

Physical and Microbiological Evaluation of Chitosan Films: Effect of Essential Oils and Storage

N. Valderrama, W. Albarracín, N. Algecira

Abstract—The effect of the inclusion of thyme and rosemary essential oils into chitosan films, as well as the microbiological and physical properties when storing chitosan film with and without the mentioned inclusion was studied. The film forming solution was prepared by dissolving chitosan (2%, w/v), polysorbate 80 (4% w/w CH) and glycerol (16% w/w CH) in aqueous lactic acid solutions (control). The thyme (TEO) and rosemary (REO) essential oils (EOs) were included 1:1 w/w (EOs:CH) on their combination 50/50 (TEO:REO). The films were stored at temperatures of 5, 20, 33°C and a relative humidity of 75% during four weeks. The films with essential oil inclusion did not show an antimicrobial activity against strains. This behavior could be explained because the chitosan only inhibits the growth of microorganisms in direct contact with the active sites. However, the inhibition capacity of TEO was higher than the REO and a synergic effect between TEO:REO was found for *S. enteritidis* strains in the chitosan solution.

Some physical properties were modified by the inclusion of essential oils. The addition of essential oils does not affect the mechanical properties (tensile strength, elongation at break, puncture deformation), the water solubility, the swelling index nor the DSC behavior. However, the essential oil inclusion can significantly decrease the thickness, the moisture content, and the L^* value of films whereas the b^* value increased due to molecular interactions between the polymeric matrix, the loosening of the structure, and the chemical modifications. On the other hand, the temperature and time of storage changed some physical properties on the chitosan films. This could have occurred because of chemical changes, such as swelling in the presence of high humidity air and the reacylation of amino groups. In the majority of cases, properties such as moisture content, tensile strength, elongation at break, puncture deformation, a^* , b^* , chroma, ΔE increased whereas water resistance, swelling index, L^* , and hue angle decreased.

Keywords—Chitosan, food additives, modified films, polymers.

I. INTRODUCTION

CHITOSAN (CH) (poly-(1,4)-2-amino-2-deoxy-P-D-glucose) is obtained from the process of deacetylation of chitin (poly-(1,4)-2-acetamide-2-P-D-deoxy-glucose) by enzymatic or chemical methods. The acetamide groups of chitin are hydrolyzed by a nucleophilic substitution mechanism. Chitin is one of the most abundant polymers extracted from crustaceans, insects, fungi, and microorganisms [1], [2]. CH is used to produce films for biomedicine,

pharmacology, ophthalmology, cosmetics, and food industry [2], [3].

The recent packaging advances have focused on the inclusion of different chemical compounds to improve the properties of CH films [4]-[9]. The essential oils have replaced synthetic antioxidants [10]. These have been included in CH polymeric matrices to improve their antimicrobial and antioxidant properties [11], [12]. The mechanical, physical, and sensory properties are not significantly different when essential oils are included at low concentrations (100 μ l/g) [13]. However, higher concentrations may affect their properties [14]. Additionally, the essential oils inclusion improves the antimicrobial properties of the films for some pathogens due to the action of phenolic compounds such as α -pinene, bornyl acetate, camphor, 1,8-cineole, thymol, carvacrol, tepineno, cymene, diterpenes, ursolic acid, and carnosol [12], [13], [15]-[17] even during storage [18], [19].

Storage conditions such as humidity, temperature, and time can modify the mechanical and barrier films properties [20], [21]. This might be due to changes such as the re-crystallization of CH, the loss of moisture and plasticizer, the slow reacylation of amino groups, the extended conformations, the free volume changes, and others [22]-[24]. The deformation-strength properties of chitosan films decrease with the storage time [23], [25]. Furthermore, temperature and humidity exert greater influence on mechanical properties [24], [26]. However, microbiological and water vapor barrier properties decrease whereas the temperature of the decomposition onset increase directly respect to the storage time [24], [27]-[29].

The physical properties of films determine the affectivity of the packaging application and consequently the quality of food. The mechanical properties assure the integrity and the conservation of the packaging. The properties such as humidity, water resistance, water vapor permeability and swelling are important because they protect the foods while it is in contact with water, as well as food with a high water activity. Some researchers have characterized films with inclusion of different compounds and have tested during the storage [4]-[25].

The aim of this article was to determine the effect of the inclusion of thyme and rosemary essential oils into chitosan films, as well as the microbiological and physical properties when storing chitosan film with and without the mentioned inclusion.

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II. MATERIALS AND METHODS

A. Films Preparation

Kitoflokk™, medium degree of deacetylation (80%), was purchased from Norwegian Chitosan, Aspertunet, Norway. CH solution was prepared by dissolving 2% (w/v) of CH in 1.0% (v/v) of lactic acid aqueous solutions using an Ultra-Turrax®. This solution was subsequently filtered at room temperature to remove impurities through a Boeco® qualitative filter (Boeckel Co., Hamburg, Germany) and sterilized at 100°C for 15 minutes.

The solutions were mixed with polysorbate 80 4% (w/w) and glycerol 16% (w/w) based in the chitosan weight. The formulation TR1 was prepared by 50/50 rate of thyme (TEO) and rosemary (REO) essential oils. The essential oils were included on proportion 1:1 (w/w) based in the chitosan weight. The film forming solutions were homogenized under aseptic conditions at 30,000 rpm for 5 min using an Ultra-Turrax® and degasified in a vacuum chamber at 25°C.

Each film were prepared with 10 g of film forming solution which ensured 1,27 mg/cm² per petri dish. Control films were prepared identically but without the addition of essential oils (EOs). After drying at 30 °C during 65 hours, the films were analyzed immediately.

B. Determination of Antimicrobial Effects

An agar diffusion test was used for determining the antibacterial effects on the non-stored film for *E. coli*, *S. enteritidis*, *S. aerus*, *L. monocytogenes* and *B. cereus* strains. This test was developed for the solutions and the films. The solutions (n = 3) were placed in wells and the films (n = 3) were cut into 1 cm diameter disks with a circular knife. Initial number of bacteria in Mueller-Hinton agar was in the range of 105-106 CFU/ml. Bacterial strains were incubated at 37°C for 48 hours. The whole zone area was calculated then subtracted from the film disk area, and this difference in area was reported as the zone of inhibition. The oxytetracycline was the control antibiotic. The technique was described as in [30].

C. Determination of the Solutions Properties

1) Turbidimetric Measurements

The turbidity of these solutions was measured at 400nm using a Genesys™ 10S UV-Vis Spectrophotometers (Thermo Scientific, Radnor, PA). All experiments were performed at room temperature in triplicate and each value is the average of the three measurements.

2) pH

The pH of solutions was determined by a standard pH meter (Orion 920A, Scientific Support, Hayward, CA, USA).

D. Determination of Physical Properties of Chitosan Films

1) Thickness

The thickness was determined using a Digital Micrometer to the nearest 0.001 mm (Coolant Proof Micrometer 293, Mitutoyo Measuring Instruments, Kanagawa, Japan). Values

were an average of at least 10 random locations along the sheets of chitosan films.

2) Tensile Strength and Elongation

TA.XTPlus Texture Analyzer (Stable Micro Systems, Ltd. Hamilton, MA) was used to measure tensile strength (TS) at break and percent elongation (%E) at break. Samples (ASTM D 638M) were cut and film thickness was measured. TS (n = 10) was calculated by dividing the maximum (peak) load by the cross-sectional area. %E (n = 10) was calculated by dividing the elongation at the moment of rupture by the initial length of specimen and multiplying by 100.

3) Puncture Strength and Deformation

The puncture strength (PS) and the puncture deformation (PD) were measured using a TA.XTPlus Texture Analyzer (Stable Micro Systems, Ltd. Hamilton, MA). Films were cut in square of 2 cm. A cylindrical probe (2 mm diameter) passed through the film at a constant speed (1 mm/s). Strength values at the puncture point (n = 10) were used to calculate the hardness of the film. The PS values were divided by the thickness of the films in order to correct for the effect of thickness variation. The PD of the films was calculated using the distance difference between the time of film contact and the time of puncture point.

4) Moisture Content

The moisture content of film pieces (n = 3) was determined measuring the weight loss of films, upon drying them in an oven at 110°C until a constant weight was reached.

5) Water Resistance

The solubility was measured by immersing weighed pieces of chitosan film (n = 3) in 50 mL of distilled water by a constant agitation using Ultra-Turrax® for 15 minutes at room temperature. Subsequently, the solutions were filtered at room temperature using Boeco® quantitative filters (Boeckel Co., Hamburg, Germany). The chitosan films and the wet filters were dried at 110°C until constant weight. Water resistance (%) was calculated as in [31].

6) Swelling Index

Films (n = 3) were weighed and subsequently immersed in water for 10 minutes. The mass of the swollen films was measured after the surface water was removed with filter paper. The swelling index (SI) of the film was calculated as in [32].

7) Color

The color parameters were measured by reflection with specular component included (RSIN) using a ColorQuest XE colorimeter (Hunterlab, Reston, VI, USA). D65 illuminant, 10° observer and white standard plate (L* = 91,99; a* = -0,75 and b* = 1,8) were used for measurements. The L* (lightness), a* (intensity of red and green), b* (intensity of yellow and blue), ΔE (color difference), C (chrome) and H (hue angle) were obtained as the average of ten measurements on each film.

8) Scanning Electron Microscopy

Chitosan films were mounted on the specimen holder with aluminum tape and then were sputtered with gold in BAL-TEC SDC-050 sputter coater (BAL-TEC AG, Balzers, Liechtenstein). All the specimens were examined with a FEI QUANTA 200 scanning electron microscope (FEI, Hillsboro, Oregon) under low vacuum condition (2×10^{-2} torr). Samples were photographed at different tilt angles to obtain the views in the cross section.

9) Differential Scanning Calorimetry (DSC)

DSC was carried out using a DSC 2910 Modulated (TA Instruments, New Castle, DE). The range of the scanning temperature was -40 to 200°C . The heating/cooling rate was $10^\circ\text{C}/\text{min}$, under a nitrogen atmosphere.

10) Storage

The films with a combination of 2% TEO:REO in solution were stored at 5, 20, 33°C (T5, T20, T33) and relative humidity 75% (H75). Control films were prepared identically but without the addition of essential oils (EOs). The physical properties of every film were evaluated after two and four weeks.

11) Statistical Analysis

Analysis of multiple-range tests were used to perform statistical analysis on all results, using Statgraphics® Centurion XV (StatPoint Technologies Inc, Warrenton, Virginia, USA). Differences between means were considered to be significant when $p \leq 0.05$.

III. RESULTS AND DISCUSSION

A. The Effect of Essential Oils Addition on the Properties of Solutions

The pH solutions range was between 3.94 and 5.9. However, the addition of essential oils did not modify the pH. Additionally, the turbidity increased from 0.43 ± 0.15 to 2.74 ± 0.16 (a.u.) due to the essential inclusion of the oils, results that suggest a slight phase separation on the chitosan dispersion [33].

B. The Effect of Essential Oils Addition on the Antimicrobial Properties

The antimicrobial activity of chitosan edible films improved with EOs against *E. coli*, *S. enteritidis*, *S. aerus*, *L. monocytogenes* and *B. cereus* was studied. This property was measured based on clear zone surrounding the circular film including diameter of the film strips. If there is no clear zone surrounding the film, then it should be assumed that there was not an inhibitory zone.

Reference [13] shows that chitosan has antimicrobial activity itself. Additionally, the incorporation of antimicrobial agents into chitosan edible film improves antimicrobial efficacy of chitosan. However, the films with essential oil inclusion did not show an antimicrobial activity significantly against strains, even though the results of the test with antibiotic inclusion in the film was positive (Table I).

Reference [34] shows that chitosan is incapable of diffusion through the adjacent agar media because only the organism in direct contact with the active sites of chitosan is inhibited.

The main components of the rosemary essential oil are Eucalyptol (39.6%), Camphor (19%), α -pinene (4.8%), Thymol (52.9%) and p-cymene (34.0%), whereas the main components of the thyme essential oil are Thymol (52.9%) and p-Cymene (34.0%) [35]. These components can inhibit microorganism growth but the incorporating of EOs into chitosan solutions. The incorporation of 1:1 p/p (REO:TEO) slightly increased the inhibitory effect of chitosan solutions against *S. enteritidis* due to a synergic effect.

TABLE I
ANTIMICROBIAL ACTIVITY OF EDIBLE CHITOSAN FILMS

Microorganism		Inhibition zone diameter (mm)	
		Wells	Disks
<i>S. aureus</i>	C	15,33± 1,53 ^a	10,33± 0,58 ^a
	TR1	18,33± 0,58 ^a	15,67± 2,08 ^b
	A	53,67± 1,53 ^c	31± 1,73 ^c
	R	17,33± 0,58 ^a	NT
	T	45± 5 ^b	NT
<i>B. cereus</i>	C	11± 1 ^a	7,67± 0,58 ^a
	TR1	13,3± 1,15 ^{ab}	10± 2,65 ^{ab}
	A	44± 3,61 ^d	29± 1 ^c
	R	16,33± 1,53 ^b	NT
	T	27± 2 ^c	NT
<i>L. monocytogenes</i>	C	8± 1 ^a	8,66± 0,58 ^a
	TR1	12± 1 ^a	10± 1 ^{ab}
	A	36,67± 8,33 ^b	11± 1 ^b
	R	12,33± 0,58 ^a	NT
	T	46± 3,61 ^c	NT
<i>E. coli</i>	C	11± 1 ^a	8± 1 ^a
	TR1	15± 1 ^a	9,33± 0,58 ^{ab}
	A	40± 7 ^c	17,33± 2,52 ^c
	R	10,67± 1,15 ^a	NT
	T	32,33± 2,52 ^b	NT
<i>S. enteritidis</i>	C	11,33± 0,58 ^a	8,33± 0,58 ^a
	TR1	15,33± 0,58 ^a	9,66± 0,58 ^{ab}
	A	35,67± 5,13 ^c	17,67± 2,52 ^c
	R	14,67± 0,58 ^a	NT
	T	29± 3,61 ^b	NT

C: control film without essential oil addition; TR1: film with essential oil inclusion, 1:1 p/p (REO:TEO); A: antibiotic; R: Rosemary essential oil; T: thyme essential oil; NR: not registered. The values are expressed as means \pm SD (n = 3). Means in columns with different superscripts are significantly different at $p < 0.05$. NT: not tested.

Additionally, the results showed that the essential oil with best inhibition against all strains was TEO whereas the REO has a slightly inhibitory effect against *B. cereus*. Reference [35] shows that lower concentrations of TEO have the same microbiological inhibition than higher concentrations of REO and the combinations of TEO and REO presented a synergistic activity.

C. Effect of Essential oil Addition and Storage on the Physical Properties of Films

1) Thickness

The thickness of the films is shown on the Fig. 1. It ranged

from 0.063 to 0.1078 mm, indicating that the thickness of the films was significantly lower ($p < 0.05$) due to essential oils inclusion. Film thickness was dependent of the film composition [36]. These results agree with those as in [37], where thickness was influenced for the film composition, however they are opposite of the results as in [30], who did not find differences on the film thickness. The storage decreased the thickness of chitosan films without essential oil addition. However, thickness of chitosan films with essential oil addition was stable. It was only until the fourth week that the highest temperature showed a significantly effect on control chitosan films.

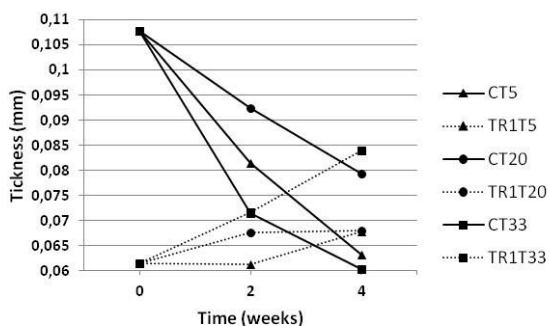


Fig. 1 Thickness of chitosan films. Data are the mean of determinations made in ten repetitions in three sample ($n = 30$)

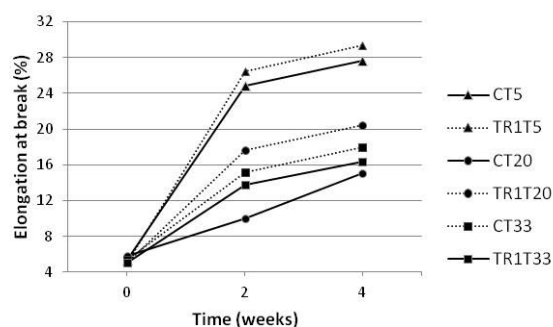


Fig. 2 Elongation at break of chitosan films. Data are the mean of determinations made in ten samples ($n = 10$)

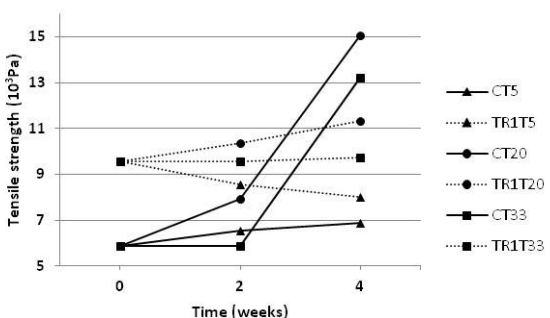


Fig. 3 Tensile strength of chitosan films. Data are the mean of determinations made in ten samples ($n = 10$)

2) Tensile Strength and Elongation

The addition of EOs does not affect the tensile strength or

the deformation at break. However, the mechanical response of the films during storage presented different trends (Figs. 2 and 3). The %E parameter decreased significantly with the storage. However, there were not significantly differences on time effect between samples on the second and fourth weeks. The higher temperatures during storage decreased significantly the elongation at break. Additionally, they also increased the tensile strength of control chitosan films at the fourth week.

3) Puncture Strength and Deformation

These mechanical properties assure the packaging integrity and the conservation of the barrier properties avoiding damages by penetration. The essential oil inclusion did not affect the puncture deformation but it increased the puncture strength (Figs. 4 and 5). The puncture deformation (PD) of films was significantly higher ($p > 0.05$) due to the storage. Additionally, the highest temperature of storage increased the puncture strength for films with and without essential oil incorporation.

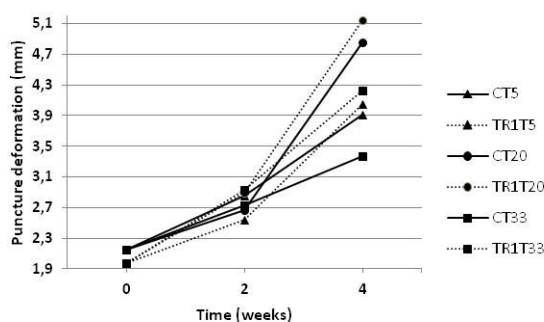


Fig. 4 Puncture deformation. Data are the mean of determinations made in triplicate ($n = 3$)

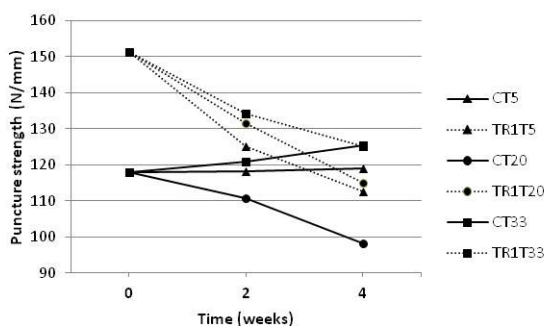


Fig. 5 Puncture strength. Data are the mean of determinations made in triplicate ($n = 3$)

4) Moisture Content

The addition of the essential oils decreased the moisture content value significantly even during storage (Fig. 6), which is attributed to an increase in hydrophobicity of films. Reference [30] shows that the essential oil inclusion causes the formation of covalent bonds between the functional groups of chitosan chains. This phenomenon leads to a decrease in the availability of hydroxyl and amino groups and limits the interactions between polysaccharide and water by hydrogen

bonding. The higher temperatures increased the moisture content for samples with essential oil inclusion. These results confirm that chitosan films behaved as a hydrophilic membrane. During storage, the water molecules interact with the cationic chitosan molecules of the polymeric matrix. This phenomenon increases the water-holding capacity, the absorption of water molecules, and therefore the moisture content [22], [29], [38]. These changes occur particularly before the second week.

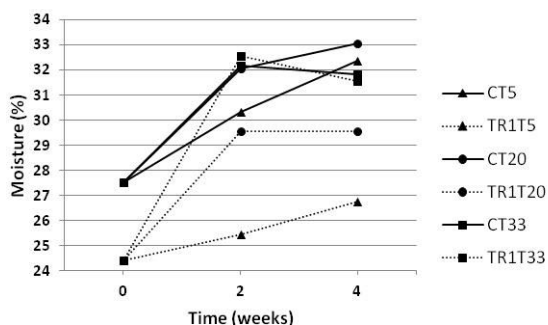


Fig. 6 Moisture of chitosan films. Data are the mean of determinations made in triplicate (n = 3)

5) Water Resistance

This property is important in food industry applications because the packaging must protect the food while it is in contact with water or foods with a high water activity [31]. The results presented in Fig. 7 showed that chitosan film presented a low solubility value which is similar as in [31]. However, when the EOs were added to the film, a significant increase in water solubility was observed. However, this result was in contrast with [39] that showed chitosan/rosemary essential oil had more water resistance compared to neat chitosan film.

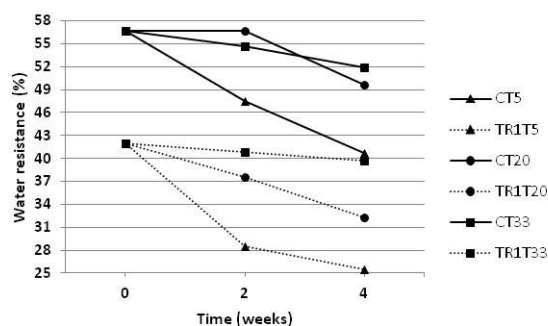


Fig. 7 Water resistance of chitosan films. Data are the mean of determinations made in triplicate (n = 3)

On the other hand, the chitosan films could suffer a swelling due to higher temperatures (Fig. 7). Reference [38] shows that the chitosan films are in a hydrogel state because it traps the water molecules when they are exposed to air with high humidity. This phenomenon produces changes in the polymer structure that occur in response to stresses generated within the film. The results demonstrated that some higher

temperatures produced an increase of the water resistance property. Reference [24] shows that storage of chitosan films produces a slow reacylation of amino groups, consequently the films lose solubility in water and acetic acid.

6) Swelling Index

This property predicts the maintenance of quality during packaging and storage of food product [40]. In some cases, a higher swelling index can be desirable to absorb extra water from outer surface of food with high moisture [11]. No significant changes were observed for the essential oil inclusion on chitosan films ($p < 0.05$) (Fig. 8). However, the result contrasted with [39] that assured that the swelling index decreased with an increase in the extract concentration. These differences probably occurred due to the extract and a lower quantity of essential oils used.

On the other hand, the results indicated that the swelling index decreased with the time storage (Fig. 8). When the hydrophilic films are stored at high humidity, some changes in the microstructure of the film occur and open up the polymer structure to increase the water interactions [41].

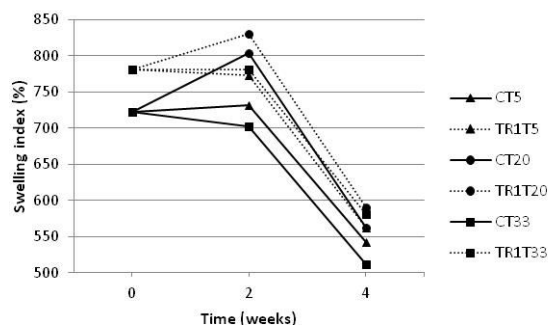


Fig. 8 Swelling index of chitosan films. Data are the mean of determinations made in triplicate (n = 3)

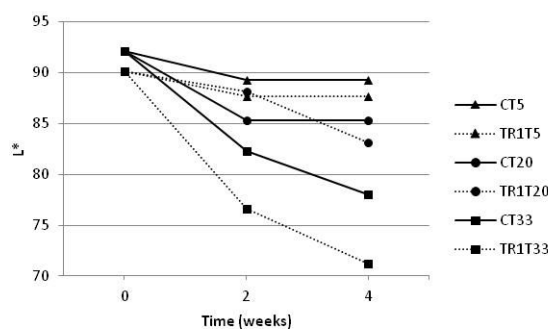


Fig. 9 L* value of chitosan films. Data are the mean of determinations made in ten repetitions in three sample (n = 30)

7) Color

Color is important because it directly affects consumer acceptability [26]. The results are presented in CIELAB rectangular coordinate system with (L^* , a^* , b^* , ΔE , C and H).

The addition of essential oils reduced the L^* even when the films were stored for four weeks (Fig. 9). Similar results as in [42], [11] that show the concentration of essential oils from cinnamon extract and grape decrease the lightness and

transparency of the films. Reference [43] defines that the inclusion of thyme essential oil decreased the surface lightness by the increasing of the roughness and the irregularities due to migration of oil to the surface.

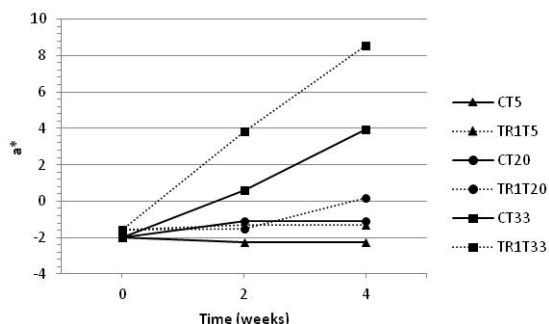


Fig. 10 a* value of chitosan films. Data are the mean of determinations made in ten repetitions in three sample (n = 30)

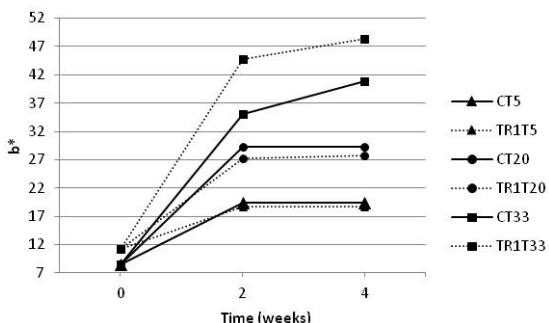


Fig. 11 b* value of chitosan films. Data are the mean of determinations made in ten repetitions in three sample (n = 30)

In addition, the increasing of time and temperature during storage decreased the values of L*. The chitosan films became matt by the reduction in lightness. This effect is probably due to the exchange on the molecular structure and chemical composition of chitosan [25], [28]. Reference [44] concluded that an increasing of time and temperature during storage induced the darkening on chitosan solutions.

On the other hand, the inclusion of essential oils, time, and temperature during storage incremented the a* and b* values (Figs. 10 and 11). The thyme essential oil inclusion increased the yellowness of the films without sensory change on the overall appearance [11]. Reference [28] shows that the increasing of the b* value on the stored chitosan films suggests that the films became yellower by non-enzymatic browning. Reference [45] shows that the effect of non-enzymatic darkening was more intense after the first week of storage and until the fifth week. However, some studies reported no significant differences in the color properties for films that still were stored for 16 weeks [29].

The essential oils inclusion increased the color difference (ΔE) significantly just on the stored samples at the highest temperature. Additionally, the time and temperature increasing during storage caused higher color difference (Fig. 12). This phenomenon is attributed to the slight decrease of the

parameter L* and the slight increase of the parameter b* [42].

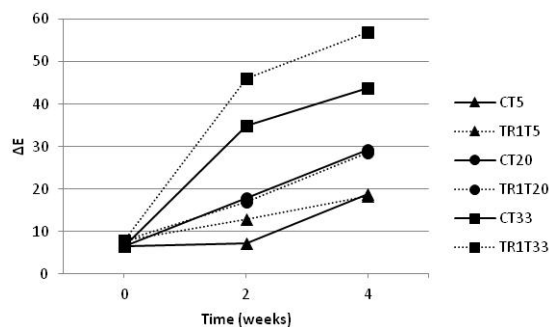


Fig. 12 ΔE of chitosan films. Data are the mean of determinations made in ten repetitions in three sample (n = 30)

The chrome value indicates the degree of saturation of color and is proportional to the strength of the color. The chrome increased significantly due to the essential oil inclusion during storage at high temperatures (Fig. 13). The hue angle showed a tendency to increase with the temperature and time increment.

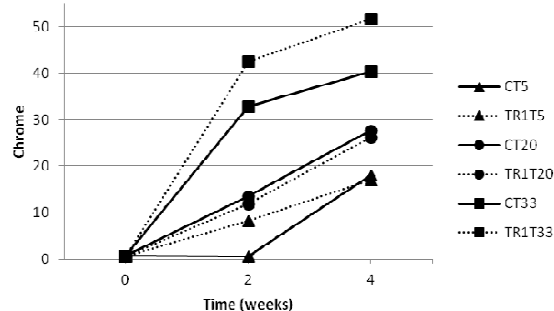


Fig. 13 Chrome. Data are the mean of determinations made in ten repetitions in three sample (n = 30).

8) Scanning Electron Microscopy

The structure was continuous for the CH film [Figs. 14 (a), (c), (e), (g)] whereas the CH film containing essential oils did not have a homogenous structure [Figs. 14 (b), (d), (f), (h)]. These results are in accordance with the results of other investigators [12], [18], [46]. Reference [46] shows that the incorporation of rosemary essential oil caused a loose structure and cracked surface in the chitosan films. This result suggests that EOs caused two phases in the polymeric solutions, lipid droplets embedded in a continuous polymer network [18]. Reference [12] shows that the fast evaporation of the thyme oil incorporated in films caused some bubbles in the upper surface (air-contacting) and it formed a porous structure distributed homogeneously. These studies agreed that the size and quantity of pores also increased with a higher oil concentration. Additionally, the conditions of storage did not show representatives changes on the surface neither in the internal structure. The chitosan film without the addition of essential oils had a smooth, homogenous, and compact structure without cracking after the storage. However, the chitosan films containing essential oils showed a non-

homogenous structure even after four weeks of storage.

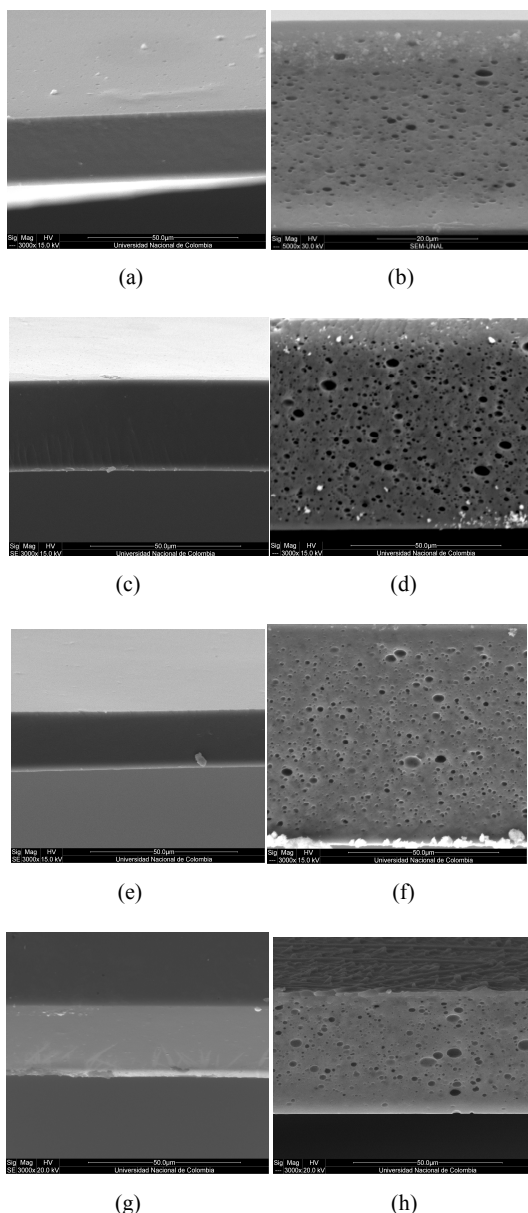


Fig. 14 SEM micrographs of the cross section of chitosan films. a) C (3000x); b) TR1 (5000x); c) CT5(2 weeks) (3000x); d) TR1T5(2 weeks) (3000x); e) CT20(two weeks) (3000x); f) TR1T20(two weeks) (3000x); g) CT33(four weeks) (3000x); h) TR1T33(four weeks) (3000x)

9) Differential Scanning Calorimetry (DSC)

The thermal properties of chitosan films have been studied using DSC from -40 to 200°C (Table II). The DSC thermograms of chitosan films were given on the Fig. 16. On the Table II, the glass transition temperature (T_g) decreased with the essential oil inclusion. Additionally, samples showed an endothermic drop was registered between the temperatures of 89°-144°C (Fig. 15). The endothermic effect corresponded to the weight loss and it was attributed to the evaporation of

water and the solvent traces [47]. The higher crystallization temperature is caused by the essential oils inclusion and the increasing of the concentration [12].

Additionally, the temperature had not a clear effect in T_g during storage. However, the films with essential oil inclusion had a higher thermal property even during the storage of the films. Reference [48] suggests that the glass transition depends on the polymer crystallinity, the chitosan degree of deacetylation, the macromolecular conformation and the water content on the film. The conformational changes in the microstructure of the film and the content of water could have modified the thermal properties of films especially in the films with the inclusion of essential oils.

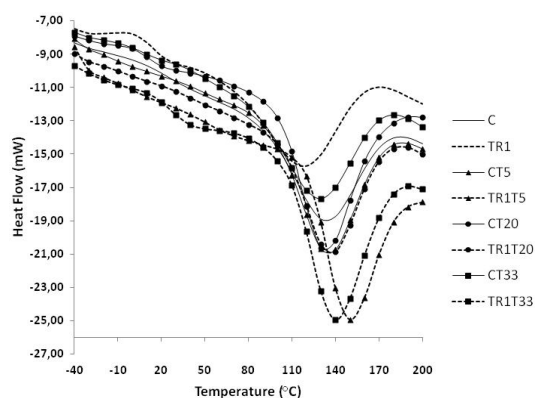


Fig. 15 DSC thermographs of chitosan after four weeks of storage

TABLE II
RESULTS OF DSC ANALYSIS AFTER FOUR WEEKS OF STORAGE

Materials	T_g (°C)	T_c (°C)
C	26,04	89,17
TR1	17,01	112,5
CT5	14,57	106,37
TR1T5	41,57	111,08
CT20	9,97	101,54
TR1T20	39,63	103,16
CT33	11,02	83,33
TR1T33	26,09	107,02

C: control film without essential oil addition; TR1: film with essential oil inclusion, 1:1 p/p (REO:TEO); T5, T20, T33: 5, 20, 33°C storage temperatures, respectively

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