JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 7499–7504 7499
DOI:10.1021/jf9002363

Antimicrobial, Mechanical, and Barrier Properties of Cassava Starch−Chitosan Films Incorporated with Oregano Essential Oil

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The physicochemical and antimicrobial properties of starch−chitosan films incorporated with oregano essential oil (OEO) have been investigated. The antimicrobial effects of starch−chitosan−OEO films against *Bacillus cereus*, *Escherichia coli*, *Salmonella enteritidis*, and *Staphylococcus aureus* were determined by the disk inhibition zone method. The film mechanical properties, water vapor permeability (WVP), Fourier transform infrared spectra (FTIR), and thermograms (TGA) were also determined. Films added with OEO effectively inhibited the four microorganisms tested and demonstrated improved barrier properties. The presence of OEO in starch−chitosan films led to the formation of more flexible films. Chitosan was not effective against the tested organisms, but it decreased film rigidity and WVP. TGA analysis demonstrated that the addition of chitosan and OEO did not affect the thermal stability of the films.

KEYWORDS: Active packaging; starch−chitosan; oregano essential oil; antimicrobial activity; permeability

INTRODUCTION

Recently, several alternatives have been investigated to minimize the environmental impact of conventional polymers, including the use of biodegradable polymers. Because of the abundance, low cost, and degradability of cassava starch (*1*), its use in the production of biodegradable films has been extensively investigated (*2*−*4*). However, technological applications of cassava starch normally require the improvement of mechanical and barrier properties, as these films are water-soluble, brittle, and difficult to process.

These problems may be solved with the addition of plasticizers. Because of the compatibility and availability of glycerol, this compound is one of the most used plasticizers. However, plasticizers have some disadvantages, such as permeability to oxygen and hydrophilicity, which lead to low film resistance to moisture (*5*).

The combination of chitosan with starch in films has been largely used, mainly to enhance the film barrier characteristics. Chitosan is a biopolymer fiber obtained by deacetylation of chitin, the most abundant polysaccharide on Earth after cellulose. This material is an excellent oxygen barrier and is relatively more hydrophobic than starch, which may favor the formation of films less permeable to water vapor. Additionally, chitosan is biodegradable, biocompatible, and a nontoxic antimicrobial (*6*), which are useful characteristics for several applications (*5*). For instance, the use of chitosan in starch-based films has great potential (*7*−*9*).

Epidemiological studies have demonstrated that the number of food-related diseases caused by pathogenic microorganisms has increased in recent years. As a form to introduce active agents to increase food preservation, the area of antimicrobial packaging has become one of the major areas of research in food packaging (*10*−*12*). The direct incorporation of a biocide into the packaging material could provide several advantages, such as the maintenance of a high concentration of the active agent directly on food surface, with low migration; the decrease of chances of active substance inactivation by food constituents; and the avoidance of the use of this substance as a food additive (*10*).

Essential oils from plant extracts are unique antimicrobial agents since they are natural compounds. Studies on the bacterial activity of several essential oils have shown that oregano (*Thymus capitatus*) is one of the most effective antibacterial agents (*11*−*13*).

Fractions of oregano essential oil (OEO) and pepper oil have been demonstrated to have activity against several species of bacteria, such as *Salmonella* (*14*, *15*) and *Escherichia coli* O157:H7 (*16*). Seydim and Sarikus (*13*) reported that edible whey protein-based films combined with OEO are more effective against *E. coli* O157:H7, *Staphylococcus aureus*, *Salmonella enteritidis*, *Listeria monocytogenes*, and *Lactobacillus plantarum* than rosemary and garlic oils.

In summary, the use of antimicrobial films has become very attractive for several applications in the food industry, particularly due to the successful results obtained so far. However, this

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Published on Web 07/23/2009

Table 1. Composition of the Biodegradable Films

	basic components proportions (g/100 g)			
formulation	starch	chitosan	glycerol	OEO added to the blend of basic components (g/100 g)
1	82	0	18	0
2	77	5	18	0
3	77	5	18	0.1
4	77	5	18	0.5
5	77	5	18	1.0

area still requires technological advances, and further studies are necessary on the potential of each antimicrobial agent, which will promote development in this field of research. Thus, the objective of this study was to investigate the properties of cassava starch−chitosan films with OEO produced by an extrusion process.

MATERIALS AND METHODS

Materials. The raw materials used in this study were native cassava starch (Indemil, Paranavaí, PR, Brazil), glycerol (Nuclear, Diadema, SP, Brazil), chitosan (average molecular mass of 100−300 kDa) (Acros Organics, Geel, Belgium), and OEO (Sigma-Aldrich, Steinheim, Germany). *Bacillus cereus* ATCC 25923, *E. coli* ATCC 25922, *S. aureus* FRI196e, and *S. enteritidis*, the last two isolates from outbreaks in the state of Paraná, Brazil, were obtained from the culture collection of the Microbiology Laboratory the Food Science and Technology Department of the Universidade Estadual de Londrina.

Film Production. The films were produced using a laboratory extruder (model EL-25, BGM, São Paulo, Brazil) with a screw diameter of $D = 25$ mm and a screw length of 28 D, four heating zones, and die with two 3 mm diameter holes for the production of pellets. For the production of tubular films, the same extruder was used, with five heating zones, 50 mm film-blowing die with internal air for the formation of the film "bubble" and a ring for cooling, two pneumatic spools, controllers, and microprocessor-based digital temperature display, proportional integral derivative control (PID) of the heating and cooling zones of the cooling tower, an automatic spooler, and a pelletizer with adjustable speed.

The proportion of 77% starch, 5% chitosan, and 18% glycerol was chosen for the incorporation of OEO because this formulation had the best mechanical properties and barrier results in a previous study aiming to develop films based on starch/chitosan blends (data not shown). The antimicrobial essential oil was added at concentrations of 0.0, 0.1, 0.5, and 1.0% in relation to the total basic formulation. A control film (without chitosan) with 82% starch and 18% glycerol was also prepared (**Table 1**). The formulation compounds (without OEO) were mixed in a home mixer (model Ciranda Classic, Arno, São Paulo, Brazil) at the lowest speed (about 780 rpm) for 5 min.

In the first stage of the extrusion process, the mixtures were extruded and pelletized with a temperature profile of 120/120/120/110 °C and a screw speed of 35 rpm. The OEO was added to the pellets, which were reprocessed. Next, the reprocessed pellets were extruded again for the formation of the film by the blow technique with a temperature profile of 120/120/120/120/130 °C and 35 rpm screw speed.

Antimicrobial Activity. The disk inhibition zone assay was used to qualitatively evaluate the antimicrobial activity of the films according to Rojas-Graü et al. (*12*), with modifications. The films produced with and without (control) OEO and chitosan were aseptically cut into 10 mm discs and placed on plates containing Mueller−Hinton (MHA) agar (Vetec Química Fina Ltd.a, Rio de Janeiro, RJ), which had been previously spread with 0.1 mL of inoculums, each containing 10^8 CFU mL^{-1} of bacterial cultures, previously standardized using the McFarland scale. The plates were incubated at 37 °C for 24 h. The diameter of the growth inhibition zones around the discs was measured using a pachymeter, and the growth under the film discs (area of contact with the agar surface) was visually examined. The tests were carried out in triplicate for each formulation.

Mechanical Properties. Tensile properties were determined with a texture analyzer, Stable MicroSystems (model TATX2i, England), according to Sarantópoulos et al. (*17*) and based on American Society for Testing and Material Standard ASTM D882-00 (2001). The film samples were cut into strips that were 100 mm in length by 25.4 mm in width and fit to the tensile grips. The initial distance between the grips was 50 mm, and the crosshead speed was set at 8.3 mm/s. The parameters determined were as follows: tensile strength (MPa), elongation at break (%), and Young's modulus (MPa). Measurements were performed in five replicates, and the samples were cut parallel to the film flow. For analysis, the samples were conditioned for 48 h in glass desiccators with a relative humidity of 64% and a temperature of 25 °C.

Table 2. Antimicrobial Activity of Cassava Starch−Chitosan Films Incorporated with OEO against the Tested Microorganisms[a]

	inhibitory zone (mm)		
	oregano oil concentration		
microorganism	0.1%	0.5%	1.0%
S. enteritidis	6.28 aA ± 0.97	14.97 aB ± 0.57	19.50 aC ± 0.50
E. coli	9.99 bA ± 0.78	18.92 bB ± 0.67	23.73 bC ± 1.91
S. aureus	13.26 cA ± 0.89	22.21 cB ± 1.53	30.81 cC ± 1.30
B. cereus	13.98 cA ± 1.15	22.84 cB ± 2.33	33.88 dC ± 1.44

[a] Small letters indicate differences between the types of microorganisms in each column, and capital letters indicate differences between OEO concentrations in each line ($p \leq 0.05$).

Water Vapor Permeability (WVP). WVP (g/Pa m s) was determined by gravimetry following standard ASTM E96-00 (*18*), with modifications. The preconditioned film (64% relative humidity, 25 °C for 48 h) was placed on the circular opening (60 mm diameter) of the permeability capsule with silicone grease to ensure that the humidity migration occurred only through the film. The capsule inside was completely filled with calcium anhydride ($CaCl_2$, 0% relative humidity), and the system was placed in a desiccator containing saturated sodium chloride solution (NaCl, 75% relative humidity). The desiccator containing the films was placed in a BOD oven at 25 °C. Each formulation was assayed in duplicate. The samples were weighed 10 times with 12 h between each weighing. The mass gain (m) was graphically determined as a function of time (t) using the angular coefficient (m/t) and calculating the water vapor permeation ratio (WVPR) with eq :

$$\mathrm{WVPR} = \frac{m}{t} \cdot \frac{1}{A} \quad (1)$$

where m/t is the angular coefficient of the curve (g water/s) and A is the sample permeation area (m^2).

WVP was determined with eq :

$$\mathrm{WVP} = \frac{\mathrm{WVPR} \cdot t}{sp(\mathrm{RH}_1 - \mathrm{RH}_2)} \quad (2)$$

where t is the mean sample thickness (m), sp is the water vapor saturation pressure at the assay temperature (Pa), RH_1 is the relative humidity of the chamber, and RH_2 is the relative humidity inside the capsule.

Fourier Transform IR Spectroscopy (FTIR). The film samples were dried in a desiccator containing $CaCl_2$ for 3 weeks before analysis. FTIR spectra were collected in a Bomem model FT-100 fit with a Universal Attenuated Total Reflectance (UATR) Pike Miracle HATR module and a diamond/ZnSe crystal with triple reflection for analysis of the films. The analysis was performed in the mean infrared region with a Fourier transform wavenumber range of 4000−500 cm^{-1} and spectral resolution of 4 cm^{-1}.

Thermogravimetric Analysis (TGA). The samples were previously conditioned at 64% relative humidity and at 25 °C for 48 h. The thermogravimetric curves were obtained in a Shimadzu analyzer model TGA50 under nitrogen flow of 50 mL/min. The samples were heated from 25 to 450 °C at 10 °C/min.

Statistical Analysis. The data were subjected to variance analysis and Tukey's test for comparison of means with Statistica Version 7.0 software (StatSoft, Inc. Tulsa, OK) at a 5% significance level.

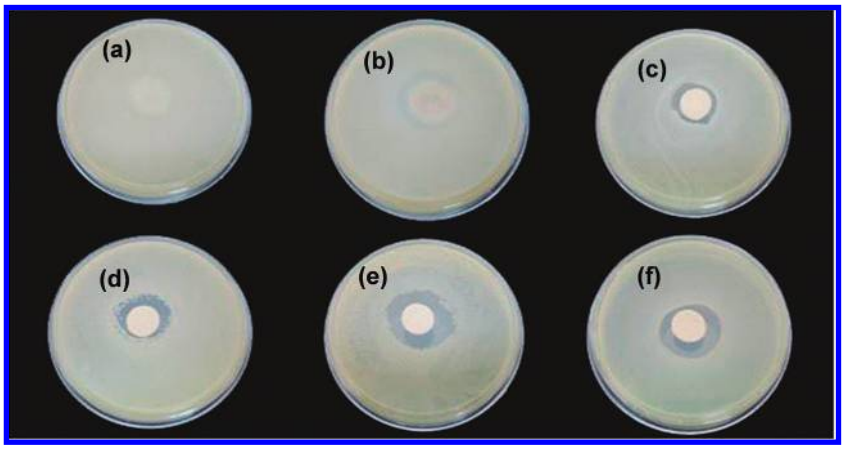

Figure 1. Inhibition zones for (**a**) cassava starch film, (**b**) cassava starch−chitosan film, and (**c**−**f**) cassava starch−chitosan films incorporated with 1% OEO against the microorganisms (**c**) *S. enteritidis*, (**d**) *E. coli*, (**e**) *B. cereus*, and (**f**) *S. aureus*. (Because in **a** and **b** no inhibition zones were observed, only one representative plate for each case is shown.)

Table 3. Effect of the Concentrations of Chitosan and OEO on Tensile Strength (*T*), Elongation at Break (*E*), Young's Modulus (*Y*), and WVP of the Biodegradable Films[a]

concentration (%)					
chitosan	oregano oil	*T* (MPa)	*E* (%)	*Y* (MPa)	WVP × 10^{-10} (g/Pa m s)
0	0	2.45 a ± 0.20	21.95 a ± 1.98	140.36 a ± 5.3	1.39 a ± 1.56
5	0	2.54 a ± 0.01	23.07 a ± 1.67	72.34 b ± 5.17	1.00 b ± 0.06
5	0.1	1.96 b ± 0.44	27.18 a ± 2.85	67.72 b ± 4.26	0.99 b ± 0.04
5	0.5	1.78 bc ± 0.25	40.73 b ± 2.40	49.36 c ± 6.43	0.74 bc ± 0.08
5	1.0	1.43 c ± 0.26	48.40 c ± 5.32	18.90 d ± 2.61	0.62 c ± 0.15

[a] The means in the same column with different letters differ significantly at $p \leq 0.05$.

RESULTS AND DISCUSSION

The cassava starch−chitosan−OEO films were homogeneous and easily manipulated. The films did not contain any bubbles or surface cracks. The film thickness ranged from 0.20 to 0.24 mm.

Antimicrobial Activity. Films were tested against microorganisms to determine antimicrobial activity. Only the ones that contained OEO presented this effect, and the inhibition zones increased significantly ($p \leq 0.05$) with increasing oil concentrations for all tested microorganisms (**Table 2**). *B. cereus* presented the largest inhibition for all tested concentrations, while *S. enteritidis* presented the lowest one.

The largest inhibition halos were observed for the Gram-positive bacteria (*B. cereus* and *S. aureus*), while the smallest ones were observed for the Gram-negative bacteria (*S. enteritidis* and *E. coli*) (**Figure 1**). Most studies investigating the action of whole essencials oils (EOs) against food spoilage organisms and food-borne pathogens agree that, generally, EOs are slightly more active against Gram-positive than Gram-negative bacteria (*19*, *20*). This result may be related to the presence of an additional external membrane surrounding the cell wall in Gram-negative bacteria, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (*19*).

According to Zivanovic and Draughon (*20*), the proposed mechanism of antimicrobial activity of phenolic compounds of EOs is in their attack on the phospholipid cell membrane, which causes increased permeability and leakage of cytoplasm, or in their interaction with enzymes located on the cell wall. Thus, the resistance of Gram-negative bacteria to the essential oils likely lies in the protective role of their cell wall lipopolysaccharides or outer membrane proteins.

Dadalioglu and Evrendilek (*21*) reported the powerful inhi bitory effect of OEO applied directly to *E. coli* O157:H7, *L. monocytogenes*, *S. typhimurium*, and *S. aureus*. They attributed the antimicrobial activity of OEO to two components: the phenolic compound carvacrol and monoterpene *p*-cimene. Burt (*19*) described the action of carvacrol as the disintegration of the external membrane of Gram-negative bacteria followed by the release of the lipopolysaccharides present, resulting in increased permeability of the cytoplasm membrane to ATP.

In another study, the incorporation of 1% OEO to whey protein isolated (WPI)-based films was effective against *E. coli* O157:H7 and *Pseudomonas* spp. on the surface of beef (*22*). The authors observed that 2% OEO was required for WPI films to reach the minimum inhibitory level against *S. aureus*, *S. enteritidis*, *L. monocytogenes*, *L. plantarum*, and *E. coli* O157:H7. The investigation presented here demonstrated that a concentration of 0.5% OEO inhibited the tested microorganisms effectively, producing halos between 14.97 and 22.84 mm. Therefore, we can conclude that the source and concentration of the active plant extract compounds and the film composition have a major effect on the biological action of films.

Despite the fact that the chitosan has demonstrated antimicrobial activity against fungi and bacteria in various studies (*6*, *23*), its presence did not inhibit the four microorganisms tested in this work (**Figure 1**). As demonstrated by studies performed before, only the soluble protonated fraction of chitosan that is released from the solid film upon liquid phase contact (on antimicrobial test) is capable of acting as a biocide (*24*). In this case, the positively charged molecules may interact with the negatively charged membranes of bacteria, resulting in membrane rupture and cell death (*25*). Thus, the antimicrobial activity of chitosan in insoluble films is negligible (*23*).

The fact that other researchers (*6*, *25*) have observed antimicrobial activity of chitosan films produced by casting is due to the previous dissolution of chitosan in acetic acid when filmogenic

solution is prepared. This action protonates the NH_2 groups of chitosan and enhancing solubility. As in the extrusion process chitosan dissolution is unnecessary, the NH_2 groups are not protonated, which explains the absence of the antimicrobial activity.

Mechanical Properties. The mechanical properties of the studied films are given in **Table 3**. A biodegradable film must be resistant to the normal stress that occurs in application, transport, and handling to maintain the integrity and properties of foods. According to **Table 3**, the tensile strength of the biofilms was affected by the addition of OEO. The presence of OEO caused the reduction of the tensile strengh in the films, which was most likely due to plasticizing capacity.

Elongation at break indicates the flexibility and elongation capacity of the films. The addition of OEO significantly affected elongation at break (**Table 3**). Concentrations of 0.1 and 0.5% OEO increased elongation at break from 27.18 to 40.73%, respectively, reaching a maximum of 48.40% for 1% OEO. Zivanovic, Chi, and Draughon (*20*) observed a decrease in tensile strength and an increase in elongation of chitosan films combined with essential oils, which corroborates the present results.

The Young's modulus indicates the rigidity of the film; a larger Young's modulus indicates a more rigid material. **Table 3** shows that both the chitosan and the OEO affected this property. The addition of chitosan and OEO led to a significant reduction of the Young's modulus ($p \leq 0.05$) and, therefore, the formation of less rigid films. The film produced with only starch and glycerol presented a higher Young's modulus, 140.36 MPa, which is related to the effect of extrusion conditions (high temperature, pressure, and shearing) in the starch, allowing for the approximation and interaction of the chains and favoring the formation of a denser and more rigid matrix.

Considering the hydrophilic nature of the films, predominantly due to starch and glycerol, water sorption processes may occur and affect the film properties with a plasticizing action. In this study, the films produced were conditioned at 64% equilibrium relative humidity, yielding moisture levels ranging from 0.09 to 0.14 g of water/g of solids. This substantive difference could also have influenced the film mechanical properties.

In general, the addition of OEO resulted in a film matrix that was less dense, which facilitated the movement of the polymer chains and improved the film flexibility. The presence of chitosan resulted in films that were less rigid.

WVP. One of the main functions of packaging is to protect foods by avoiding or reducing the transfer of moisture, forming a barrier to prevent or reduce the contact of foods with the external environment (*17*). **Table 3** shows that the WVP of the films decreased significantly ($p \leq 0.05$) with the addition of chito san. The cassava starch film (control) had the highest WVP value, 1.39×10^{-10} g/Pa m s, which may be attributed to the larger number of free hydroxyl groups and, consequently, the increased interaction with water, favoring permeability. However, the addition of chitosan resulted in an increased interaction with starch, due to the formation of hydrogen bonds between the NH_2 present in chitosan and the OH^- of cassava starch, reducing the availability of the hydrophilic groups and decreasing the WVP to 1.00×10^{-10} g/Pa m s.

WVP also decreased significantly ($p \leq 0.05$) with an increase in the concentration of OEO, which is consistent with the results of previous studies (*12*, *20*). The WVP for the maximum concentration of OEO (1.0%) was 0.62×10^{-10} g/Pa m s.

As essential oils are highly hydrophobic and complex mixtures, the increase in film hydrophobicity will reduce the water absorption. Similarly, WVP will decrease with an increase of the hydrophobic compound fraction, as the water vapor transference occurs through the hydrophilic portion of the film; thus, WVP depends on the hydrophilic−hydrophobic ratio of the film constituents (*12*). Therefore, the addition of OEO increases the antimicrobial efficiency and enhances the barrier properties of the cassava starch−chitosan film.

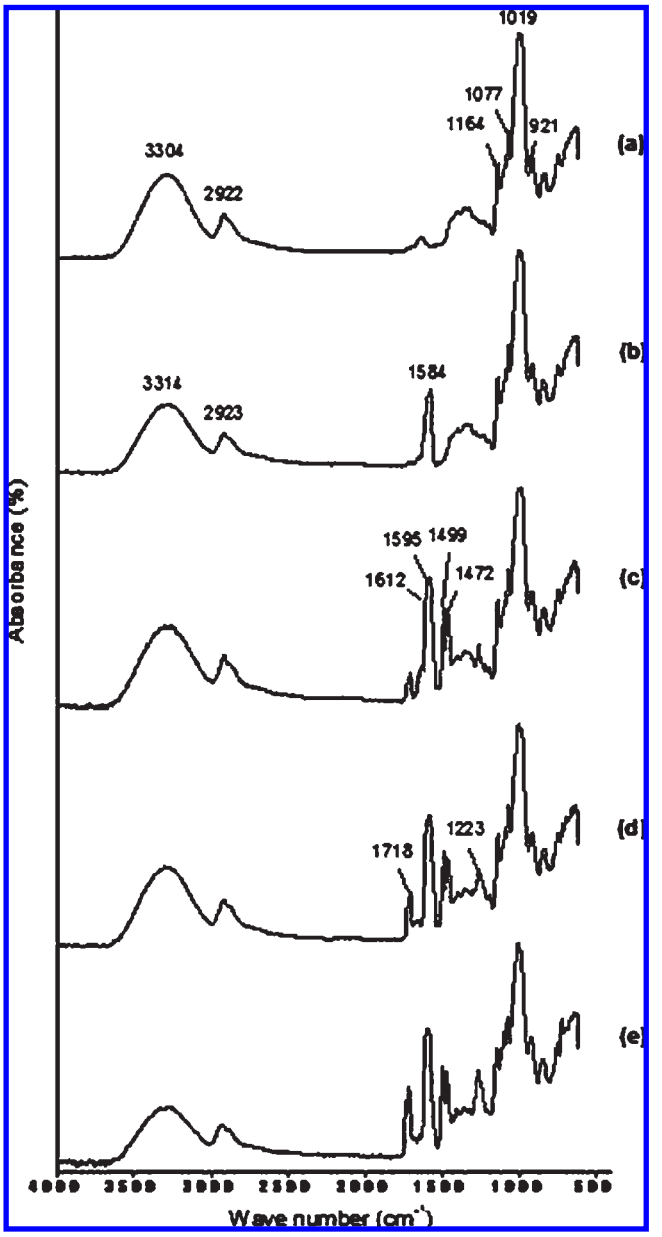

Figure 2. FTIR spectra of the films (**a**) starch, (**b**) starch−chitosan, and (**c**−**e**) starch−chitosan with (**c**) 0.1% OEO, (**d**) 0.5% OEO, and (**e**) 1.0% OEO.

FTIR. FTIR spectroscopy was used on the film samples to investigate the starch, chitosan, and OEO interactions. The infrared spectra of the starch, starch−chitosan, and starch−chitosan incorporated with 0.1, 0.5, and 1.0% OEO are given in **Figure 2**.

The broad band at 3304 cm^{-1} in the starch film spectrum (**Figure 2a**) is due to hydrogen bonds formed with starch hydroxyl groups and glycerol (*26*). The band at 2922 cm^{-1} corresponds to the C−H stretching (*26*), and the bands at 1164 and 1077 and 1019 and 921 cm^{-1} result from the stretching of C−O in C−O−H and C−O in C−O−C bonds, respectively (*27*).

Xu et al. (*9*) reported that the chitosan film spectrum has a broad band at 3351 cm^{-1} attributed to the OH stretching,

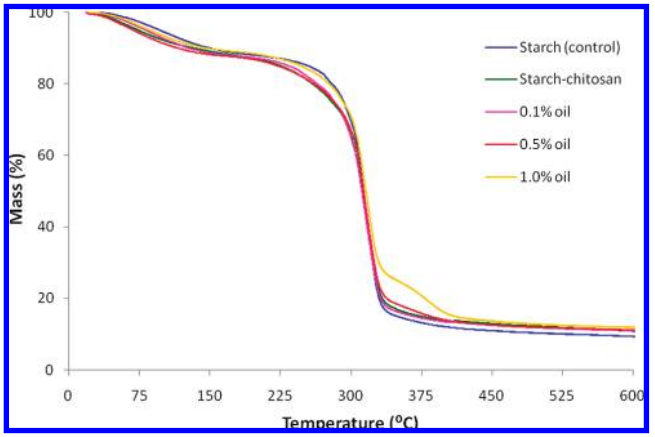

Figure 3. TGA curves of the films starch, starch−chitosan, and starch−chitosan−OEO with 0.1, 0.5, and 1.0% OEO.

which overlaps with the stretching of NH in the same region. The C−H stretching observed at 2923 cm^{-1} and the band at 1578 cm^{-1} represent the deformation of the NH stretching plane.

The physical combinations as compared to the chemical interactions of two or more mixed substances are reflected by characteristic changes of the spectrum bands. The spectrum of the starch−chitosan film (**Figure 2b**) shows that the band relative to the amino group shifted to a high wavenumber from 1578 to 1584 cm^{-1}, with the addition of starch. This result indicates the interaction of the hydroxyl groups of starch with the amino groups of chitosan (*9*). The data obtained in this work suggest the compatibility of the two components, as well as their interaction. However, the band relative to the hydroxyl groups cannot be used to evaluate the interactions, due to the effects of the glycerol and water contents.

The addition of OEO resulted in the appearance of six new bands. Four bands, between 1600 and 1400 cm^{-1} (**Figure 2c**), are attributed to the benzene ring insaturations and one to the actual ring. These bands are from the aromatic hydrocarbons of *p*-cimene, thymol, and *y*-terpinene, in addition to the phenolic compound carvacrol in the composition of the OEO. Also, the abnormal stability resulting from the benzene ring resonance explains the preservation of this functional group in the extrusion process (*26*). The band at 1223 cm^{-1} corresponds to the C−O−H stretching of the phenolic compound, carvacrol, while the band at 1818 cm^{-1} indicates the presence of the carbonyl group (C═O) (**Figure 2d**). During the extrusion process, the OEO may have acted as a catalyst in the oxidation of the hydroxyl radicals of the starch, chitosan, or glycerol, enhancing the formation of the carbonyl group.

The comparison of the films of starch−chitosan−OEO (**Figure 2c**−**e**) demonstrates an increase in the absorbance of the bands at 1223 and 1718 cm^{-1} (15.36 and 1.12, 29.39 and 18.45, and 42.76 and 34.90% for 0.1, 0.5, and 1.0% OEO, respectively). As the absorbance provides quantitative data on the bonds, this increase is directly related to an increase in the concentration of the OEO, indicating a larger number of characteristic bonds of this component in the mixture.

TGA. TGA was performed to evaluate the thermal stability of the starch, starch−chitosan, and starch−chitosan−OEO films. The TGA curves and their derivatives (DTG) are shown in **Figures 3** and **4**. The maximum decomposition temperature ($T_{d\,max}$) and the percentage of residues are given in **Table 4**. The $T_{d\,max}$ values were determined from the maximum temperatures of the peaks in the TGA curve derivatives.

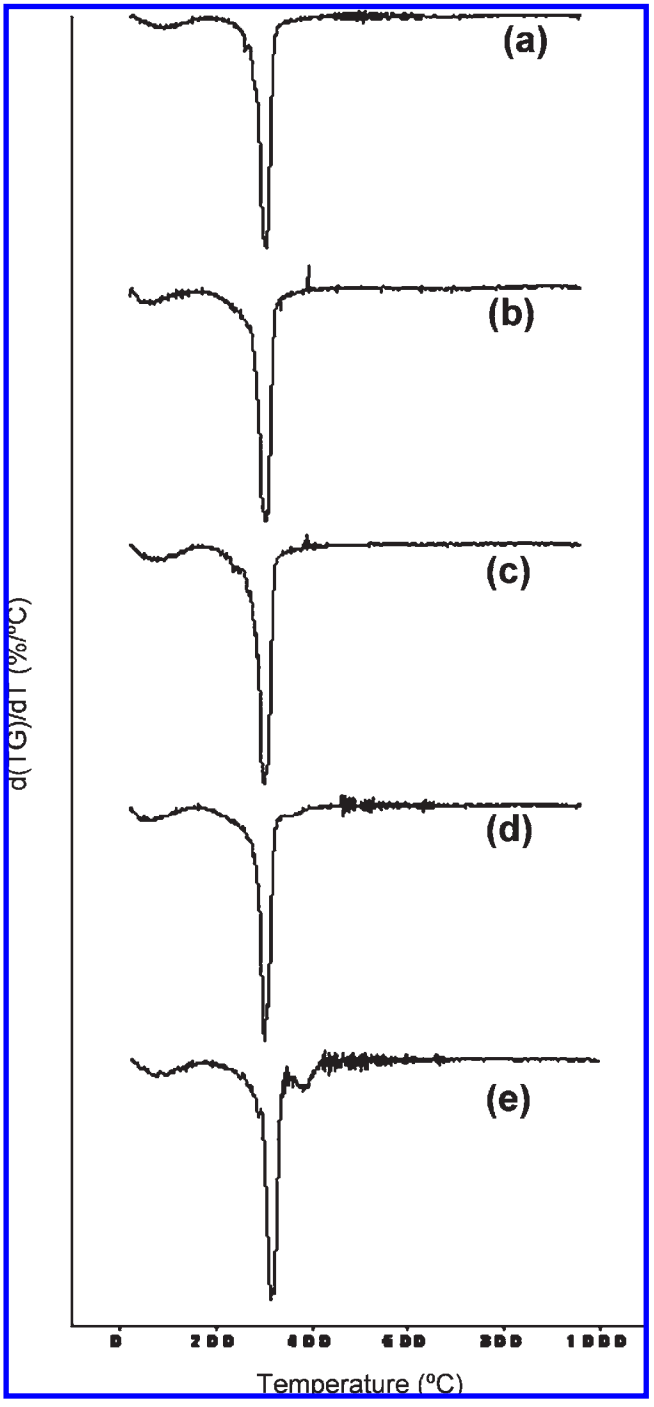

Figure 4. DTG curves of the films (**a**) starch, (**b**) starch−chitosan, and (**c**−**e**) starch−chitosan with (**c**) 0.1% OEO, (**d**) 0.5% OEO, and (**e**) 1.0% OEO.

The starch−chitosan−OEO 1% film presented three mass loss steps, while the other films presented only two (**Figure 3**). The aromatic structures present in the OEO are highly stable due to the resonance of the benzene ring, which results in the decomposition of these compounds at higher temperatures (380 °C). Decomposition may also have occurred in the films with 0.1 and 0.5% OEO but was not detected due to the low contents.

The first step, between $T_{d\,max}$ of 72 and 99 °C, is attributed to the evaporation of water absorbed by starch, chitosan, and glycerol, along with the evaporation of the low molecular weight compounds. Decomposition of starch and chitosan occurs at approximately 250−350 °C. Similar results have been reported

Table 4. TGA and DTG Curve Parameters of the Films

concentration (%)		$T_{d\,max}$ (C)			
chitosan	oregano oil	1° stage	2° stage	3° stage	residue (%)
0	0	99	318		6.44
5	0	78	318		6.34
5	0.1	80	315		9.09
5	0.5	72	314		8.79
5	1.0	78	314	380	10.99

for these materials (*28*−*30*). The addition of chitosan and OEO did not influence the thermal stability of these films, as demonstrated by the small variation in the $T_{d\,max}$ values during the second step (314−318 °C). However, an increase in percent residue after the incorporation of the OEO was also observed.

ACKNOWLEDGMENT

We thank the financial support provided by CAPES and SETI-PR.

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Received January 21, 2009. Revised manuscript received June 4, 2009. Accepted July 14, 2009.