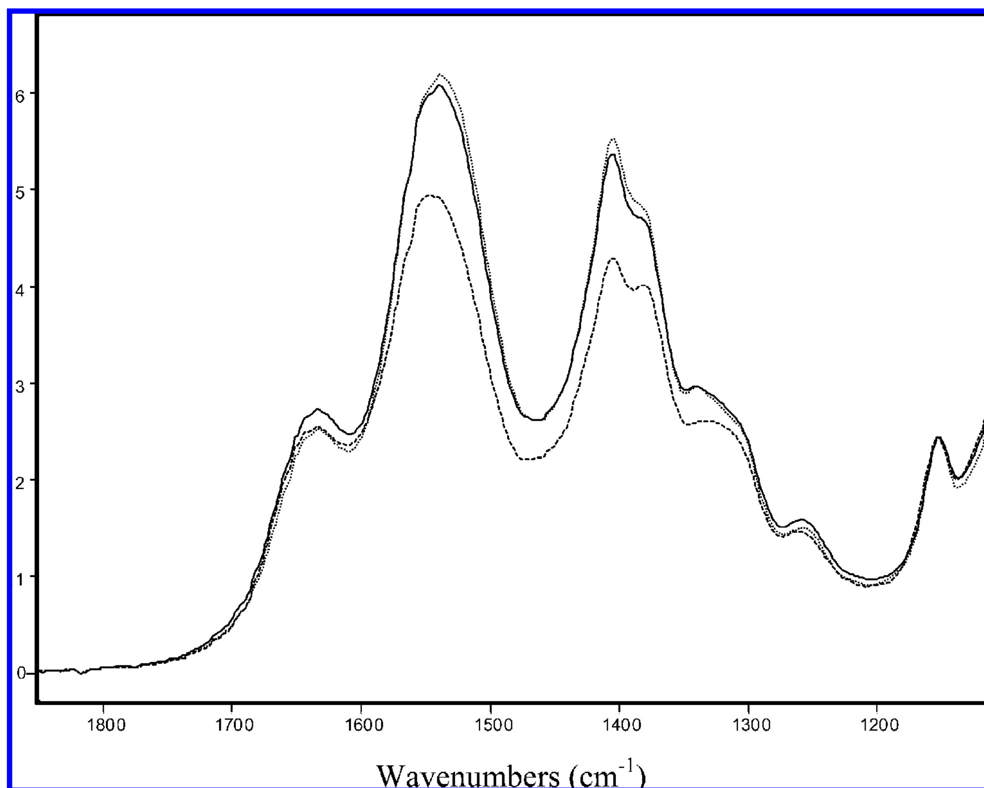

Figure 6. ATR-FTIR spectra of conditioned chitosonium acetate matrices after storage at 23 °C and different relative humidity conditions, 0% RH (- - -) and 75% RH (—), for 60 days. (— — —) Spectrum of a conditioned film that was stored at 75% RH and immersed in water for 24 h.

films maintained at both 75 and at 0% RH showed a larger intensity of the antimicrobial species. Because the films were previously conditioned, such intensity must not to be due to the interference with the sorbed solvent. However, later it will be shown that the films stored at 75% RH did not exhibit measurable biocide properties.

To explain this counterintuitive behavior, the stored samples were immersed in water for 24 h. In these experiments all of the specimens maintained at 0% RH dissolved completely in water, whereas the film stored at 75% RH (23 °C) did not change shape or size. After immersion in water, the sample was equilibrated at dry conditions and was subsequently measured

Table 4. Antimicrobial Activity of Chitosonium Acetate Films Formed at Different Temperatures against the Growth of *S. aureus* and *Salmonella* Species

T (°C) of casting	*S. aureus* (CFU/mL)	*Salmonella* spp. (CFU/mL)	pH of 60 mg film MHB	pH of 40 mg film MHB
control	1.88×10^8 (0.20)[a] A	3.16×10^8 (0.12) A	6.2	6.2
120	5.01×10^7 (0.05) A	2.25×10^7 (0.10) B	6.07	6.15
80	4.67×10^4 (0.28) B	1.07×10^6 (0.01) C	5.70	5.82
37	7.58×10^4 (0.37) B	9.12×10^5 (0.12) C	5.66	5.85

[a] Each value represents the mean of three replicate counts. Standard deviation is given in parentheses. Different letters in the same column indicate significantly different groups determined by Tukey's test ($p < 0.05$).

by ATR-FTIR spectroscopy. The spectrum of this particular sample is also shown in **Figure 6** (dashed spectrum). In a manner analogous to that for the films formed at 120 °C, this matrix preserved to a significant extent the carboxylate bands, suggesting that chemical developments have taken place which inhibit the migration of the protonated active groups to the solution. In good agreement with our results, Ritthidej et al. (*20*) reported that after exposure of chitosan films to moist heat (60 °C, 75% RH) the formation of amide groups between the chitosan and the acetate acid was suggested by FTIR spectroscopy, for which the presence of water is required. This is also in agreement with the strong yellowness measured, which is associated with double-bond formation and other chemistry developments in the materials by some authors (*21, 22, 28*). In contrast, the samples maintained in a dry environment did not appear to show such chemical changes and therefore maintained their hydrophilic behavior, even when stored at 37 °C. Some previous studies have demonstrated that an amidation process still takes place in the chitosan films after a thermal treatment even at dry conditions (*19, 21*). Nevertheless, in the latter works the applied temperatures were always higher than the 37 °C used in this work. Evidence for amidation is supported by the relative increase in the amide I band in **Figure 6**.

To elucidate the influence of all of the above alterations on the biocide properties of the chitosonium acetate films, susceptibility tests were performed against two bacteria types, that is, *S. aureus* and *Salmonella* spp. Preliminary assays performed in our group on the assessment of the minimal inhibitory concentration (MIC) of just-formed low molecular weight chitosonium acetate films against these microorganisms revealed values of 40 mg/10 mL for *S. aureus* and 60 mg/10 mL for *Salmonella*. Consequently, such quantities were employed in the current work for each bacterial strain.

Whereas the thickness of the chitosan film influenced the color parameters as indicated above, a previous work demonstrated that this variable did not affect significantly the biocide properties of high molecular weight chitosan films in a range from 25 to 100 μm (*13*). Consequently, the differences obtained in the susceptibility tests could be comparable between all of the tested samples regardless of the thickness of the matrix.

Table 4 shows the final bacterial counts obtained when the effect of the casting temperature was evaluated. The table also illustrates the pH values of the nutrient broth after film immersion and before microbial inoculation. From these results, it is shown that samples formed at 120 °C presented weak biocide properties for both bacteria, being uniquely significant for *Salmonella* spp. test. Alternatively, film specimens obtained at 37 and 80 °C showed a strong antibacterial action for both strains, and no significant differences between these temperatures were found. It is important to mention that the amount of the just-formed chitosonium acetate film evaluated in this work for the case of *Salmonella* (i.e., 60 mg) was less toxic than the

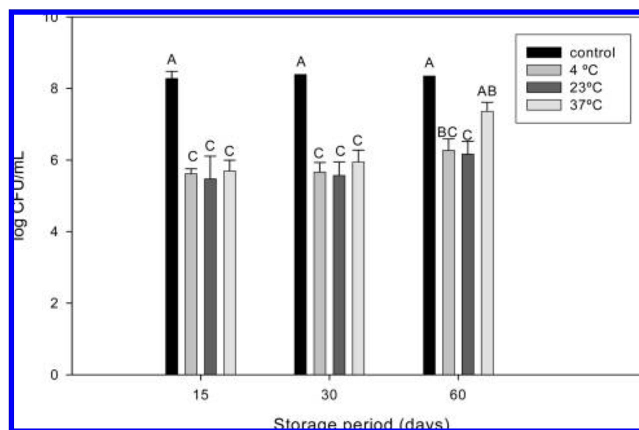

Figure 7. Antimicrobial properties of chitosonium acetate films stored at different temperature conditions against *S. aureus*. Different letters indicate significantly different groups determined by Tukey's test ($p < 0.05$).

weight employed for the case of *S. aureus*. Thus, whereas the inhibition led to final counts of ca. 9.12×10^5 CFU/mL for the former case, the final growth was 7.58×10^4 CFU/mL for *S. aureus*, corroborating the results previously reported by other authors about a major susceptibility of Gram-positive bacteria to chitosan salts.

The nutrient broth experienced considerable reductions of pH (5.66−5.85) when tested on films obtained at lower temperatures (i.e., 37 and 80 °C), suggesting that a residual content of free acetic acid could be present in these samples. This is in agreement again with the ATR-FTIR observation in these samples of a residual band at ca. 1706 cm^{-1}. In contrast to this, when the films were cast at 120 °C, which exceeds the boiling point of the acid (i.e., 117.9 °C), the free acid vanished from the matrix and, therefore, neither the pH reduction nor the specific band was detected. The pH drop in the former cases could play a role in the inhibition of the bacterial growth. Nevertheless, previous trials performed by the authors have shown that both bacteria grow optimally in MHB until a pH of the medium of ca. 5.3 (results not shown). Therefore, because the pH reduction did not exceed this threshold in any case, the biocide properties of the film materials should be, therefore, exclusively attributed to the presence of the biocide species of chitosan. It is worth mentioning that the film specimen formed at 120 °C presented a significant drop in biocide properties, which should be a result of the previous ATR-FTIR results that proved that much lower active species migration takes place in connection with potential cross-linking and/or amidation effect during the film-forming process. This lower migration could also be behind the higher pH obtained in the solution after contact with this sample.

Figures 7 and **8** show the results of the susceptibility tests carried out against the growth of *S. aureus* and *Samonella* spp. for the films stored at the various temperatures (4, 23, and 37 °C, 0% RH). As can be seen from **Figure 7**, the films preserved significant biocide properties against *S. aureus* when maintained at 4 and 23 °C. Nevertheless, for the same microorganism, the samples stored at 37 °C presented an important loss in their antimicrobial capacity after 60 days, being not able to exert a significant reduction in final bacterial population. Alternatively, the observed effects were quite different for the case of *Salmonella* spp. (**Figure 8**). Thus, for this particular microorganism, the films were not able to perform a significant reduction of the final bacterial counts after a storage period at 4 or 23 °C for 30 days. Moreover, also in this strain, the samples maintained at 37 °C lost their detectable antimicrobial properties after 15

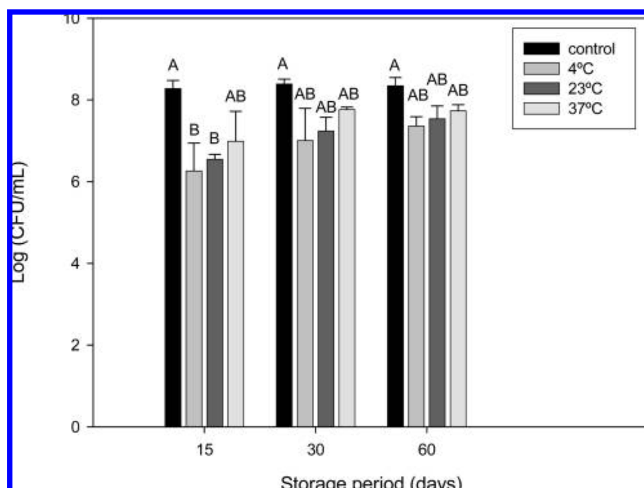

Figure 8. Antimicrobial properties of chitosonium acetate films stored at different temperature conditions against *Salmonella* spp. Different letters indicate significantly different groups determined by Tukey's test ($p < 0.05$).

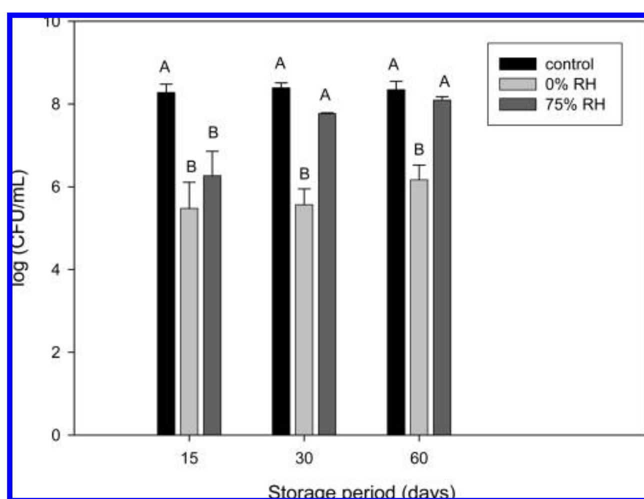

Figure 9. Antimicrobial properties of chitosonium acetate films stored at different humidity conditions against *S. aureus*. Different letters indicate significantly different groups determined by Tukey's test ($p < 0.05$).

days of storage. All of these results are in accordance with the chemical evolution of the films observed in the current work by ATR-FTIR, in which a higher reduction of the carboxylate antimicrobial groups was detected when the sample was stored at the latter temperature (**Figure 5**).

When the influence of the relative humidity on storage was evaluated (**Figures 9** and **10**), a good preservation of the biocide character against the growth of *S. aureus* was seen when samples were stored at 0% RH (23 °C) but not at 75% RH (23 °C). Thus, in the latter case, after 30 days of storage, the results did not reveal a significant antimicrobial effect. This finding is again in agreement with results observed by the infrared technique. Concerning *Salmonella* spp., such preservation was not observed in any case, and after 30 days of storage, no significant inhibitory effect was observed for both storage conditions. In this particular test, even for the samples maintained at 0% RH a sufficient loss in antimicrobial species over time has occurred to exhibit biocide performance against this particular microorganism.

Despite the disparities found between bacteria types, a trend in biocide performance was observed with storage in all cases. Although such a trend seems to be more evident for *S. aureus*, the results of the susceptibility tests may not be comparable

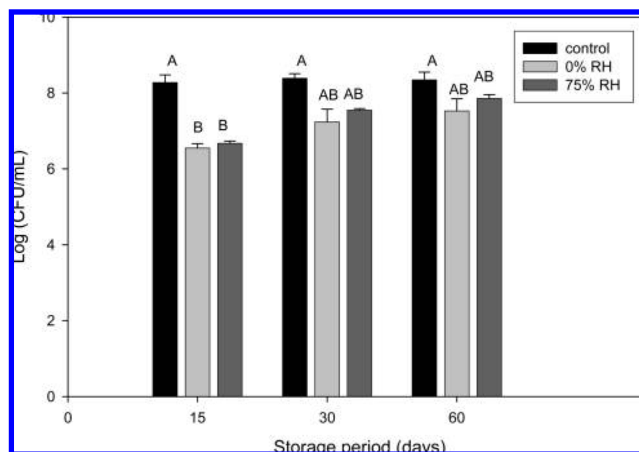

Figure 10. Antimicrobial properties of chitosonium acetate films stored at different humidity conditions against *Salmonella* spp. Different letters indicate significantly different groups determined by Tukey's test ($p < 0.05$).

Table 5. Antimicrobial Effectiveness against the Growth of *S. aureus* and *Salmonella* of the Chitosonium Acetate Films Stored under Various Relative Humidity and Temperature Conditions

		antimicrobial effectiveness (%) against	
storage conditions	storage time (days)	*S.aureus*	*Salmonella* spp.
4 °C, 0% RH	15	78.71	87.00
	30	77.36	55.68
	60	59.79	41.34
23 °C, 0% RH	15	82.77	74.97
	30	80.09	46.01
	60	62.75	33.80
37 °C, 0% RH	15	76.50	56.55
	30	69.16	23.92
	60	28.26	23.92
23 °C, 75% RH	15	59.90	69.63
	30	16.45	32.96
	60	7.01	19.97

between strains in view of the fact that the bacterial inhibition of a just-formed film was considerably higher for *S. aureus* (see **Table 4**). Therefore, for a better understanding of the results obtained, the antimicrobial effectiveness of the samples with storage was calculated by the increment of the final bacterial counts with respect to the microbial inhibition when a just-formed film was tested (eq 2). The values obtained by this relationship (**Table 5**) confirm the major conservation of the antimicrobial effectiveness in samples stored at 4 or 23 °C in dry conditions. Nevertheless, this efficiency was lower for *Salmonella* spp., probably due to the higher resistance that this microorganism offers to chitosan.

When samples were maintained at 37 °C (0% RH), the biocide effectiveness dropped drastically over time, especially for *Salmonella* spp., in which such reduction started from the first 15 days of storage, as previously observed in **Figure 4**. In any case, the least appropriate storage conditions corresponded to 75% RH (23 °C), which led to effectiveness reductions to 7 and 19% for *S. aureus* and *Salmonella*, respectively, after 60 days of storage.

Finally, glucosamine release from the film to the nutrient broth was also measured as a direct parameter of the solubility of the films in order to support the results of the susceptibility assays. The values obtained are shown in **Table 6**. From this

Table 6. Matrix Dissolution from the Just-Formed and Stored Chitosonium Acetate Films to the Nutrient Broth after Incubation at 37°C for 24 h

sample	released chitosan (mg/10 mL)	film dissolution (%)
just-formed film, 37 °C	20.31 (1.38)[a]	50.80 (3.45)
just-formed film, 80 °C	19.83 (1.00)	49.58 (2.52)
just-formed film, 120 °C	6.88 (0.45)	17.21 (1.13)
stored for 60 days , 4 °C, 0% RH	11.16 (2.36)	27.91 (5.90)
stored for 60 days, 23 °C, 0% RH	11.85 (2.39)	29.63 (5.98)
stored for 60 days, 37 °C, 0% RH	8.59 (1.24)	21.49 (3.11)
stored for 60 day, 23 °C, 75% RH	4.31 (0.51)	10.78 (1.28)

[a] Each value represents the mean of three replicate counts with the standard deviation given in parentheses.

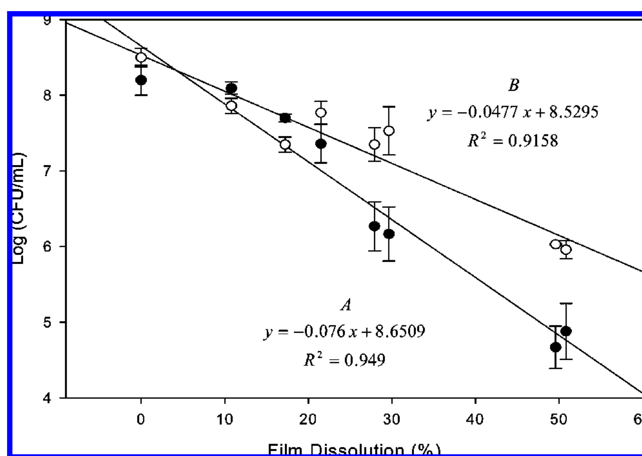

Figure 11. Relationship between chitosan film dissolution (percent) and the growth of *S. aureus* (●, *A*) and *Salmonella* spp. (○, *B*).

table, it is noteworthy to observe that a decrease of the glucosamine release takes place for the samples with the weaker biocide properties, that is, for the films formed at 120 °C or stored at 75% RH (23 °C) or at 37 °C (0% RH) for 60 days. These results are also in agreement with the FTIR results. The percentage of dissolution of the film, calculated from the released chitosan, is also indicated in the table and plotted versus the growth of *S. aureus* and *Salmonella* spp. (**Figure 11**). From this figure, a good linear relationship between film solubility and antimicrobial effectiveness is clearly observed ($R^2 = 0.95$ for *S. aureus* and 0.90 for *Salmonella* spp.).

In summary, the current work has demonstrated that just-formed low molecular weight chitosonium acetate films present significant biocide properties against *S. aureus* and *Salmonella* when cast at 37 or 80 °C, whereas this capacity is reduced when films are cast at 120 °C. In addition, the films preserved significant biocide properties against the growth of Gram-positive bacteria when maintained at low temperatures and dry conditions (4, 23 °C, 0% RH) during the storage periods studied. On the other hand, when the same films were maintained at high relative humidity conditions (i.e., 75%) or higher temperatures (i.e., 37 °C), the samples presented a progressive yellow coloration and a gradual loss of their antimicrobial capacity, due to water resistance induced by chemical and/or physical alterations in the films. These alteration processes result in a substantial loss in the release of antimicrobial protonated species as a result of cross-linking and/or other molecular entanglement process and/or by chemical alterations of a more hydrophobic nature in the biomaterial. As a result, an appropriate control of the formation and storage conditions of chitosan films or coating must be carried out to obtain satisfactory results in terms of product safety or anti-infective properties. This research highlights for the first time essential aspects of the use of chitosan as a biocide that can have important technological consequences in various applications including food technology and biomedical and pharmaceutical products.

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